Aspects of the Molecular Systematics, Taxonomy and Population Genetics of *Otomops* (Chiroptera: Molossidae) in Africa and Madagascar.

By

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Submitted in fulfillment of the academic
requirements for the degree of
Doctor of Philosophy in the
School of Life Sciences,
University of KwaZulu-Natal,
Westville Campus

December 2015

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ABSTRACT

Otomops (Chiroptera: Molossidae), commonly known as the large-eared, free-tailed bat, has an Old World distribution which includes areas from the Afrotropical region: mainland Africa, the Arabian Peninsula and Madagascar. This study, based on mitochondrial and nuclear sequence data and microsatellites, examines aspects of the molecular systematics and population genetics of Afro-Malagasy members of the genus on a variety of levels, including: its position within the family; relationships among species; phylogeography; and relationships among and within colonies.

In initial investigations, mitochondrial sequence markers (cytochrome *b* gene and D-loop) were used to assess phylogenetic relationships and phylogeography in a sample from mainland Africa and Madagascar. Results suggested the existence of two reciprocally-monophyletic *Otomops* clusters/clades (Madagascar and Africa). The African group was further subdivided into two reciprocally-monophyletic clusters/clades: the north/east Africa (NEA) clade, comprising samples from Ethiopia, Kenya and Yemen, and the south/west Africa (SWA) clade, comprising samples from South Africa, Ivory Coast, Zimbabwe, Tanzania and Burundi, which were separated by a cytochrome *b* genetic distance of 2.1%. The clade from Madagascar was recognized as *O. madagascariensis* and was separated by average genetic distances of 3.1% and 3.5% from the African SWA and NEA clades, respectively. Separation of the clades was also confirmed by an AMOVA which indicated that a higly significant 80% of variance is between the geographically defined species-groups. Genetic distance between the Ivory Coast sample was identical to that observed between the SWA and NEA clades indicating that this population may be undergoing speciation. Population genetic analysis of D-loop data suggests that the population from Madagascar may have been expanding for the last 27 388 to 52 242 years. Sequence data suggests that Asian *Otomops* species are ancestral to Afro-Malagasy species, consistent with Asia being the centre of diversity of *Otomops*.

Results from this initial species-level study warranted further investigation into the taxonomy of *Otomops* on the African mainland since there appeared to be two clades/clusters present. In addition to mitochondrial data, nuclear sequence and microsatellite data analyses show the separation of individuals from the African mainland and the Arabian Peninsula into two reciprocally-monophyletic groups, suggesting that the southeast, central and west (SECW) African and northeast (NEA) African lineages should rather be classified as separate species (cytochrome *b* genetic distance: 2.1%). Morphometric results were congruent with molecular-based findings where principal component analyses revealed a similar separation of samples according to geographical locality. Thus we proposed that the SECW clade retain the designation *O. martiensseni*, since a sample from the type locality is found within this grouping, and we described the NEA grouping as a new Afro-Arabian

species, *Otomops harrisoni*. Ecological niche modelling suggests that the distribution range of the *O. harrisoni* extends from the Arabian Peninsula through to Eritrea and south to Ethiopia and Kenya.

Nuclear RAG2 sequence data was combined with mitochondrial data to ascertain the phylogenetic position of *Otomops* within the Molossidae and resolve genus-level relationships among the other molossid genera. Results showed strong support for the monophyly of the family, with *Mormopterus* (origin 36.56 MYA) occupying a basal position within the clade. *Otomops* formed a strongly-supported, discrete, monophyletic clade (diverged 21.31 MYA). *Sauromys* also appears as a distinct genus. *Chaerephon* and *Mops* were not supported as monophyletic genera, but formed a nested clade in which some paraphyly was present (diverged 19.07 MYA), and in which *Mops* taxa were generally ancestral to *Chaerephon* taxa. Additional investigation may find *Chaerephon* and *Mops* better referred to a single genus. *Tadarida* does not exhibit monophyly and the status of this genus warrants further investigation with a more extensive taxonomic and genetic sample. Molossid groupings found in this study were substantially different from those suggested by Freeman (1981) and Legendre (1984), but showed the greatest similarity to those proposed by Simmons (2005) with the exception of the classification of *Chaerephon* and *Mops* as two separate genera.

Genetic analysis at the population- and colony-level was based on genotype data from six nuclear microsatellites and mitochondrial sequence data. STRUCTURE and haplotype network analysis confirmed the existence of three groupings corresponding to the *Otomops martiensseni*, *O. harrisoni* and *O. madagascariensis* species, which are separated by significant ($p < 0.01$) pairwise F_{ST} values. A Mantel test revealed that lineage separation is not as a result of isolation by distance. Kinship analysis revealed 70% of individuals within and among colonies were part of either parent/offspring, full-sibling or half-sibling relationships. No kinship associations were exhibited across the three main lineages. Results suggest that strict colony faithfulness is not observed, and that individuals engage in extra-colony matings within their respective lineages. This results in a lack of inbreeding, high genetic diversity and the observed predominance of half-sibling associations. There is also little evidence to support the existence of a social system based on male or female philopatry in any of the Afro-Malagasy *Otomops* spp.

PREFACE

The experimental work described in this dissertation was carried out at the School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban. This work was carried out from April 2008 to December 2015, under the supervision of Prof. Jenny M. Lamb, Dr. Peter J. Taylor and Prof. Steven M. Goodman.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly noted in the text.

DECLARATION 1 - PLAGIARISM

I, Taryn Marietta Cecilia Ralph declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

DECLARATION 2 - PUBLICATIONS

PLEASE NOTE: CHAPTER 2 OF THIS THESIS COMPRISES MY MSc DISSERTATION. THIS WAS EXAMINED AND PASSED IN 2008.

Publication 1 (Chapter 4)

Ralph, T.M.C. and Lamb, J.M. (2013). Cross-genus amplification and characterisation of microsatellite loci in the large-eared free-tailed bat *Otomops* (Chiroptera: Molossidae) from Africa and Madagascar. *African Journal of Biotechnology* **12**: 4233-4237.

The ideas were conceived by T.M.C. Ralph and J.M. Lamb. The data were collected and analysed by T.M.C. Ralph. The writing was led by T.M.C. Ralph.

Publication 2 (Chapter 5)

Ralph, T.M.C and Lamb, J.M. (2015). Examination of the population genetics of Afro-Malagasy *Otomops* (Chiroptera: Molossidae) via microsatellite analysis. *African Zoology* (in preparation).

The ideas were conceived by T.M.C. Ralph and J.M. Lamb. The data were collected and analysed by T.M.C. Ralph. The writing was led by T.M.C. Ralph.

Please note: this chapter has been presented in chapter format for the purposes of thesis submission and examination.

Publication 3 (Chapter 6)

Ralph, T.M.C., Richards, L.R., Taylor, P.J., Napier, M.C. and Lamb, J.M. (2015). Revision of Afro-Malagasy *Otomops* (Chiroptera: Molossidae) with the description of a new Afro-Arabian species. *Zootaxa* **4057**: 1-49.

This is a collaborative project involving morphometric and genetic assessments of phylogenetic and phylogeographic relationships in Afro-Malagasy *Otomops* spp.

The ideas were conceived by T.M.C. Ralph, L.R. Richards, P.J. Taylor and J.M. Lamb. The molecular data were collected and analysed by T.M.C. Ralph. The morphometric data were collected and analysed by L.R. Richards. The writing was done jointly by T.M.C. Ralph and L.R. Richards.

Publication 4

Lamb, J.M., Ralph, T.M.C., Goodman, S.M., Bogdanowicz, W., Fahr, J., Gajewska, M., Bates, P.J.J., Eger, J., Benda, P. and Taylor, P. (2008). Phylogeography and predicted distribution of African-Arabian and Malagasy populations of giant mastiff bats, *Otomops* spp. (Chiroptera: Molossidae). *Acta Chiropterologica* **10**: 21-40.

Molecular work on *Otomops* spp. samples was carried out by T.M.C. Ralph under the supervision of J.M. Lamb.

Publication 5

Lamb, J.M., Ralph, T.M., Naidoo, T., Taylor, P.J., Ratrimomanarivo, F., Stanley, W.T. and Goodman, S.M. (2011). Toward a molecular phylogeny for the Molossidae (Chiroptera) of the Afro-Malagasy region. *Acta Chiropterologica* **13**: 1-16.

Molecular work on *Otomops* spp. and additional molossid samples was carried out by T.M.C. Ralph under the supervision of J.M. Lamb.

Publication 6

Lamb, J.M., Abdel-Rahman, E.H., Ralph, T.M.C., Fenton, M.B., Naidoo, A., Richardson, E.J., Jacobs, D.S., Denys, C. and Taylor, P.J. (2006). Phylogeography of southern and northeastern African populations of *Otomops martiensseni* (Chiroptera: Molossidae). *Durban Museum Novitates* **31**: 42-53.

Molecular work on additional *Otomops* spp. samples that were added to the original dataset (collected by E.H. Abdel-Rahman) was carried out by T.M.C. Ralph under the supervision of J.M. Lamb.

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ACKNOWLEDGMENTS

I wish to thank my supervisor, Prof. Jennifer M. Lamb, and co-supervisors, Prof.. Peter J. Taylor and Prof. Steven M. Goodman, for providing me with the opportunity to research this topic and for providing constant guidance, support and assistance throughout the duration of this project.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF. Additional thanks to the University of KwaZulu-Natal for their financial assistance over the duration of this project.

My sincere appreciation goes to those who provided and/or helped acquire sample material used in this study: Dr. Peter Taylor, Prof. Steven Goodman, Dr. Leigh Richards and Dr. Corrie Schoeman among others, and to the various museums and institutions.

Thanks to my friends and colleagues, Dr. Leigh Richards and Dr. Theshnie Naidoo, for all of their support and assistance throughout the project and for undertaking the complementary sections of the study. Additional thanks to my friends, Muhammad Nakhooda, Tarin Ramsaroop, Michel Bengtson, Leelin Govender and Sanjivan Singh for all of your encouragement.

And special thanks go to my family: my mother, brother and uncle for all of their constant and unwavering support during these many years. It’s finally done.

LIST OF ABBREVIATIONS

π - nucleotide diversity

AEC - Adaptive Evolutionary Conservation

AMOVA - Analysis of Molecular Variance

AR - aspect ratio

β-fib / *fgb7* - 7th intron of the fibrinogen gene

BSC - Biological Species Concept

BW - bandwidth

CBD - Convention on Biological Diversity

Chd1 intron - nucleosome remodelling factor

CMS - Convention on the Conservation of Migratory Species of Wild Animals

COI - cytochrome oxidase subunit I

Dur - duration

EDTA - ethylenediaminetetraacetic acid

EMBO - European Molecular Biology Organization

ESA - USA Endangered Species Act

ESU - evolutionary significant unit

EtBr - ethidium bromide

EUROBATS - The Agreement on the Conservation of Bats in Europe

F_{max} - frequency (maximum)

F_{min} - frequency (minimum)

GSL - greatest skull length

GTR - general time reversible

h - haplotype diversity

I - incisor

IPI - inter-pulse interval

ISSR - Inter Simple Sequence Repeat

IUCN - International Union for Conservation of Nature

IUCN/ SSC - International Union for Conservation of Nature’s Species Survival Commission

kHz - kilohertz

LGM - Last Glacial Maximum

M - molar

MCO - Miocene Climatic Optimum

mtDNA - mitochondrial DNA

MU - management unit

MYA - million years ago

MYR - million years

NCA - nested clade analysis

NCBI - National Center for Biotechnology Information

ncDNA - nuclear DNA

ND1 - NADH dehydrogenase subunit 1

NEA - north/east Africa

NLM - National Library of Medicine

nt - nucleotides

OC - canine

OTU - operational taxonomic unit

PCMM - Program for the Conservation of Migratory Bats of Mexico and the United States

PCR - polymerase chain reaction

PF - peak frequency

PM - premolar

PRCK1 - protein-kinase C1

PSC - Phylogenetic Species Concept

RAG - recombination activating gene

RAPD - Random Amplified Polymorphic DNA

RF - resistance frequency

RFLP - Restriction Fragment Length Polymorphisms

rg - raggedness

S/d - expansion co-efficient

SMRS - specific-mate recognition system

sp. nov. - species nova

SSR - Simple Sequence Repeat

SWA - south/west Africa

TBE - tris-borate/EDTA

TG - thyroglobulin

THY - thyrotrophin

TNE - tris/NaCl/EDTA

UNEP - United Nations Environment Programme

WIO - Western Indian Ocean

WL - wing loading

Yr BP - years before present

zfy - zinc finger protein on the Y chromosome

CHAPTER ONE:

General Introduction

GENERAL INTRODUCTION

Chiroptera

Bats comprise the second largest mammalian order, are one of the most diverse groups of placental mammals, with over 1200 recorded species, and have a global distribution (Wilson and Reeder, 2005; Jan *et al*., 2012). There are currently 20 recognized bat families within the order Chiroptera: Cistugonidae, Craseonycteridae, Emballonuridae, Furipteridae, Hipposideridae, Megadermatidae, Miniopteridae, Molossidae, Mormoopidae, Mystacinidae, Myzopodidae, Natalidae, Noctilionidae, Nycteridae, Phyllostomidae, Pteropodidae, Rhinolophidae, Rhinopomatidae, Thyropteridae and Vespertilionidae (Eick *et al*., 2005; Simmons, 2005; Miller-Butterworth *et al*., 2007; Lack *et al*., 2010). Historically, classifications of the order were based on morphological data, which saw the separation of families into two sub-orders, the Megachiroptera (mainly non-echolocating pteropodid bats) and the Microchiroptera (echolocating bats), which was then revised by subdivision of the order into Yinochiroptera and Yangochiroptera (Hutcheon and Kirsch, 2006). With the advent of molecular techniques, these groupings were called into question and updated, where the names Yangochiroptera and Yinochiroptera were replaced with the subordinal names Vespertilioniformes (including Cistugonidae, Emballonuridae, Furipteridae, Miniopteridae, Molossidae, Mormoopidae, Mystacinidae, Myzopodidae, Natalidae, Noctilionidae, Nycteridae, Phyllostomidae, Thyropteridae and Vespertilionidae) and Pteropodiformes (including Craseonycteridae, Hipposideridae, Megadermatidae, Pteropodidae, Rhinolophidae and Rhinopomatidae), respectively (Eick *et al*., 2005; Hutcheon and Kirsch, 2006).

Eick *et al*. (2005) (Fig. 1), Teeling *et al*. (2005) and Miller-Butterworth *et al*. (2007) have reported on phylogenetic associations within the Chiroptera using DNA sequence data from a combination of various nuclear DNA regions. The structure is largely congruent across all three studies, save for the position of Myzopodidae. In Miller-Butterworth *et al*. (2007), Myzopodidae is associated within the Noctilionoidea and in Eick *et al*. (2005) it is associated with the Vespertilionoidea. Although *Cistugo* appears in Eick *et al*. (2005), it was not recognised as a separate family at the time. Lack *et al*. (2010) subsequently established that *Cistugo* should be recognised as a separate family within the Vespertilionoidea.

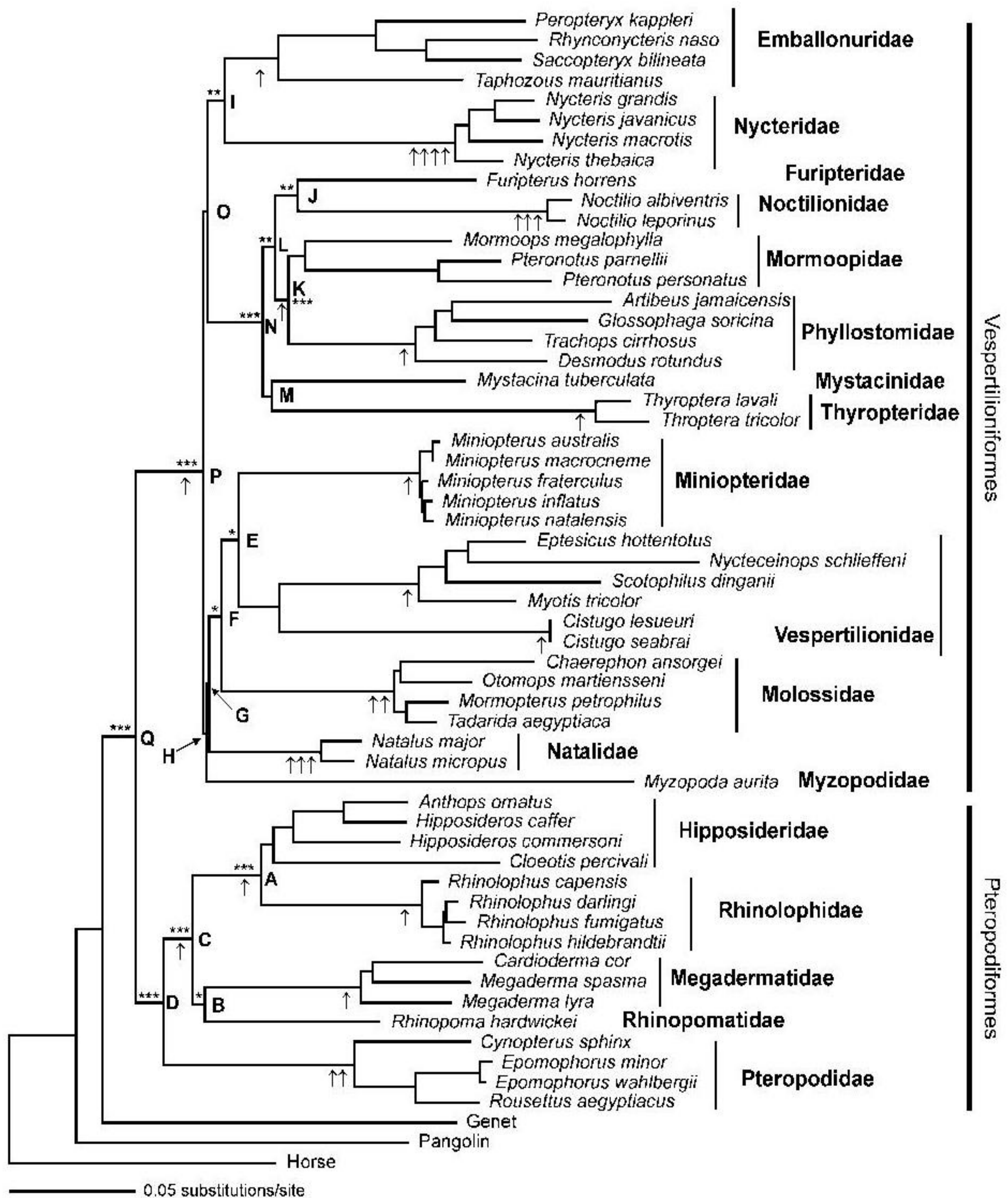

Figure 1. Maximum likelihood tree (Eick *et al.*, 2005) depicting the phylogenetic structure within the Chiroptera. Maximum parsimony, maximum likelihood and Bayesian inference analysis was performed using data from a nuclear intron supermatrix (genes: PRKC1, SPTBN, STAT5A and THY) (model: GTR+I+G). Bootstrap values ≥70% and posterior probabilities ≥0.95 are indicated by asterisks for presence in one (*), two (**) or all three (***) analysis methods.

Molossidae

The superfamily Vespertilionoidea (suborder Vespertilioniformes) comprises a number of families, including the Molossidae, Cistugidae, Vespertilionidae, Natalidae and Miniopteridae (Eick *et al*., 2005; Hutcheon and Kirsch, 2006; Lack *et al*., 2010). Members of the Molossidae are more commonly known as either free-tailed or mastiff bats and are widely distributed in tropical and subtropical regions of both the New and Old Worlds (Taylor, 2005; Jung *et al*., 2014). The family Molossidae Gervais, 1856 currently consists of 16 genera: *Chaerephon* Dobson, 1872; *Cheiromeles* Horsfield, 1824; *Cynomops* Thomas, 1920; *Eumops* Miller, 1906; *Molossops* Peters, 1865; *Molossus* Geoffroy, 1805; *Mops* Lesson, 1842; *Mormopterus* Peters, 1865; *Myopterus* Geoffroy, 1818; *Nyctinomops* Miller, 1902; *Otomops* Thomas, 1913; *Platymops* Thomas, 1906; *Promops* Gervais, 1855; *Sauromys* Robert, 1917; *Tadarida* Rafinesque, 1814 and *Tomopeas* Miller, 1900. The Molossidae are the fourth largest family of bats, comprising more than 100 species (Simmons, 2005).

Higher-level classifications within the Molossidae were traditionally based on morphological data, e.g. Freeman (1981) placed molossid genera into two major groups: (1) *Mormopterus*, *Myopterus*, *Cheiromeles* and *Molossops* and (2) *Tadarida*, *Chaerephon*, *Mops*, *Otomops*, *Nyctinomops*, *Promops*, *Molossus* and *Eumops,* using a multivariate approach, which included morphometrics. Legendre's (1984) use of dental morphology grouped the genera into three subfamilies: (1) Molossinae (*Molossus*, *Eumops*, *Molossops*, *Cynomops*, *Neoplatymops*, *Myopterus* and *Promops*), (2) Tadarinae (*Tadarida*, *Mormopterus*, *Nyctinomops*, *Otomops* and *Rhizomops*) and (3) Cheiromelinae (*Cheiromeles*). Gregorin and Cirranello (2015) used craniodental, external morphology, tongue and penis characteristics to separate the Molossidae into two clades: (1) *Mormopterus*, *Platymops*, *Sauromys*, *Neoplatymops*, *Molossops*, *Cynomops*, *Cheiromeles*, *Molossus* and *Promops* and (2) *Tadarida*, *Otomops*, *Nyctinomops*, *Eumops*, *Chaerephon* and *Mops*. Simmons (2005) combined morphological and molecular data, separating genera into two subfamilies: (1) Molossinae (*Chaerephon*, *Cheiromeles*, *Cynomops*, *Eumops*, *Molossops*, *Molossus*, *Mops*, *Mormopterus*, *Myopterus*, *Nyctinomops*, *Otomops*, *Platymops*, *Promops*, *Sauromys* and *Tadarida*) and (2) Tomopeatinae (*Tomopeas*). There has been a relative paucity of studies on the phylogeny of the Molossidae (Gregorin and Cirranello, 2015). Whereas Lamb *et al*. (2011) and Ammerman *et al*. (2012) used mitochondrial and nuclear sequence data in their phylogenetic reconstructions, Freeman (1981), Legendre (1984) and Gregorin and Cirranello (2015), reported morphologically-based structure.

Difficulty in obtaining samples of these high-flying bats, which inhabit often-inaccessible parts of the world has led to inconsistency in published phylogenies. For example, the genera *Chaerephon* and

Tadarida are not monophyletic, whereas *Chaerephon* and *Mops* appear to form a monophyletic clade; this has raised issues regarding the validity of these genera (Lamb *et al*., 2011; Ammerman *et al*., 2012; Gregorin and Cirranello, 2015). Members of the molossid genus *Otomops*, which are distributed within the Afrotropical region and Arabian Peninsula, are the focus of this study.

Otomops

Originally placed in the now defunct *Nyctinomus* genus, *N. martiensseni* Matschie (1897) and *N. wroughtoni* Thomas (1913) were described as a new genus, *Otomops*, based on morphological criteria (Chubb, 1917; Long, 1995; Peterson *et al*., 1995). The genus *Otomops* currently includes seven recognized species (Simmons, 2005) and has a wide distribution within the Old World, including the Afrotropical, Indo-Malayan, Australasian and Palaearctic regions (Freeman, 1981; Hutson *et al*., 2001). *Otomops formosus* Chasen, 1939 was first found in Tijibadak in West Java (Indonesia) and can be found throughout Java (Fig. 2). *Otomops johnstonei* Kitchener *et al.,* 1992 is also known from Indonesia (Desa Apui, Alor Island, Nusa Tenggara), with a predominant distribution on Alor Island (Fig. 2). *Otomops papuensis* Lawrence, 1948 and *O. secundus* Hayman, 1952 were both first reported from Papua New Guinea; *Otomops papauensis* inhabits SE New Guinea (Gulf Province) whereas *O. secundus* can be found in NE New Guinea (Madang Province) (Fig. 2). As of 2003, the IUCN had listed these four species as Vulnerable (facing a high risk of extinction in the wild in the medium-term future) (Hutson *et al*., 2001). *Otomops wroughtoni* (Thomas, 1913), the first *Otomops* species described, was found in Barapede Cave (near Talewadi, Kanara, Mysore) in southern India. Unlike other Asian *Otomops* species, *O. wroughtoni* has a fairly wide distribution, and is found in south and northeast India as well as in Cambodia (Fig. 3) (Thabah and Bates, 2002; Ruedi *et al*., 2014).

Figure 2. Distribution of Oriental *Otomops* species, taken from Kitchener *et al*. (1992), depicting *Otomops johnstonei* (■) – Indonesia, *O. formosus* (▼) – West Java, *O. papuensis* (●) – SE New Guinea and *O. secundus* (★) – NE New Guinea.

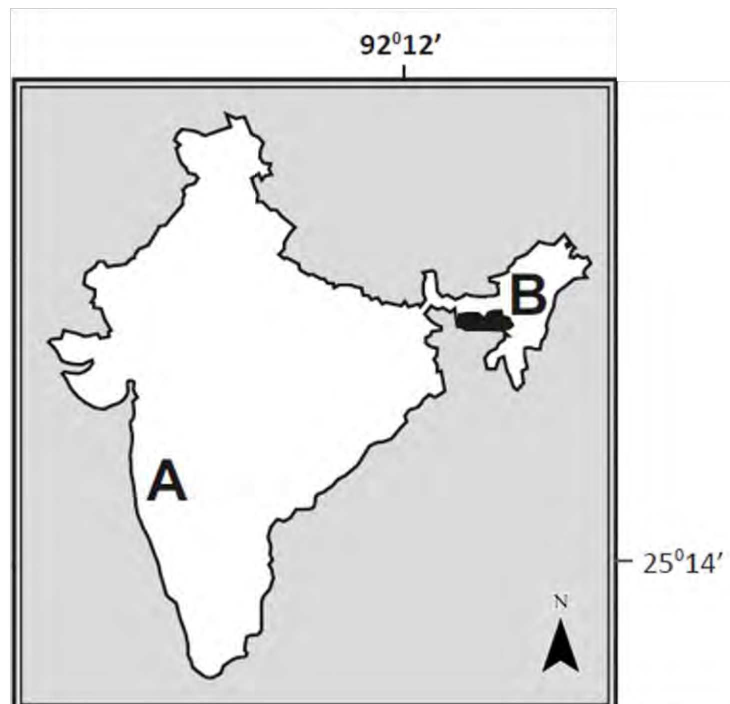

Figure 3. Distribution of *O. wroughtoni*, adapted from Ruedi *et al*. (2014), within Indian localities. A – Karnataka, Western Ghats, B – Meghalaya province.

The type locality of *Otomops martiensseni* (Matschie, 1897) is the Magrotto Plantation (SE Usambara Mountains, west of Tanga) in Tanzania. *Otomops martiensseni* is widely distributed, and has also been reported from Angola, Botswana, Burundi, Central African Republic, Democratic Republic of Congo, Dijibouti, Eritrea, Ethiopia, Ghana, Ivory Coast, Kenya, Malawi, Rwanda, South Africa, Uganda, Zambia and Zimbabwe from mainland Africa and Yemen from the Arabian Peninsula (Al-Jumaily, 1999; Simmons, 2005; Taylor, 2005; Adams *et al*., 2015). In the past, some authors classified *O. martiensseni* into three subspecies; *O. m. martiensseni*, *O. m. icarus* and *O. m. madagascariensis* (Long, 1995; Taylor, 2005). *Otomops icarus* Chubb (1917) was first reported from Durban, South Africa and has, over the years, been treated as a separate species (Chubb, 1917; Peterson *et al.,* 1995), a subspecies of *O. martiensseni* (Ellerman *et al*., 1953; Hayman and Hill, 1971; Ansell, 1974; Freeman, 1981), or a synonym of *O. martiensseni* (Harrison, 1957; Simmons, 2005; Mickleburgh *et al*., 2008). Most recent literature on *Otomops* refers to Simmons' (2005) classification, which recognises *O. icarus* as a synonym of *O. martiensseni* rather than a species or subspecies. *Otomops madagascariensis* Dorst, 1953 was first found in the Réserve Naturelle Intégrale de Namoroka (S of Soalala, Province de Mahajanga) in Madagascar. As with *O. icarus*, *O. madagascariensis* was initially classified as a subspecies of *O. martiensseni* (Freeman, 1981; Long, 1995; Al-Jumaily, 1999; Simmons, 2005) but was subsequently reclassified as a separate species (Peterson *et al*., 1995; Simmons, 2005). *Otomops madagascariensis* can be found in the northern (Province d'Antsiranana), southern (Province de Fianarantsoa) and western (Province de Mahajanga and Province de Toliara) parts of the island (Peterson *et al*., 1995; Goodman and Cardiff, 2004; Goodman *et al*., 2005). In recent years, *O. martiensseni* and *O. madagascariensis*, with type specimens classified via morphological characteristics, have been considered as the only two species within the Afrotropical region.

Historically, the taxonomy of *Otomops* was inferred through traditional taxonomic methods where separation of the species was based on size differences determined using multivariate analyses of craniodental and external characters, e.g. *O. madagascariensis* has a relatively smaller cranium and narrower, more elongate rostrum than *O. martiensseni* (Peterson *et al*., 1995; Richards *et al*., 2012). General characteristics, i.e. morphology, ontogeny, reproduction, distribution, ecology, behaviour and karyotype of *Otomops* (Afro-Malagasy and Oriental) have been described by a number of other authors, including Hill and Carter (1941), Harrison (1957, 1965), Verschuren (1957), Hayman and Hill (1971), Kingdon (1974), Ansell (1974, 1978), Freeman (1981), Crawford-Cabral (1986), Ansell and Dowset (1988), Ansell (1989), Monfort (1992), Long (1995), Peterson *et al*. (1995), Richardson and Taylor (1995), Decher *et al*. (1997), Grubb *et al*. (1998), Taylor (2000, 2005), Hutson *et al*. (2001), Fenton *et al*. (2002), Russ *et al*. (2003), Thabah and Bates (2002), Goodman and Cardiff (2004), Simmons (2005) and Prié (2011). *Otomops martiensseni* and *O. madagascariensis* have also been used in a number of additional (including comparative) studies on karyotypes (Đulic and Mutere,

1973; Warner *et al*., 1974; Rautenbach *et al*., 1993), haematology (Kinoti, 1973), reproduction (Mutere, 1973; Racey, 1982; McWilliam, 1987; Cumming and Bernard, 1997), parasites and diseases affecting bats (Tong *et al*., 2009; Laudisoit *et al*., 2012; Tao *et al*., 2013; Conrardy *et al*., 2014; Kobayashi *et al*., 2014; de Sales Lima *et al*., 2015), dentition, diet and eating habits (Freeman, 1979, 1984; Rydell and Yalden, 1997), roosting ecology (Kunz, 1982; Kofoky *et al*., 2007), cervical vertebrae (Fenton and Crerar, 1984), osmetrichia (Hickey and Fenton, 1987), cochlea structure (Pye, 1980), brain allometry (Hutcheon *et al*., 2002), wing and leg allometry (Norberg, 1981, 1987), morphology (Swartz *et al*., 2003; Ranivo and Goodman, 2007; Swartz and Middleton, 2008; Muldoon *et al*., 2009; Richards *et al*., 2012), echolocation (Fenton, 1982, 2003; Obrist *et al*., 1993; Russ *et al*., 2003; Fenton *et al*., 2002, 2004; Debaeremaeker and Fenton, 2003; Taylor *et al*., 2005; Veselka *et al*., 2010; Schoeman and Goodman, 2012) and conservation status (Hutson *et al*., 2001; Hutson, 2002; Singaravelan *et al*., 2009). Genetic diversity of *O. martiensseni*, *O. madagascariensis*, *O. wroughtoni* and *O. formosus* has been investigated by Lamb *et al*. (2006, 2008) and *Otomops* samples have also been included in phylogenetic studies of the Molossidae (Lamb *et al*., 2011; Ammerman *et al*., 2012; Gregorin and Cirranello, 2015) and between the various families within the Chiroptera (Jones *et al*., 2002; Agnarsson *et al*., 2011).

Otomops species are distinguished by their distinctively large, long ears and their characteristic fur. The ears are attached along the length of the head and fused on the extended snout; they range from ~30 – 40 mm in length (*O. martiensseni* ~40 mm, *O. madagascariensis*: 30 – 35 mm) (Kingdon, 1974; Long, 1995; Peterson *et al.,* 1995; Taylor, 2000, 2005; Russ *et al*., 2003). The fur is short and fine, brown in colour (dorsal pelage is darker than ventral) and has a thin pale band across the shoulders and a narrow white band from the shoulders to the knee (Harrison, 1957; Ansell, 1974; Long, 1995; Peterson *et al*., 1995; Russ *et al*., 2003; Taylor, 2005). Variations in pelage colouration are sometimes used for identification/differentiation between *Otomops* species. The presence of a gular gland, found on the lower throat, is characteristic of all male *Otomops* species, and is most notable in well-developed adults (Harrison, 1957; Hayman and Hill, 1971; Long, 1995; Russ *et al*., 2003; Taylor, 2005; Ruedi *et al*., 2014).

The body of *Otomops* individuals is more streamlined and slender than those of other Molossidae (Kingdon, 1974; Long, 1995). *Otomops martiensseni* is the largest member of the genus (Chubb, 1917; Ansell, 1974; Long, 1995; Rydell and Yalden, 1997; Al-Jumaily, 1999; Taylor, 2000, 2005; Fenton *et al*., 2002). Craniodental measurements of Afro-Malagasy *Otomops* provide evidence of sexual dimorphism, as females are larger than males (Richardson and Taylor, 1995; Richards *et al*., 2012). Norberg (1981, 1987) showed that the wings of *O. martiensseni* have a high aspect ratio (10.04) and a wingspan range from 450 – 480 mm, which allows for fast, high and powerful flight. Forearm length has been used as an identifying feature in *Otomops*; *O. martiensseni*, *O.*

madagascariensis and *O. wroughtoni* have longer forearms (~63 – 68 mm) than *O. formosus* (58 mm<, but <60 mm), *O. secundus* (54 mm< but <59 mm), *O. papuensis* (<54 mm) and *O. johnstonei* (<54 mm) (Freeman, 1981; Thabah and Bates, 2002; Long, 1995).

Studies on dentition, diet and eating habits of *Otomops* have also been conducted (Freeman, 1979, 1984; Rydell and Yalden, 1997) revealing a dental formula (I 1/2 OC 1/1 P 2/2 M 3/3), which is common among Molossidae (Long, 1995; Peterson *et al*., 1995; Al-Jumaily, 1999). *Otomops martiensseni* and *O. madagascariensis* appear to feed predominantly on soft-bodied insects such as Lepidoptera and some Coleoptera (Freeman, 1981, 1984; Long, 1995; Rydell and Yalden, 1997; Fenton *et al*., 2004; Andriafidison *et al*., 2007). Echolocation frequencies of *O. martiensseni* range between 10 – 17 kHz (human hearing range) and are a mixture of echolocation and colony-specific and/or individual-specific social calls (Fenton and Bell, 1981; Fenton *et al*., 2002; Fenton, 2003).

Otomops spp. live in various habitats, depending on structures available for roosting: *O. martiensseni* (northeast Africa), *O. madagascariensis* and *O. wroughtoni* can be found in cave structures, e.g. lava caves of Mount Suswa and Ithundu in Kenya (Mutere, 1973), limestone karst caves in Madagascar (Goodman *et al*., 2005) and caves near the Pynurkba, Umlatdoh and Thansah villages in northern India (Ruedi *et al*., 2014), respectively. By comparison *O. formosus*, *O. johnstonei* and *O. papuensis* were first discovered roosting in the hollows of trees (Kitchener *et al*., 1992; Bonaccorso and Hamilton, 2008; Hutson *et al*., 2008a, 2008b) and *O. martiensseni* (South Africa) is known for roosting in urban areas (Fenton *et al*., 2002). The specific roosting habits of *O. secundus* are not known at present, but individuals have been sighted in both forested and urban areas (Bonaccorso and Reardon, 2008). The number of individuals found in roosting sites also varies: *O. martiensseni* cave colonies can number several hundred (Mutere, 1973; Kingdon, 1974; Long, 1995; Kock *et al*., 2005), whereas roosts found in urban structures can contain 1 – 30 individuals (Fenton *et al*., 2002). Andriafidison *et al*. (2007) found up to 62 *O. madagascariensis* individuals roosting within the Anjohikinakina Cave (Province de Mahajanga) at one time. Ruedi *et al*. (2014) documented between 12 – 82 individuals in the caves housing *O. wroughtoni* and the Barapede caves are known to house between 40 – 100 individuals (Francis *et al*., 2008). Roost sizes of southeast Asian *Otomops* species (*O. formosus*, *O. johnstonei*, *O. papuensis* and *O. secundus*) are not well documented and, in some cases, comprise a single individual, as in the case of *O. wroughtoni* from Cambodia (Walston and Bates, 2001). It has also been suggested that *O. martiensseni* (especially within South Africa) displays a harem colony structure with male to female ratios ranging from 1:2 to 1:11 among some colonies (Fenton *et al*., 2002). Although such structures are easily observable in urban-dwelling *O. martiensseni*, determining the existence of a harem among cave-dwelling individuals can be difficult, as the aggregation of individuals in large numbers does not allow for the identification of distinct colonies and/or the association of a dominant male with females and sub-adults (Fenton *et al*., 2002;

Andriafidison *et al*., 2007). As a result of the difficulties associated with observation, roost structure in southeast Asian *Otomops* species is yet to be thoroughly researched and reported (Bonaccorso and Hamilton, 2008; Bonaccorso and Reardon, 2008; Hutson *et al*., 2008a, 2008b).

Lack of information on population numbers, as well as the range, threats and ecological requirements of oriental *Otomops* (*O. formosus*, *O. johnstonei*, *O. papuensis*, *O. secundus* and *O. wroughtoni*) has led to their listing as Data Deficient on the IUCN Red List of Threatened Species (Bonaccorso and Hamilton, 2008; Bonaccorso and Reardon, 2008; Francis *et al*., 2008; Hutson *et al*., 2008a, 2008b). *Otomops madagascariensis* is currently listed as Least Concern due to its relatively widespread distribution (it is believed to be present in more areas than are currently recorded) and because the rate of population decline is not sufficient to warrant a higher threat category listing (Andriafidison *et al*., 2008). *Otomops martiensseni* is listed as Near Threatened since there has been an increase in population numbers in southern Africa. However, the decrease in numbers, especially in east Africa, is significant (~30% over 10 years) and the lack of information regarding population numbers from central and west Africa all contribute to its Near Threatened status (Mickleburgh *et al*., 2008).

Studies using molecular methods to investigate the genetic diversity of *Otomops* have been relatively limited. Prior to results from this study, only Lamb *et al*. (2006) had published a study based on PCR-RAPDs and mitochondrial cytochrome *b* and D-loop sequence data, focussing specifically on the phylogeographic structure of *O. martiensseni* populations from South Africa and Kenya. Phylogenetic analysis showed the division of samples in two well-supported lineages from northeast Africa and South Africa (2.5% cytochrome *b* divergence). There was a significant gap in the literature on the genetics of *Otomops*. This study sought to fill this by carrying out analyses based on mitochondrial and nuclear DNA sequencing and microsatellites (SSRs) to investigate relationships within and among Afro-Malagasy *Otomops* at various taxonomic levels; these studies included phylogenetics, phylogeography, molecular dating, population genetics and the taxonomic classification of Afro-Malagasy *Otomops*. Based on findings by Lamb *et al*. (2006), we hypothesize that there may be more than one species level grouping of *Otomops* in mainland Africa. By examining the phylogenetics, phylogeography and population genetics of Afro-Malagasy *Otomops*, we hope to gain a better understanding of the relatedness, divergence and gene flow among various populations throughout the region. In addition to species-level investigation, we also examine the phylogenetic position of *Otomops* in relation to other molossid genera, since this family has remained relatively under-studied (Ammerman *et al*., 2012). Inconsistencies in the taxonomic classification within the Molossidae are evident in the literature (Freeman, 1981; Legendre, 1984; Simmons, 2005), which has traditionally been based on morphological data. Hence, there is a need for a study using molecular data to refine the morphology-based taxonomy. This would allow the dating of the origin of major lineages. Fenton *et al*. (2002) and Andriafidison *et al*. (2007) have carried out colony- and population-level observation

studies on Afro-Malagasy *Otomops*. We aim to complement this work by examining gene flow and relatedness within and among populations and colonies of *Otomops* individuals using microsatellite genotyping. Based on Fenton *et al*. (2002) and Andriafidison *et al*. (2007), we hypothesized that *Otomops* may exhibit a harem colony structure with females remaining faithful to the roost and genetic diversity maintained by male-mediated gene flow.

Molecular studies in Afro-Malagasy bats

The Afrotropical region mainly encompasses sub-Saharan Africa but extends to the southwestern region of the Arabian Peninsula in the north and also includes Madagascar and its neighbouring oceanic islands: Comoro Archipelago (Grand Comore, Mohéli, Anjouan and Mayotte), La Réunion and Mauritius of the Mascarene Islands and Aldabra in the western Seychelles (Fig. 4) (Olson *et al*., 2001; Hoffman *et al*., 2009).

Figure 4. Representation of the eight global biogeographical realms, including the Afrotropical region, taken from Olson *et al*. (2001).

This region has a relatively high diversity of bats (13 families; 70 genera), with the highest diversity occurring in southeastern Africa, Madagascar and the forests of West Africa, East African Arc and the East African coast (ACR, 2014). Despite this high diversity, very few of the extant bat species in this region has been studied in detail, which may in part be due to the challenges associated with sample collection given the difficulty in locating roosts and catching nocturnal, fast-flying animals (Hill *et al*.

2015). In addition, political instability within certain countries can also prove to be a hindrance to sample collection. A result of inadequate data in the region is that determination of the true number of families, genera and species numbers in the region has been underestimated (Hoffman *et al*., 2009; Goodman *et al*. 2012b). The addition of new specimen material and the use of new techniques have allowed researchers to re-evaluate existing classifications, with new bat species described from the Afrotropical region (Hoffman *et al*., 2009).

Historically, studies utilising molecular techniques to investigate Afro-Malagasy bats had been relatively limited, but has seen a steady increase in recent years (Table 1). These studies have focussed on investigating the phylogeny, biogeography, diversity, divergence and evolutionary history of species from various families. Dispersal, radiation and/or colonisation by various bat species has also been a topic of interest, e.g. results from Ruedi *et al*. (2012) estimated that Old World emballonurine lineages diverged ~30 MYA into two paraphyletic groups: one comprising Malagasy *Emballonura*, *Coleura* and *Mosia* and the other comprising *Emballonura* which radiated throughout the Indo-Pacific region through at least one long-distance dispersal event. The ability to resolve cryptic species, especially within the Miniopteridae, has also improved in recent years with the introduction of molecular data, e.g. Goodman *et al*. (2009b) used both molecular and morphological data to separate the *M. manavi* complex into three species: *M. manavi* s.s., *M. griveaudi* and *M. aelleni*. Although these species display much morphological similarity, they are genetically divergent, monophyletic and are able to be diagnosed on the basis of certain external and craniodental characters. New genus and species identification in the Afrotropical region has also seen an increase in recent years across the various families, e.g. Emballonuridae (Goodman *et al*., 2006, 2012a), Miniopteridae (Goodman *et al*., 2008a, 2011, 2015b; Monadjem *et al*., 2013a), Rhinolophidae (Taylor *et al*., 2012b) and Vespertilionidae (Goodman *et al*. 2012b, 2015a; Monadjem *et al*., 2013c). Excluding papers resulting from this study, most work on Afro-Malagasy molossids has been done on *Chaerephon* (Table 1). Although progress has been made in recent years, members of the Molossidae remain relatively understudied (Ammerman *et al*., 2012) despite their abundance.

Table 1. Molecular technique-based studies on bats from a variety of families, including Molossidae, from the Afrotropical region.

Family	Author(s)	Journal article title	Technique(s)
Emballonuridae	Goodman *et al*. (2006)	A new species of *Emballonura* (Chiroptera: Emballonuridae) from the dry regions of Madagascar.	Mitochondrial cytochrome *b* & D-loop sequencing; morphological comparison (cranial, dental, total length, tail length, hind foot length, ear length & forearm length measurements)
- " -	Goodman *et al*. (2012a)	Phylogeny of the Emballonurini (Emballonuridae) with descriptions of a new genus and species from Madagascar.	Mitochondrial cytochrome *b* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length, tragus & mass measurements); bioacoustic variable comparisons (PF, BW, Dur & IPI)
- " -	Ruedi *et al*. (2012)	Biogeography of Old World emballonurine bats (Chiroptera: Emballonuridae) inferred with mitochondrial and nuclear DNA.	Mitochondrial cytochrome *b* sequencing; nuclear RAG2 sequencing
Hipposideridae	Russell *et al*. (2008a)	Coalescent analyses support multiple mainland-to-island dispersals in the evolution of Malagasy *Triaenops* bats (Chiroptera: Hipposideridae).	Mitochondrial cytochrome *b* sequencing
- " -	Vallo *et al*. (2008)	Variation of mitochondrial DNA in the *Hipposideros caffer* complex (Chiroptera: Hipposideridae) and its taxonomic implications.	Mitochondrial cytochrome *b* sequencing

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Hipposideridae	Monadjem *et al*. (2013b)	Diversity of Hipposideridae in the Mount Nimba massif, West Africa, and the taxonomic status of *Hipposideros lamottei*.	Mitochondrial cytochrome *b* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & mass measurements); bioacoustic variable comparisons (F_{max})
Miniopteridae	Miller-Butterworth *et al*. (2005)	Genetic and phenotypic differences between South African long-fingered bats, with a global miniopterine phylogeny.	Mitochondrial cytochrome *b* sequencing; nuclear microsatellite genotyping; bioacoustic variable comparisons (PF, F_{max}, F_{min}, Dur, BW & IPI); morphological comparisons (mass & forearm length measurements), diet comparisons
- " -	Goodman *et al*. (2007)	Specific status of populations on Madagascar referred to *Miniopterus fraterculus* (Chiroptera: Vespertilionidae), with description of a new species.	Mitochondrial cytochrome *b* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & tragus measurements)
- " -	Juste *et al*. (2007)	Taxonomy of little bent-winged bats (*Miniopterus*, Miniopteridae) from the African islands of Sao Tome, Grand Comoro and Madagascar based on mtDNA.	Mitochondrial cytochrome *b* sequencing

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Miniopteridae	Goodman *et al*. (2008a)	A new species of *Miniopterus* (Chiroptera: Miniopteridae) from lowland southeatern Madagascar	Mitochondrial cytochrome *b* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & tragus measurements)
- " -	Weyeneth *et al*. (2008)	The biogeography of *Miniopterus* bats (Chiroptera: Miniopteridae) from the Comoro Archipelago inferred from mitochondrial DNA.	Mitochondrial cytochrome *b* & D-loop sequencing
- " -	Goodman *et al*. (2009a)	The use of molecular phylogenetic and morphological tools to identify cryptic and paraphyletic species: examples from the diminutive long-fingered bats (Chiroptera: Miniopteridae: *Miniopterus*) on Madagascar.	Mitochondrial cytochrome *b* sequencing; morphological comparison (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & tragus measurements)
- " -	Goodman *et al*. (2009b)	The use of molecular and morphological characters to resolve the taxonomic identity of cryptic species: the case of *Miniopterus manavi* (Chiroptera, Miniopteridae).	Mitochondrial cytochrome *b* sequencing; morphological comparison (cranial, dental, total length, tail length, hind foot length, ear length, forearm length, tragus & mass measurements)

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Miniopteridae	Goodman *et al*. (2011)	Morphological, bioacoustical, and genetic variation in *Miniopterus* bats from eastern Madagascar, with the description of a new species.	Mitochondrial cytochrome *b* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & tragus measurements); bioacoustic variable comparisons (PF, F_{max}, F_{min}, Dur & IPI)
- " -	Ramasindrazana *et al*. (2011)	Identification of cryptic species of *Miniopterus* bats (Chiroptera: Miniopteridae) from Madagascar and the Comoros using bioacoustics overlaid on molecular genetic and morphological characters.	Mitochondrial cytochrome *b* sequencing; morphological comparisons (forearm length measurements, pelage colouration & tragus shape); bioacoustic variable comparisons (PF, F_{max}, F_{min}, Dur & IPI)
- " -	Weyeneth *et al*. (2011a)	Wings or winds: inferring bat migration in a stepping-stone archipelago.	Mitochondrial D-loop sequencing; nuclear microsatellite genotyping
- " -	Wood *et al*. (2011)	Development and characterisation of 20 microsatellite loci isolated from the large bent-wing bat, *Miniopterus schreibersii* (Chiroptera: Miniopteridae) and their cross-taxa utility in the family Miniopteridae.	Nuclear microsatellite genotyping

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Miniopteridae	Monadjem *et al*. (2013a)	A cryptic new species of *Miniopterus* from south-eastern Africa based on molecular and morphological characters.	Mitochondrial cytochrome *b* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & mass measurements)
- " -	Christidis *et al*. (2014)	Insights into the evolution of a cryptic radiation of bat: dispersal and ecological radiation of Malagasy *Miniopterus* (Chiroptera: Miniopteridae).	Mitochondrial cytochrome *b* sequencing
- " -	Goodman *et al*. (2015b)	Description of a new species of the *Miniopterus aelleni* group (Chiroptera: Miniopteridae) from upland areas of central and northern Madagascar.	Mitochondrial cytochrome *b* sequencing; morphological comparison (cranial, dental, total length, tail length, hind foot length, ear length, forearm length, mass & tragus measurements and pelage colouration)
Molossidae	Jacobs *et al*. (2004)	Genetic similarity amongst phenotypically diverse little free-tailed bats, *Chaerephon pumilus*.	Mitochondrial cytochrome *b* sequencing
- " -	Lamb *et al*. (2006)	Phylogeography of southern and northeastern African populations of *Otomops martiensseni* (Chiroptera: Molossidae).	Mitochondrial cytochrome *b* & D-loop sequencing; PCR-RAPD band scoring

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Molossidae	Ratrimomanarivo *et al*. (2007)	Morphological and molecular assessment of the specific status of *Mops midas* (Chiroptera: Molossidae) from Madagascar and Africa.	Mitochondrial cytochrome *b* & D-loop sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, tibia length, forearm length & mass measurements)
- " -	Goodman *et al*. (2008b)	Specific status of populations in the Mascarene Islands referred to *Mormopterus acetabulosis* (Chiroptera: Molossidae), with description of a new species.	Mitochondrial control region sequencing; nuclear THY & *β-fib* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & mass measurements)
- " -	Ratrimomanarivo *et al*. (2008)	Morphological and molecular variation in *Mops leucostigma* (Chiroptera: Molossidae) of Madagascar and the Comoros: phylogeny, phylogeography, and geographic variation.	Mitochondrial cytochrome *b* & D-loop sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & mass measurements)
- " -	Ratrimomanarivo *et al*. (2009a)	Geographic and phylogeographic variation in *Chaerephon leucogaster* (Chiroptera: Molossidae) of Madagascar and the western Indian Ocean islands of Mayotte and Pemba.	Mitochondrial cytochrome *b* & D-loop sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & mass measurements)

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Molossidae	Ratrimomanarivo *et al*. (2009b)	Morphological and genetic variation in *Mormopterus jugularis* (Chiroptera: Molossidae) in different bioclimatic regions of Madagascar with natural history notes.	Mitochondrial cytochrome *b* & D-loop sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & mass measurements)
- " -	Taylor *et al*. (2009)	Cryptic lineages of little free-tailed bats, *Chaerephon pumilus* (Chiroptera: Molossidae) from southern Africa and the western Indian Ocean islands.	Mitochondrial cytochrome *b* & D-loop sequencing; morphological comparisons (cranial & dental measurements)
- " -	Goodman *et al*. (2010a)	Patterns of morphological and genetic variation in western Indian Ocean members of the *Chaerephon* '*pumilus*' complex (Chiroptera: Molossidae), with the description of a new species from Madagascar.	Mitochondrial cytochrome *b* & D-loop sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & mass measurements)
- " -	Taylor *et al*. (2012a)	Wing loading correlates negatively with genetic structuring of eight Afro-Malagasy bat species (Molossidae).	Mitochondrial cytochrome *b* sequencing; nuclear RAG2 sequencing; morphological comparisons (body size & wing – WL & AR)
- " -	Naidoo *et al*. (2013a)	Cross-genus amplification and characterisation of microsatellite loci in the little free tailed bat, *Chaerephon pumilus* s.l. (Molossidae) from south eastern Africa.	Nuclear microsatellite genotyping

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Molossidae	Naidoo *et al*. (2013b)	Stable Pleistocene-era populations of *Chaerephon pumilus* (Chiroptera: Molossidae) in southeastern Africa do no use different echolocation calls.	Mitochondrial cytochrome *b* & control region sequencing; morphological comparisons (mass & forearm length); bioacoustic variable comparisons (PF, F_{max}, F_{min}, Dur & BW)
Myzopodidae	Russell *et al*. (2008b)	Population genetic analysis of *Myzopoda* (Chiroptera: Myzopodidae) in Madagascar.	Mitochondrial cytochrome *b* & D-loop sequencing
Pteropodidae	O'Brien *et al*. (2009)	Multiple colonisations of the western Indian Ocean by *Pteropus* fruit bats (Megachiroptera: Pteropodidae): the furthest islands were colonised first.	Mitochondrial cytochrome *b*, 12S rRNA & control region sequencing
- " -	Goodman *et al*. (2010b)	Phylogeny and biogeography of western Indian Ocean *Rousettus* (Chiroptera: Pteropodidae).	Mitochondrial cytochrome *b* sequencing; nuclear microsatellite genotyping
- " -	Chan *et al*. (2011)	Increased population sampling confirms low genetic divergence among *Pteropus* (Chiroptera: Pteropodidae) fruit bats of Madagascar and other western Indian Ocean islands.	Mitochondrial cytochrome *b* sequencing
- " -	Shi *et al*. (2014)	A deep divergence time between sister species of *Eidolon* (Pteropodidae) with evidence for widespread panmixia.	Mitochondrial cytochrome *b* sequencing; nuclear *β-fib*, RAG1 & RAG2 sequencing

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Rhinolophidae	Stoffberg *et al*. (2010)	Molecular phylogenetics and historical biogeography of *Rhinolophus* bats.	Mitochondrial cytochrome *b* sequencing; nuclear TG, THY & PRKC1 intron sequencing
- " -	Stoffberg *et al*. (2012)	Correlated genetic and ecological diversification in widespread southern African horseshoe bat.	Mitochondrial control region sequencing; morphological comparisons (wing – WL & AR); bioacoustic variable comparisons (RF)
- " -	Taylor *et al*. (2012b)	Four new bat species (*Rhinolophus hildebrandtii* complex) reflect Plio-Pleistocene divergence of dwarfs and giants across an Afromontane archipelago.	Mitochondrial cytochrome *b*, 12s rRNA & control region sequencing; nuclear Chd1 intron sequencing; morphological comparison (cranial, dental, noseleaf & baculum measurements)
- " -	Jacobs *et al*. (2013)	Phenotypic convergence in genetically distinct lineages of a *Rhinolophus* species complex (Mammalia, Chiroptera).	Mitochondrial cytochrome *b* sequencing; nuclear THY sequencing; morphological comparisons (cranial, tail length, ear length, tibia length, forearm length, noseleaf & baculum measurements); bioacoustic variable comparisons (RF)
Vespertilionidae	Stadelmann *et al*. (2004)	Phylogeny of African *Myotis* bats (Chiroptera: Vespertilionidae) inferred from cytochrome *b* sequences.	Mitochondrial cytochrome *b* sequencing

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Vespertilionidae	Trujillo *et al*. (2009)	Molecular phylogenetics of the bat genus *Scotophilus* (Chiroptera: Vespertilionidae): perspectives from paternally and maternally inherited genomes.	Mitochondrial cytochrome *b* sequencing; nuclear *zfy* sequencing
- “ -	Weyeneth *et al*. (2011b)	Do diversification models of Madagascar’s biota explain the population structure of the endemic bat *Myotis goudoti* (Chiroptera: Vespertilionidae)?	Mitochondrial cytochrome *b* & D-loop sequencing
- “ -	Goodman *et al*. (2012b)	The genus *Neoromicia* (Family Vespertilionidae) in Madagascar with the description of a new species.	Mitochondrial 12S rRNA & 16S rRNA sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length, tragus & baculum measurements)
- “ -	Koubínová *et al*. (2013)	Hidden diversity in Senegalese bats and associated findings in the systematics of the family Vespertilionidae.	Mitochondrial cytochrome *b*, $tRNA^{Thr}$, 12S rRNA, $tRNA^{Val}$, 16S rRNA, ND1 sequencing; nuclear RAG1 & RAG2 sequencing; chromosome comparisons
- “ -	Monadjem *et al*. (2013c)	High diversity of pipistrelloid bats (Vespertilionidae: *Hypsugo*, *Neoromicia*, and *Pipistrellus*) in a West African rainforest with the description of a new species.	Mitochondrial *COI* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length & baculum measurements)

Table 1 continued.

Family	Author(s)	Journal article title	Technique(s)
Vespertilionidae	Vallo *et al*. (2013)	Conflicting mitochondrial and nuclear paraphyly in small-sized West African house bats (Vespertilionidae).	Mitochondrial cytochrome *b* sequencing; nuclear *zfy* & *fgb7* sequencing; morphological comparison (cranial, dental & forearm length measurements)
- " -	Goodman *et al*. (2015a)	An integrative approach to characterize Malagasy bats of the subfamily Vespertilioninae Gray, 1821, with the description of a new species of *Hypsugo*.	Mitochondrial cytochrome *b* sequencing; morphological comparisons (cranial, dental, total length, tail length, hind foot length, ear length, forearm length, tragus, mass & baculum measurements); bioacoustic variable comparisons (PF, F_{max}, F_{min}, Dur & IPI)
- " -	Vallo *et al*. (2015)	Phylogeographic position of the giant house bat *Scotophilus nigrita* (Chiroptera, Vespertilionidae).	Mitochondrial cytochrome *b* sequencing; nuclear *zfy* sequencing

AR – aspect ratio, *β-fib* / *fgb7* – 7th intron of the fibrinogen gene, BW – bandwidth, Chd1 intron – nucleosome remodelling factor, *COI* – cytochrome oxidase subunit I, Dur – duration, F_{max} – frequency (maximum), F_{min} – frequency (minimum), IPI – inter-pulse interval, ND1 – NADH dehydrogenase subunit 1, PF – peak frequency, PRCK1 – protein-kinase C1, RAG – recombination activating gene, RF – resistance frequency, TG – thyroglobulin, THY – thyrotrophin, WL – wing loading, *zfy* – zinc finger protein on the Y chromosome.

Rationale and scope of study

Although *Otomops* species have been included in family-level, e.g. Lamb *et al*. (2011) and Ammerman *et al*. (2012), and order-level, e.g. Jones *et al*., (2002) and Agnarrson *et al*. (2011), studies, molecular-based investigations into Afro-Malagasy *Otomops* have been limited. Prior to findings from this study, molecular investigation had only been conducted by Lamb *et al*. (2006), making *Otomops* the least studied of the Afro-Malagasy molossids (Table 1). Additionally, the 2006 study focussed on African *Otomops* and did not include Malagasy *Otomops* samples and may thus be considered an incomplete assessment of the taxonomic status and relationships between *Otomops* species in the Afrotropical region. This is especially relevant considering that *Otomops* is capable of long distance flight, which may enable interaction and possible gene flow between geographically distant areas. *Otomops* is renowned for being difficult to capture and/or to observe in its natural habitat due to its fast and high-flying ability, roost type and nocturnal lifestyle (Fenton *et al*., 2002). This has led to a relative paucity of data, resulting in inconsistencies in *Otomops* taxonomy. Based on an increased sample, we sought to investigate and make inferences relating to the phylogenetic position of *Otomops* within the Molossidae, its evolutionary history, phylogenetic / phylogeographic structure, kinship associations, behavioural patterns (migration/dispersal), and social structure (the possible existence a harem structure and philopatry). By using multiple markers from both mitochondrial and nuclear genomes, and distribution data we sought to reassess the number of species of Afro-Malagasy *Otomops*.

Chapter-specific aims

This thesis is divided into a number of chapters, following this introductory chapter and organised as follows:

<u>Genetic diversity and number of *Otomops* operational taxonomic units (OTUs) within the Afrotropical region (Chapter 2)</u>

Chapter 2, entitled "Analysis of the genetic diversity of *Otomops* (Chiroptera: Molossidae) in Africa and Madagascar" comprises my Master of Science (Biology) dissertation, which was written, examined and passed in 2008. This degree was banked rather than conferred, an option offered to students at the University of KwaZulu-Natal. This option allows students to convert the degree of MSc to PhD through the inclusion of additional work. The MSc dissertation is in its original 2008 form, and literature cited was therefore published prior to

2008. Chapter 2 investigates the genetic diversity of *Otomops* spp. from Africa and the Arabian Peninsula and Madagascar, assessed using sequence data from two mitochondrial molecular markers: the slower-evolving cytochrome *b* gene and the faster-evolving D-loop region. Phylogenetic patterns are inferred using both phenetic and cladistic methods and the phylogeographic relationships/distribution among haplotypes also assessed.

Taxonomic association and evolutionary history between members of the Molossidae family from the Afrotropical region (Chapter 3)

Chapter 3, presented in thesis format, examines the genetic structure of various genera within the Molossidae, based on available representative, and the relative position of *Otomops* within this family. Although the Molossidae is the 4th largest family of bats, investigation into the higher-level associations among genera within the family is still lacking. Historically, classification within Chiropteran families was determined using morphological data. Stanley (2008), Gonzalez-Ruiz *et al*. (2010) and Goodman *et al*. (2010) reported on the phylogenetics of species of *Mops*, *Molossus* and *Chaerephon*, respectively. The aim of this chapter was to use a DNA sequence based approach to test the validity of the associations proposed by Freeman (1981), Legendre (1984) and Simmons (2005) and reassess phylogenetic associations among molossid genera, including *Otomops*. Concatenated sequence data from 2 markers: the nuclear Recombination Activating Gene 2 (RAG2) and mitochondrial cytochrome *b* gene are used to assess molossid genetic diversity and infer the position of *Otomops* therein by analysing the phenetic and phylogenetic relationships among the representative samples of *Otomops*, *Chaerephon*, *Mops*, *Mormopterus*, *Sauromys* and *Tadarida*. Additionally, the evolutionary history of the family is investigated by dating the divergence of the major, supported clades.

Adaptation and assessment of microsatellite markers for use in Afro-Malagasy *Otomops* species (Chapter 4)

Chapter 4, presented in published paper format, is a method-based chapter describing the development and testing of *Otomops*-specific microsatellite protocols adapted from *Tadarida brasiliensis*-specific microsatellite primers (Russell *et al*., 2005). Microsatellites (short tandem repeats), which display high levels of polymorphism, are useful for estimating parameters such as gene flow, inbreeding, migration rates, population size and kinship (Selkoe and Toonen, 2006) making them a popular choice of marker in recent years for population genetic studies of various organisms, including bats. Development of novel,

species-specific microsatellite markers is a relatively expensive and time-consuming process (Zane *et al*., 2002; Abdelkrim *et al*., 2009) whereas testing of microsatellite primers which have already been developed for organisms in the same genus or family is cost-effective (Barbará *et al*., 2007). Nine markers developed by Russell *et al*. (2005) for American *T. brasiliensis* (family: Molossidae) were tested on Afro-Malagasy *Otomops* species (family: Molossidae), and recommendations regarding their suitability for population-level analysis of *O. martiensseni*, *O. harrisoni* and *O. madagascariensis* are made on the basis of these characteristics. Studies on genetic variation, gene flow and kinship may provide insights that can contribute to the understanding of the behaviour, including roosting ecology, of Afro-Malagasy *Otomops*.

Interaction and association between and within Afro-Malagasy *Otomops* at regional (population) and local (colony) level with inferences regarding social structure (Chapter 5)

Chapter 5 has been prepared for submission to a peer-reviewed journal but is presented here in traditional chapter format and reports on the use of microsatellites to analyse the population genetic structure of *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. Preliminary work carried out in Chapter 4 identifies suitable nuclear microsatellite primers for amplification and genotyping: TabrA10, TabrA30, TabrD10, TabrD15, TabrH6 and TabrH12. Based on analyses of allele distribution among these loci, inferences are made regarding migration and gene flow at species and colony level, as well as parentage, kinship and philopatry. Results from this study are novel contributions to the body of work on the *Otomops*. Mitochondrial cytochrome *b* and D-loop data are also used to create a statistical parsimony network to enable comparison of genetic structure revealed by nuclear vs. mitochondrial markers.

Species-level conflict within Afro-Malagasy *Otomops*, including revision of the number of species within the region (Chapter 6)

Chapter 6, presented in paper format, reassesses the taxonomic status of *Otomops* from the Afrotropical region, and provides molecular (mitochondrial and nuclear sequence), morphological and ecological evidence for the recognition of a new species from northeast Africa and the Arabian Peninsula, namely *O. harrisoni*. Craniodental measurements are used to explore morphological variation and shape changes among lineages. Ecological niche modelling is used to predict the distribution of *Otomops* specimens from northeast Africa and from southern and central Africa. Based on findings from these various analyses, description

of a new species, *O. harrisoni*, is presented, including details on the new holotype (including paratypes and referred specimens), type locality, paratypes, referred specimens, etymology, diagnosis and systematic description and comparisons, including external, cranio-dental and molecular characteristics, biology, distribution and conservation status. Identification of a new species has implications for conservation and will require the reassessment of current species action plans and recommendations for future action.

General aims and objectives

The aim of this study was to investigate the genetic diversity in Afro-Malagasy *Otomops* at a variety of scales: genus, species, population, colony and individual. Mitochondrial and nuclear gene sequencing and microsatellite genotyping were used to:

1. Investigate genetic diversity to resolve the potential number of *Otomops* species within the Afrotropical region (Chapter 2) by analysing phylogenetic relationships, phylogeographic patterns, and population expansion/stasis.
2. Make conservation recommendations on the basis of the findings in objective 1 (Chapter 2).
3. Investigate genetic structure among various genera within the Molossidae, including the relative position of *Otomops* in the family (Chapter 3).
4. Test the validity of the family structure proposed by Freeman (1981), Legendre (1984) and Simmons (2005) for the Molossidae using molecular methods (Chapter 3).
5. Reassess phylogenetic positions and higher-level relationships among molossids based on the findings in objective 3 (Chapter 3).
6. Investigate the evolutionary history of molossid genera, including divergence dating (Chapter 3).
7. Identify polymorphic *Otomops*-specific microsatellite primers and protocols for population genetic analysis (Chapter 4).
8. Report on the population genetics of Afro-Malagasy *Otomops* at inter- and intra-specific levels by examining gene flow (Chapter 5) to:
    a. determine relatedness, migration and dispersal patterns;
    b. establish kinship associations, including parent-offspring and sibships;
    c. make inferences regarding *Otomops*' roosting behaviour within the various habitats, including the existence of a harem roost structure and philopatry.
9. Reassess the taxonomic status of Afro-Malagasy *Otomops* species on the basis of molecular, morphometric and ecological data (Chapter 6).

10. Describe a new *Otomops* species from northeast Africa and the Arabian Peninsula (Chapter 6).

Arrangement and style of thesis

Chapters 2 and 3 in this thesis have been presented in traditional chapter format according to the style that has been outlined and required by the College of Agriculture, Engineering and Science at the University of KwaZulu-Natal. Chapter 2 comprises the original MSc dissertation on which the conversion to PhD was based. As such, this chapter must remain in its original format and will therefore reflect findings and facts based on literature published prior to 2008. Results from Chapters 2 and 3 have subsequently contributed to articles published in peer-reviewed journals: Lamb *et al*. (2008) and Lamb *et al*. (2011). Chapters 4, and 6 presented in this thesis have been published in peer-reviewed journals and have thus been prepared and presented according to the format of the relevant journal. Chapter 5 has been prepared as a paper, which is to be submitted to a peer-reviewed journal, however for the purposes of this thesis this paper will be presented in traditional chapter format. Consequently, formatting across the chapters varies and has also resulted in some repetitive text, as is unfortunately inevitable given the thesis format stipulated by the University of KwaZulu-Natal. Each chapter comprises an introduction to the topic and issue(s) being addressed, a description of the sample materials, laboratory techniques and methods of analyses used, a description of the results, a discussion on the findings, a reference list and an appendix (where applicable). Figure and table labels correspond to the individual chapters in which they appear and are not labelled in continuity throughout the thesis. Pages are sequentially numbered and an appendix is provided containing declared publications.

Chapter 1 – General introduction

Chapter 2 – *Otomops* species-level investigations

Analysis of the genetic diversity of *Otomops* (Chiroptera: Molossidae) from Africa and Madagascar.

Chapter 3 – Molossid genus-level investigations

Investigation into the molecular phylogeny of Molossidae (Chiroptera) from Africa and Madagascar.

Chapter 4 – *Otomops*-specific technique development

Ralph, T.M.C. and Lamb, J.M. (2013). Cross-genus amplification and characterisation of microsatellite loci in the large-eared free tailed bat, *Otomops* (Chiroptera: Molossidae) from Africa and Madagascar. *African Journal of Biotechnology* **12**: 4233-4237.

Chapter 5 – *Otomops* population- and colony-level investigations

Examination of the population genetics of Afro-Malagasy *Otomops* (Chiroptera: Molossidae) via microsatellite analysis. (in preparation).

Chapter 6 – *Otomops* species-level investigations and description of a new *Otomops* species

Ralph, T.M.C., Richards, L.R., Taylor, P.J., Napier, M.C. and Lamb, J.M. (2015). Revision of Afro-Malagasy *Otomops* (Chiroptera: Molossidae) with the description of a new Afro-Arabian species. *Zootaxa* **4057**: 1-49.

Chapter 7 – Summary and general conclusions

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CHAPTER TWO:

Analysis of the Genetic Diversity of *Otomops* (Chiroptera: Molossidae) from Africa and Madagascar

ABSTRACT

Otomops, or the large-eared, free-tailed bat, is part of the *Molossidae* family. There are two species in the Afrotropical/Malagasy region, namely *O. martiensseni*, from mainland Africa, and *O. madagascariensis*, from Madagascar. *Otomops icarus* Chubb (1917) from Durban, South Africa is sometimes considered to be a synonym of *O. martiensseni*, or a separate species (Freeman, 1981; Meester *et al*., 1986; Al-Jumaily, 1999). This study was carried out to provide genetic information which might assist in determining whether two species (*O. martiensseni* and *O. madagascariensis*) or three species (*O. martiensseni*, *O. madagascariensis* and *O. icarus*) exist within the Afro-Malagasy region.

DNA sequencing of the mitochondrial cytochrome *b* and D-loop regions was used to estimate the genetic diversity of a sample of 61 *Otomops* individuals derived from populations found in Africa, Madagascar and the Arabian Peninsula. Neighbour-joining and Bayesian analyses suggest the existence of two reciprocally-monophyletic *Otomops* clusters/clades (Madagascar and Africa), with the African group further divided into two reciprocally-monophyletic clusters/clades: the north/east Africa (NEA) clade which comprises samples from Ethiopia, Kenya and Yemen, and the south/west Africa (SWA) clade which comprises samples from South Africa, Ivory Coast, Zimbabwe, Tanzania and Burundi.

The clade from Madagascar is recognized as *O. madagascariensis*. However, NEA and SWA lineages might be better classified as major clades or a subspecies, since divergence levels between these groups are low (cytochrome *b*, 2.1%), relative to the Madagascar versus NEA (cytochrome *b*, 3.5%) and SWA divergences (cytochrome *b*, 3.1%). Genetic divergence values between the Ivory Coast sample and the rest of the SWA species group (cytochrome *b*, 2.1%; s.d.: 0.004) is equivalent to the divergence observed between the NEA and SWA groups (cytochrome *b*, 2.1%; s.d.: 0.005); therefore Ivory Coast *Otomops* could be a speciating population in this region and might be regarded as a potential MU or ESU. Phylogenies obtained from analysis of the D-loop are congruent with those from the cytochrome *b* region. Generally, for both cytochrome *b* and D-loop data, haplotype diversity (h) is high whilst nucleotide diversity (π) is low. Values obtained for the three geographically and genetically defined groups are (1) Cytochrome *b*, SWA (h: 0.9900; π: 0.0082), NEA (h: 0.8760; π: 0.0036), Madagascar (h: 0.9450; π: 0.0072) and (2) D-loop, SWA (h: 0.9270; π: 0.0337), NEA (h: 0.9520; π: 0.0302) Madagascar (h: 0.9680; π: 0.0196).

The Bayesian cytochrome *b* tree suggests that the Asian species are ancestral to the Afro-Malagasy ones, which is consistent with Asia being the centre of diversity of *Otomops*. The cytochrome *b* haplotype network suggests that modern-day Afro-Malagasy species dispersed simultaneously from Asia to Madagascar and north/east Africa, followed by a southward dispersal to South Africa and western Africa. Population genetic analysis of D-loop data suggests that Madagascar contains a population that has been expanding for the last 27 388 to 52 242 years. AMOVA indicates that most variance occurs between geographically defined species-groups (80%). There appears to be little congruence between temperature, precipitation and haplotype network structure, except that the NEA clade does appear restricted to higher altitudes and drier climates. Lack of a relationship between sample localities and haplotype network structure indicates that *Otomops* does not show strict colony faithfulness and does not form genetically-stable harems.

1. INTRODUCTION

1.1 Otomops *species background*

1.1.1 Current taxonomy

The name *Otomops* is derived from the Greek word *oto* meaning long or large ear, and *mops* which is Malaysian for bat (Long, 1995). The genus *Otomops* is part of the family Molossidae which is part of the suborder Vespertilioniformes (Koopman and Cockrum, 1967; Koopman, 1984; Mindell *et al*., 1991; Nowak, 1997; Hutcheon *et al*., 1998; Taylor, 2005; Teeling *et al*., 2005; Simmons 2005; Hutcheon and Kirsch, 2006). There are currently seven recognized species within the genus *Otomops* (Simmons, 2005). There are also fourteen other genera found in this family including *Chaerephon*, *Cheiromeles*, *Cynomops*, *Eumops*, *Molossops, Molossus*, *Mops*, *Mormopterus*, *Myopterus*, *Nyctinomops*, *Platymops*, *Promops*, *Sauromys* and *Tadarida* (Simmons, 2005).

Nyctinomus was a genus, belonging to the family Molossidae, that is no longer recognized. Some species of the genus, now known as *Otomops*, were originally placed in *Nyctinomus*, i.e. *N. martiensseni* Matschie (1897) and *N. wroughtoni* Thomas (1913) (Chubb, 1917; Long, 1995; Peterson *et al*., 1995). These species exhibited some morphological differences from other species in the genus *Nyctinomus*, and therefore it was decided that a new genus should be formed for these species (Chubb, 1917). *Otomops*, as a genus, was thought to be divided into seven species, one of which comprised three subspecies (Hayman and Hill, 1971; Freeman, 1981; Nowak, 1997; Al-Jumaily, 1999). These species include *O. martiensseni* (Matschie, 1897) from mainland Africa and Yemen, *O. wroughtoni* (Thomas, 1913) from southern India, *O. formosus* Chasen, 1939 from Java, *O. papuensis* Lawrence, 1948 from Papua New Guinea, *O. secundus* Hayman, 1952 from New Guinea, *O. madagascariensis* Dorst, 1953 from Madagascar and *O. johnstonei* Kitchener *et al.,* 1992 from Indonesia (Walker *et al*., 1975; Freeman, 1981; Peterson *et al*., 1995; Nowak, 1997; Al-Jumaily, 1999; Hutson *et al*., 2001, Simmons 2005). *Otomops martiensseni* (large-eared giant mastiff bat) and *O. madagascariensis* (Malagasy giant mastiff bat) are the subject of this investigation due to the relative availability of samples and the need for a more thorough investigation into the variety and taxonomy of the species in these regions (Richardson and Taylor, 1995; Nowak, 1997; Simmons, 2005; Taylor, 2005). In addition, other members of the genus are difficult to obtain due to the rarity of the species.

In recent years, there has been much controversy concerning the number of *Otomops* species, and possible subspecies, said to exist within Africa and Madagascar. *Otomops m. martiensseni* from eastern Africa and *O. m. madagascariensis* from Madagascar are considered by most authorities to be species (as stated above) and not subspecies (as implied by the taxonomic name) (Harrison, 1957; Freeman, 1981; Meester *et al*., 1986; Ansell, 1989; Peterson *et al.,* 1995; Al-Jumaily, 1999; Taylor, 2005). *Otomops m. icarus* Chubb (1917) from Durban, South Africa is sometimes considered to be a synonym of *O. m. martiensseni*, or a separate species, i.e. *O. icarus*, as suggested by Chubb, 1917 (Ansell, 1974; Freeman, 1981; Meester *et al*., 1986; Gelderblom *et al*., 1995; Al-Jumaily, 1999). It has been generally accepted, however, that *O. martiensseni* and *O. madagascarensis* can only be found within the confines of the African continent, the Arabian Peninsula (Yemen) and Madagascar (Kingdon, 1974, Long, 1995; Richardson and Taylor, 1995; Nowak, 1997; Peterson *et al.,* 1995; Al-Jumaily, 1999; Fenton *et al*., 2002).

1.1.2 Previous studies

Historically, most conclusions about the taxonomy of *Otomops* were deduced through traditional taxonomic methods. Morphological investigations involved examination of features such as head, body and forearm length, mass, cranial measurement, dentition, fur colour, distinctive markings or morphology and histology to name but a few. A comprehensive summary of previous studies of the general characteristics of *O. martiensseni* individuals, including morphology, ontogeny and reproduction, distribution, ecology and behaviour and genetics (in the form of karyotyping), is given by Long (1995). The general morphology of type specimens was first investigated by naming authorities and/or in first records. The type specimen of *O. martiensseni*, described by Matschie (1897), was first found in Tanzania (W of Tanga, SE Usambara Mtns, Magrotto Plantation) and is currently housed in the Berlin Museum (catalogue number unknown). *Otomops icarus*, described by Chubb (1917), was first found in Durban, South Africa, and is housed in the British Museum of Natural History (no. BM 16.10.9.1). *Otomops madagascariensis*, described by Dorst (1953), was first found in Madagascar (S of Soalala, Namoroka, Réserve naturelle intégrale no. 8), and is housed in the Muséum National d'Histoire Naturelle, Paris (no. CG 1953-1). Separation of the species was initially based on the criterion of size differentiation. From these observations, it appeared that individuals of *O. madagascariensis* have a relatively smaller cranium and narrower, more elongate rostrum than those of *O. martiensseni* (Peterson *et al*., 1995). Other accounts, general descriptions (anatomical notes) and comparisons between species and/or genera have also been made by Hill and Carter (1941), Harrison (1957, 1965), Verschuren (1957), Ansell (1974, 1978), Crawford-Cabral (1986), Ansell and

Dowset (1988), Monfort (1992), Richardson and Taylor (1995), Decher *et al*., (1997), Grubb *et al*. (1998), Fenton *et al*. (2002), Thabah and Bates (2002) and Goodman and Cardiff (2004) and in a number of textbooks and field guides including Koopman and Cockrum (1967), Hayman and Hill (1971), Kingdon (1974), Walker *et al*. (1975), Meester (1976), Lawlor (1979), Freeman (1981), Nowak and Paradiso (1983), Koopman (1984), Hill and Smith (1985), Corbet and Hill (1986), Meester *et al*. (1986); Smithers (1986), Stuart and Stuart (1988), Ansell (1989), Peterson *et al*. (1995), Nowak (1997), Garbutt (1999), Taylor (2000b, 2005), Hutson *et al*. (2001), Russ *et al*. (2001) and Simmons (2005).

Other features of *Otomops* have also been investigated, in either an individual or a comparative context. Some topics of study include karyotypes (Đulic and Mutere, 1973; Warner *et al*., 1974; Rautenbach *et al*., 1993), haematology (Kinoti, 1973), reproduction (Mutere, 1973; Racey, 1982; McWilliam, 1987; Cumming and Bernard, 1997), dentition, diet and eating habits (Freeman, 1979, 1984; Rydell and Yalden, 1997). Others include roosting ecology (Kunz, 1982), cervical vertebrae in relation to roosting posture (Fenton and Crerar, 1984), osmetrichia (scent-dispersing hairs) (Hickey and Fenton, 1987), cochlea structure (Pye, 1980), brain allometry (Hutcheon *et al*., 2002) and wing and leg allometry (Norberg, 1981, 1987). In addition, studies on echolocation of *O. martiensseni* (Fenton, 1982, 2003; Obrist *et al*., 1993; Russ *et al*., 2001; Fenton *et al*., 2002, 2004; Debaeremaeker and Fenton, 2003), genetic diversity (Lamb *et al*., 2006, 2008) and conservation status (Hutson *et al*., 2001; Hutson, 2002) have also gained interest in recent years.

Past taxonomic work is partially based on morphological and ecological data, therefore subjectivity may become an influencing factor since this work is partially based on observation, e.g. pelage colour and banding patterns in karyotype studies, however, one cannot simply rule out the inclusion of morphological and ecological data (Kiefer *et al*., 2002). Possible subjectivity in the collection of data may result in different outcomes regarding species and subspecies naming within the *Otomops* genus and hence, may account for the discrepancies seen in the literature and the continued debates thereafter e.g. classification of *O. icarus* as a species by Chubb (1917) based on pelage colour was questioned by Harrison (1957) who determined, after examining the pelage colour of three *O. icarus* specimens, that “the colour characters of *O. icarus* are variable and can only be regarded as of subspecific status”.

1.1.3 General morphology

The most distinctive feature of *Otomops*, as the common name would suggest, is its ears. The ears of *O. martiensseni* are large and long (approximately 40 mm in length), whereas the ears of *O. madagascariensis* are 30 – 35 mm in length (Kingdon, 1974; Rydell and Yalden, 1997; Long, 1995; Al-Jumaily, 1999). The ears of both species have a series of small spines along the anterior border, are attached along the whole length of the head and are fused on the extended snout (Kingdon, 1974; Walker *et al*., 1975; Nowak and Paradiso, 1983; Meester *et al*., 1986; Long, 1995; Peterson *et al.,* 1995; Nowak, 1997; Taylor, 2000b, 2005; Hutson *et al*., 2001; Russ *et al*., 2001).

The bodies of both *Otomops* species being investigated are covered in short, fine, velvety-brown fur and the dorsal pelage, which is a darker brown than the ventral, is characterized by a thin band of pale fur across the shoulders (Harrison, 1957; Ansell, 1974; Kingdon, 1974; Walker *et al*., 1975; Nowak and Paradiso, 1983; Meester *et al*., 1986; Long, 1995; Peterson *et al.,* 1995; Al-Jumaily, 1999; Russ *et al*., 2001; Taylor, 2005). The ventral pelage is a lighter brown and there is a very narrow band of white fur from the shoulders to the knee on the dorsal side (Harrison, 1957; Ansell, 1974; Kingdon, 1974; Long, 1995; Al-Jumaily, 1999; Russ *et al*., 2001).

The name *O. icarus* has been regarded as a synonym of *O. martiensseni* and, as expected, characteristics of *O. icarus* are similar to those described for *O. martiensseni*, e.g. ear, forearm, weight and head and body measurements (see next paragraph) (Peterson *et al*., 1995; Simmons, 2005). The colouring of *O. icarus* has been suggested as its distinguishing feature, although this issue remains contentious (Harrison, 1957). It has been suggested that, in *O. icarus*, the pale mantle across the shoulders is less prominent than in *O. martiensseni* and that these colour characters, being variable, give subspecific status to *O. icarus* (*O. m. icarus*) (Chubb, 1913; Harrison, 1957). In addition, it was also observed that *O. martiensseni* of Durban, South Africa (possibly *O. icarus*) is smaller than the Kenyan counterpart and that sexual dimorphism seems more pronounced in Durban populations of *O. martiensseni* (Richardson and Taylor, 1995). For purposes of this study, descriptions of *O. martiensseni* will include *O. icarus*.

Adult individuals of *O. martiensseni* weigh 22.2 – 37.2 g and 30.1 – 33.4 g for males and females respectively (Mutere, 1973; Skinner and Smithers, 1990 *loc. cit*. Long, 1995; Rydell and Yalden, 1997; Al-Jumaily, 1999). However, males and females of *O. madagascariensis* weigh approximately 23 – 29 g and 20 – 26 g respectively, suggesting that males are larger

than females in this species and that *O. martiensseni* is the larger of the two species (Peterson *et al*., 1995; Russ *et al*., 2001). Head and body measurements of *O. martiensseni* are 78 – 93 mm and 94 – 103 mm for males and females respectively, thereby making this species the largest member of the genus (Chubb, 1917; Ansell, 1974; Kingdon, 1974; Walker *et al*., 1975; Nowak and Paradiso, 1983; Stuart and Stuart, 1988; Long, 1995; Richardson and Taylor, 1995; Nowak, 1997; Rydell and Yalden, 1997; Al-Jumaily, 1999; Taylor, 2000b, 2005; Hutson *et al*., 2001; Fenton *et al*., 2002). Peterson *et al*. (1995) observed that, on average, the greatest skull length (GSL) of *O. madagascariensis* was smaller than that of *O. martiensseni* for both males and females. The greatest skull length of *O. madagascariensis* is 26.17 mm for males and 24.03 mm for females, whereas the GSL of *O. martiensseni* males and females is 28.77 mm and 27.32 mm respectively (Peterson *et al*., 1995; Al-Jumaily, 1999). Forearm lengths of *O. martiensseni* vary from 52 – 58 mm and 62 – 64 mm for males and females respectively (Mutere, 1973; Kingdon, 1974; Meester *et al*., 1986; Skinner and Smithers, 1990 *loc. cit*. Long, 1995; Rydell and Yalden, 1997; Al-Jumaily, 1999; Taylor, 2005). Forearm length of *O. madagascariensis* individuals is, on average, 60 – 65 mm for males and females (Peterson *et al*., 1995l Russ *et al*., 2001). The body of members of the *Otomops* genus is, however, more streamlined and their proportions more slender than those of other members of the Molossid family (Kingdon, 1974; Long, 1995).

The wings of individuals of *O. martiensseni* have a high aspect ratio of 10.04 and the wingspan ranges from 450 to 480 mm, both of which indicate the ability for fast, high and energy-efficient flight (Kingdon, 1974; Norberg, 1981; Peterson *et al*., 1995; Rydell and Yalden, 1997; Al-Jumaily, 1999; Hutson *et al*., 2001; Jones *et al*., 2003). The wingspan of *O. madagascariensis* individuals ranges from 420 to 450 mm and it can be assumed that they would have a similar aspect ratio to *O. martiensseni* individuals, since comparable wing characteristics are also found in other Molossids (Kingdon, 1974; Freeman, 1981; Peterson *et al*., 1995).

The skull of *O. martiensseni* individuals is characterized by its large size and robust build. The top of the braincase is domed, but has a depression in the frontoparietal region (Al-Jumaily, 1999; Long, 1995; Taylor, 2005). By comparison, individuals of *O. madagascariensis* have been described as having relatively narrower, longer and lighter skulls, and also possess particularly flattened heads (Russ *et al*., 2001). Individuals of *O. martiensseni* have moderately well-developed sagittal crests, however lamboidal crests are absent in females and slightly developed in males (Al-Jumaily, 1999; Long, 1995; Taylor, 2005). *Otomops martiensseni* individuals also have very deep basisephenoid pits which may play a role in echolocation (Al-Jumaily, 1999; Debaeremaeker and Fenton, 2003).

The muzzle of both *O. martiensseni* and *O. madagascariensis* is characterized by the wrinkling of the upper lips to form flaps at the corners of the mouth (Walker *et al*., 1975; Long, 1995; Al-Jumaily, 1999). Wrinkled and flap-like lips suggest the ability to expand and thus play a role in echolocation calls and manipulation of food, i.e. insects such as moths, grasshoppers and beetles (Kingdon, 1974; Freeman, 1981; Long, 1995; Al-Jumaily, 1999). Individuals of *Otomops* also possess a particularly wide gape that can exceed 90° (Freeman, 1979, 1981, 1984; Long, 1995; Al-Jumaily, 1999).

The dentition of *O. martiensseni* and *O. madagascariensis*, like other Molossids, is made up of 30 teeth with the dental formula: I 1/2 OC 1/1 P 2/2 M 3/3 (Long, 1995; Peterson *et al*., 1995; Al-Jumaily, 1999). According to Long (1995), there is an extrusive vertical protrusion on the zygomatic outgrowth, and a diastema separating the canine and the first premolar, a characteristic not found in any other species in the genus. The diet of *Otomops* comprises predominantly soft-bodied insects such as moths (Freeman, 1981, 1984; Long, 1995; Rydell and Yalden, 1997; Al-Jumaily, 1999; Fenton *et al*., 2004; Taylor, 2005).

Both *O. martiensseni* and *O. madagascarensis* are characterized by the presence of a gular gland found on the lower throat. This gland is not present in females; it is poorly-developed in sub-adult males but becomes well developed in adult males to indicate sexual maturity (Harrison, 1957; Hayman and Hill, 1971; Kingdon, 1974; Walker *et al*., 1975; Nowak and Paradiso, 1983; Long, 1995; Russ *et al*., 2001; Taylor, 2005).

According to karyotype studies, *O. martiensseni* is distinguished by a diploid number (2*n*) of 48 and a fundamental number of 58 (Đulic and Mutere, 1973; Warner *et al*., 1974; Taylor, 2005). *Otomops martiensseni* shares much in common with other western hemisphere molossid species such as *Tadarida femorosacca* in both diploid and fundamental numbers, as well as chromosome structures (Warner *et al*., 1974). Haematological studies of *O. martiensseni* reveal a high erythrocyte count with very high haemoglobin content, indicating a high metabolic rate (Kinoti, 1973). It has been suggested that, because members of *Otomops* are strong fliers, they require large amounts of energy in order to sustain flight, hence the development of a strong oxygen-transport system (Kinoti, 1973).

Otomops martiensseni individuals produce echolocation calls that are within the range of human hearing, with the lowest frequency being 10 kHz, the highest frequency 17 kHz and maximum energy 13 – 15 kHz (Fenton and Bell, 1981). *Otomops martiensseni* and *O. madagascariensis* both have very long and shallow FM calls, with a very high inter-pulse interval of about 320 ms (Fenton, 1982; Rydell and Yalden, 1997; Taylor, 2005). The distress

calls of *O. madagascariensis* are observed to be a low frequency FM sweep repeated at regular intervals (Russ *et al*., 2001). According to Fenton *et al*. (2002) and Fenton (2003), the calls of flying *O. martiensseni* are a mixture of echolocation and social calls, functioning in communication that can be colony-specific or individual-specific. These individually distinct calls enhance interactions between group members, which are important for nocturnal, flying animals when foraging (Fenton *et al*., 2004). Individual-specific calls suggest a more complex social structure in *Otomops*, compared with many other bat species.

1.1.4 Social structure and behaviour

Individuals of *O. martiensseni* use a number of day and night roost sites (Fenton *et al*., 2002; Taylor, 2005). Once a site is disturbed, it is temporarily vacated and the bats move on to another site, only to return a few days later. Individuals from the same community can locate the roost they share by use of vocalization calls, which are a mixture of echolocation and social calls, enabling individuals to communicate with each other (Fenton *et al*., 2002, 2004). These calls help synchronize activity when individuals are away from the roost, thereby facilitating interactions between group members. Lone or independent bats that are not associated with any particular colony, could be dispersing individuals and/or incapable of attracting suitable mates/companions (Verschuren, 1957).

It has been observed that *O. martiensseni* individuals must drop for a short distance before they can take to full flight because, like other molossids, they cannot take off from a horizontal surface (Kunz, 1982; Richardson and Taylor, 1995; Taylor, 2005). When coming into or moving out of the roost, bats must land first and then crawl into or out of the roost (Kunz, 1982; Fenton *et al*., 2002). Unlike bats from other genera, *Otomops* emerge individually from the roost, and it has been suggested that these individual emergences, which are unpredictable, may serve the same purpose as simultaneous emergence in other genera, i.e. minimizing the risk of predation (Fenton *et al*., 2002).

Based on findings from recent studies of different bat genera, including *Otomops*, it has been suggested that there exists a harem social structure within the roost, i.e. the presence of one dominant male and a number of reproductive females and their dependant young (Richardson and Taylor, 1995; Fenton *et al*., 2002, 2004; Heckel and von Helversen, 2003; Taylor, 2005). Sex ratios within a colony tend to fluctuate; male: female ratios range from 1:1 to 1:4 in eastern African colonies and 1:2 to 1:11 among colonies sampled in urban areas of Durban (Mutere, 1973; Fenton *et al*., 2002). Nothing has been published on the social organization of *O. madagascariensis*. Past observations suggest that harem organization appears to be more

prominent in the Durban area, with a maximum colony size of 30 being recorded (Taylor, 2005). It has been suggested that these changes in ratio can be attributed to the eviction of sub-adult or sub-dominant males once maturity is reached in the latter (Richardson and Taylor, 1995).

Dominant males usually roost about 1 m away from the rest of the colony and are identified by the presence of a well-developed chest gland and, during mating season, enlarged testes (Fenton *et al*., 2002, 2004; Taylor, 2005). Upon investigation, it was found that the ratio of adult females to young is 1:1, i.e. lactating females usually give birth to a single young during the spring/summer months (October to December) with an approximate gestation period of three months (Mutere, 1973; Walker *et al*., 1975; Long, 1995; Richardson and Taylor, 1995; Fenton *et al*., 2002; Taylor, 2005).

1.1.5 Distribution and ecology

On a global scale, *Otomops* as a genus has a fairly wide distribution and is referred to as being an Old World bat (found in the Afrotropical, Indo-Malayan, Australasian and certain parts of the Palaearctic regions), with a suggested elevation in numbers of individuals in eastern African countries (Warner *et al*., 1974; Freeman, 1981; Hutson *et al*., 2001). Other species of *Otomops* have been reported in southeast and northeast India, New Guinea, Indonesia and the southeast Asian islands (Lawrence, 1948; Freeman, 1981; Nowak and Paradiso, 1983; Corbet and Hill, 1986; Nowak, 1997; Al-Jumaily, 1999; Thabah and Bates, 2002). *Otomops martiensseni* and *O. madagascariensis* are predominantly distributed throughout eastern Africa and the western parts of Madagascar respectively (Mutere, 1973; Kingdon, 1974; Long, 1995; Peterson *et al.,* 1995; Richardson and Taylor, 1995; Nowak, 1997; Al-Jumaily, 1999, Hutson *et al*., 2001; Fenton *et al*., 2002; Kock *et al*., 2005).

In Africa, *O. martiensseni* has a wide range extending from the Ivory Coast, Ghana and Democratic Republic of Congo in the west to Ethiopia and Kenya in the east (Al-Jumaily, 1999; Hutson *et al*., 2001; Taylor, 2005). Bats referable to this species are also found in Yemen, Djibouti, Rwanda, Tanzania, Malawi, Zambia, Angola, Botswana, Zimbabwe, Mozambique and eastern parts of South Africa in northern KwaZulu-Natal (Fig. 1) (Hayman and Hill, 1971; Meester *et al*., 1986; Smithers, 1986; Long, 1995; Nowak, 1997; Al-Jumaily, 1999; Hutson *et al*., 2001).

Otomops madagascariensis can be found in the provinces of Antananarivo, Antsiranana, Fianarantsoa, Mahajanga and Toliara in Madagascar. A substantial portion of the range of *O.*

madagascariensis falls within the drier western portions of the island, which increase in aridity from north to south (Fig. 1) (Peterson *et al*., 1995; Dufils, 2003; Du Puy and Moat, 2003; Wells, 2003; Goodman and Cardiff, 2004; Goodman *et al*., 2005). One of the major geological strata of western Madagascar is limestone. This limestone has been eroded by water action into karst formations that contain a multitude of caves and canyons which provide ideal roosting sites for bats (Goodman *et al*., 2005).

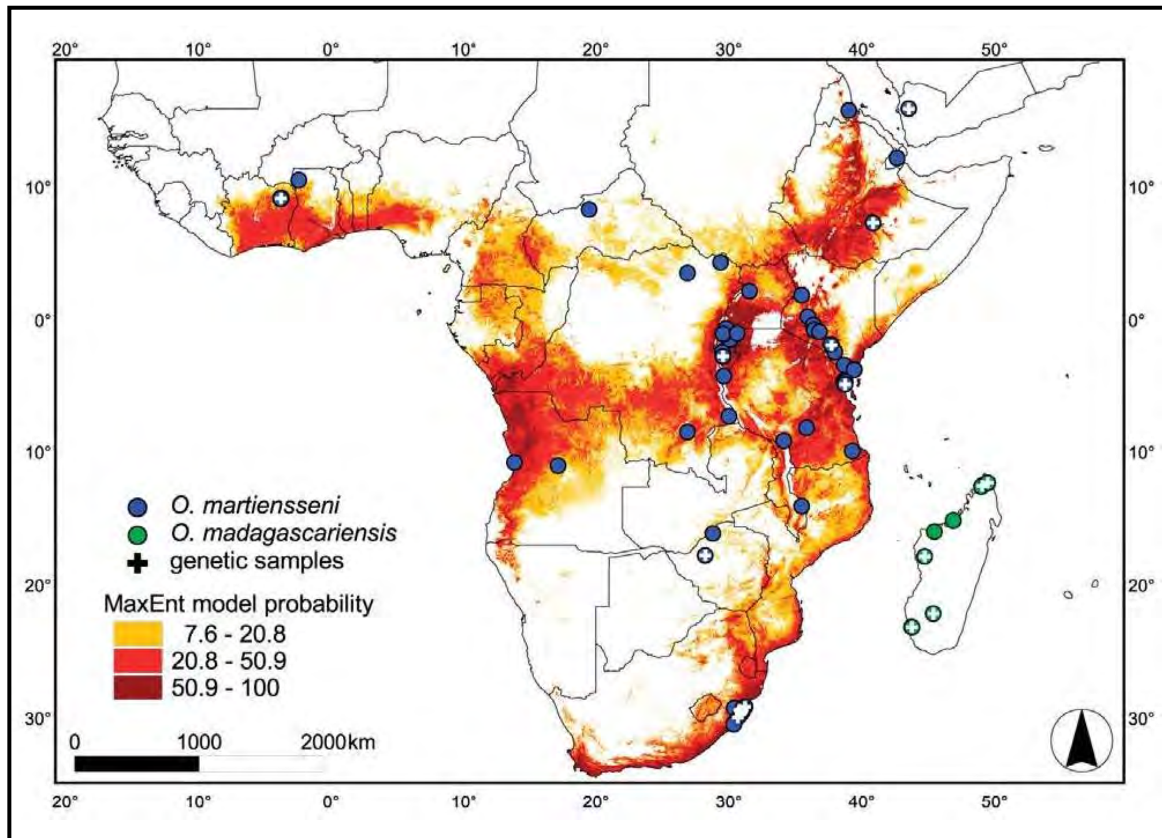

Figure 1. Map of Africa depicting sites at which individuals of the bat genus *Otomops*: *O. martiensseni* (*O. icarus*) and *O. madagascariensis* were collected for this study (genetic samples) (Lamb *et al*., 200). MaxEnt version 2.3 (Phillips *et al*., 2006) was used to plot the predicted distribution of the species found in mainland Africa based on observed distribution and habitat selection patterns. Yemen and Madagascar excluded*.

On a finer scale, the natural environment and/or habitat of *O. martiensseni* range(s) from semi-arid and savannah woodland areas to montane forests which can be found anywhere

* Yemen contains an isolated population from an arid environment and may therefore skew results regarding predicted distribution of *O. martiensseni* according to environmental conditions. Madagascar contains a different *Otomops* species (*O. madagascariensis*); it has a different climate from mainland Africa and should therefore be modelled independently.

between sea level, e.g. in Durban, South Africa, and 2000 metres above sea level in places such as Kenya (Long, 1995; Taylor, 2000b, 2005; Hutson *et al*., 2001).

It has been observed that individuals of *O. martiensseni* from eastern Africa are found in fairly large colonies of several hundred, packed tightly together, and that they tend to roost in dark and poorly-ventilated caves (Kinoti, 1973; Mutere, 1973; Kingdon, 1974; Walker *et al*., 1975; Nowak and Paradiso, 1983; Smithers, 1986; Stuart and Stuart, 1988; Long, 1995; Peterson *et al*., 1995; Richardson and Taylor, 1995; Nowak, 1997; Al-Jumaily, 1999; Hutson *et al*., 2001; Kock *et al*., 2005). Other known habitats include crevices within rocks, behind the loose bark of trees, within hollow trees and within certain man-made structures (Warner *et al*., 1974; Walker *et al*., 1975; Stuart and Stuart, 1988; Peterson *et al*., 1995; Taylor, 2005). The majority of mainland African *Otomops* tend to be found in caves, such as the Ithundu and Mt. Suswa lava caves in Kenya (Hayman and Hill, 1971; Đulic and Mutere, 1973; Kinoti, 1973; Mutere, 1973; Nowak and Paradiso, 1983; Long, 1995; Kock *et al*., 2005). In contrast, in the urban areas of Durban, South Africa, the species tends to be found dwelling in comparatively smaller numbers in the roofs of homes, as well as other buildings, although relatively few colonies are known (Smithers, 1986; Gelderblom *et al*., 1995; Richardson and Taylor, 1995; Taylor, 1999a, 2005; Hutson *et al*., 2001, Fenton *et al*., 2002, 2004). A combination of darkness, a protected environment (similar to a cave) and room for clustering makes this type of habitat suitable as a day-time roost (Fenton *et al*., 2002). The Malagasy species is known to dwell in sea caves and the hollows of trees, much like *O. martiensseni* of eastern Africa (Peterson *et al*., 1995).

Otomops is not limited to one particular habitat however, as members of this genus possess the ability of powerful flight and are known to be swift and high fliers (Kingdon, 1974; Freeman, 1981; Long, 1995). It is the high aspect ratio of the wing that imparts this ability for fast, strong flight, thereby enabling individuals to catch prey whilst in flight as well as to fly from one locality to another, e.g. in cases of long-distance foraging or in order to migrate to areas of greater food abundance during the dry season (Kingdon, 1974; Freeman, 1981; Norberg, 1981; Long, 1995; Rydell and Yalden, 1997; Hutson *et al*., 2001; Fenton *et al*., 2004). In Kenya, caves that house bats are regularly mined for guano which is used as fertilizer (Mutere, 1973; Long, 1995; Hutson *et al*., 2001). It has been suggested that these disturbances can lead to changes in 'physical characteristics' and 'microclimate' to the point where recruitment of individuals in the caves decreases, at least temporarily (Long, 1995; Taylor, 2000b; Fenton *et al*., 2002; Kock *et al*., 2005). It has been found that when roosts are disturbed, bats tend to move to new locations (Taylor, 2000b). The ability for high and swift flight makes *Otomops* a challenge to study, hence the scarcity of captures and documented

records. Flight also allows bats to move considerable distances, especially when they and the habitats in which they live become threatened (Fenton *et al*., 1998).

1.2 Conservation

1.2.1 Threats to bats

Bats are the second most diverse order of mammals worldwide; there are approximately 1100 bat species that represent approximately 25% of all mammalian species (Taylor, 1999a, 2000b; Kerth *et al*., 2002b; Mickleburgh *et al*., 2002; Jones *et al*., 2005; Simmons, 2005). About 22% of bat species are considered "threatened" and a further 25% considered "near-threatened" (Mickleburgh *et al*., 1992, 2004; Hilton-Taylor, 2000; Hutson *et al*., 2001). Bats, like many other animals, are considered to be key components of biological diversity, playing both a fundamental ecological role and having economic value, especially in tropical and arid areas where they contribute to ecosystem structure and function (Erlich and Wilson, 1991; Fujita and Tuttle, 1991; Fenton, 1997; Taylor, 1999a, 2000b; Hutson, 2002; Mickleburgh *et al*., 2002). In certain ecological settings, bats are the major faunal component responsible for pollination and seed dispersal, and are thus keystone species, whereas other species may be important for insect pest control (Hutcheon, 1994; Fenton, 1997; Taylor, 1999a; Burland and Worthington Wilmer, 2001; Cox *et al*., 1992 *loc. cit*. Mickleburgh *et al*., 2002; Andriafidison *et al*., 2006).

In spite of their importance to ecosystems, there has been a documented, worldwide decline in bat numbers over the years, mainly due to a lack of education and associated persecution of bats by humans. For example, in areas of Britain, the greater horseshoe bat, which was once very common, has become extinct and several cave-dwelling colonies in North America have declined in number by 99.9% (Taylor, 2000b; Burland and Worthington Wilmer, 2001; Hutson *et al*., 2001). Human population increases result in an augmented demand for and utilization of land, resources and food which, in turn, leads to the degradation and/or destruction of certain habitat types, e.g. forests, key landscape elements (tree lines, hedges, canals etc.) and aquatic habitats, all of which are used by bats (Erlich and Wilson, 1991; Erwin, 1991; Gelderblom *et al*., 1995; Forester and Machlis, 1996; Fenton, 1997; Fenton *et al*. 1998; Taylor, 2000b; Balmford *et al*., 2001; Burland and Worthington Wilmer, 2001; Hutson *et al*., 2001; Russ *et al*., 2001 Mickleburgh *et al*., 2002; Jones *et al*., 2003; Burgess *et al*., 2004). The loss of buildings and alterations to such sites result in roost site disturbance, whilst the chemical treatments used to maintain building materials, e.g. timber, can be poisonous and

have been thought to have a severe impact on some populations (Taylor, 1999a, 2000b; Burland and Worthington Wilmer, 2001; Hutson *et al*., 2001). In some cases, construction of new buildings can be advantageous, especially for those bat species that are adapted to an urban environment and create roosts in these structures, e.g. *O. martiensseni* populations found in Durban, South Africa. Underground bat habitats can also be affected. Activities such as quarrying, mineral mining, uncontrolled guano mining, the increasingly popular sport of caving, cave exploitation for tourism, deliberate disturbance of roosts as a means of eradication and bird nest collection, all lead to the disturbance of roost sites (Hutson *et al*., 2001; Mickleburgh *et al*., 2002; Jones *et al*., 2003). Cave-ins and sealing off mines are also responsible for blocking bat roosts (Hutson *et al*., 2001; Mickleburgh *et al*., 2002).

1.2.2 General conservation of bats

A general lack of records/information about many bat species results in their exclusion from conservation plans, but, in recent years, there has been a rapid increase in scientific and public interest in bats and their conservation (Gelderblom *et al*., 1995; Fenton, 1997; Taylor, 1999a, 2000b; Hutson *et al*., 2001). There are many international treaties protecting fauna and flora, some of which indirectly protect bats or their habitats (Hutson *et al*., 2001) including the Convention Concerning the Protection of the World Cultural and Natural Heritage 1972 (World Heritage Convention) that permits certain natural features to be designated as World Heritage Sites based on their value, scientific or otherwise and the Convention on the Conservation of Migratory Species of Wild Animals 1979 (Bonn Convention) that protects species migrating across political boundaries, among others.

1.2.3 Conservation within Africa and Madagascar

Biodiversity is under threat in Africa due to the endangerment of species and their habitats (section 1.2.1); it is for this reason that conservation priorities need to be set (Sarkar and Margules, 2002). Mainland Africa contains many important centres of endemism that are found mainly in the forested mountains across the tropical belt of Africa, the western Cape of South Africa and the Horn of Africa; it is the conservation of these areas that holds the potential for preventing the extinction of many African species (Balmford *et al*., 2001; Burgess *et al*. 2004). Most of these biomes remain unprotected by official wildlife reserves, with less than 5% being in protected areas (as at 2004) (Burgess *et al*. 2004). This indicates that there is a definite need to increase the number of protected areas for conservation. Of all the islands, Madagascar has the greatest number of endemic and unique species as well as ancient lineages and therefore is a critical area for conservation, i.e. a biodiversity hotspot

(Ganzhorn *et al*., 2001; Burgess *et al*. 2004; Goodman and Benstead, 2005; Goodman, 2006; Yoder and Nowak, 2006).

A number of intergovernmental processes and conventions have been put in place over the past 20 years to help governments manage their local biodiversity, the most important of which has been the Convention on Biological Diversity (CBD), established in 1992. The CBD requires countries to create and implement their own biodiversity strategies and action plans at the level of national government. Agencies, such as the United Nations Environment Programme (UNEP) and the World Conservation Union, provide help for these countries to implement the CBD.

An initiative by the Convention on the Conservation of Migratory Species of Wild Animals (CMS) was/is being implemented in order to encourage activities to protect endangered bat species and to ascertain the need and/or opportunity for agreements to be developed for bat conservation outside of Europe (Hutson, 2002). Within Africa, twelve countries have been considered as parties to CMS (Angola, Botswana, Democratic Republic of Congo, Lesotho, Malawi, Mozambique, Namibia, South Africa, Swaziland, United Republic of Tanzania, Zambia and Zimbabwe), although only three have agreed to become parties to CMS (Democratic Republic of Congo, South Africa and United Republic of Tanzania) (Hutson, 2002). According to the Gauteng & Northern Regions Bat Interest group, there are 56 species of bat throughout South Africa (http://www.batsgauteng.org.za/SABats.htm).

For several centuries, the island of Madagascar has been of interest to environmentalists, due to its species richness and endemism (Goodman *et al*., 2003; Goodman and Benstead, 2005). As at 2005, there were approximately 30 species of bats known to exist on Madagascar, 60% of which are endemic (Goodman *et al*., 2005; Goodman and Benstead, 2006). The current number of bat species on Madagascar is most probably closer to 45 (S.M Goodman *pers. comm.*[1]). A review by Eger and Mitchell (2003) revealed that the drier western formations have a higher species richness of bats than the more humid eastern portions, although it must be noted that a substantial part of the island remains unexplored (Goodman *et al*., 2005).

As at 2002, Madagascar had 46 legally protected areas, making up ~3% of the total land area (Randrianandianina *et al*., 2003). Goodman and Benstead (2005) report that in 2003, during the World Parks Congress in Durban, South Africa, it was declared that the Malagasy government would endeavour, over the next five years, to increase the coverage of its

[1] Dr. S.M Goodman. The Field Museum, Division of Mammals. 1400 S. Lake Shore Drive, Chicago, Illinois.

protected areas from what was around 17 000 km^2 in 2002 to 60 000 km^2. Areas that were previously under-represented, or not represented at all, whould be added to the currently protected area network in order to perpetuate conservation in Madagascar (Goodman and Benstead, 2005).

1.2.4 Conservation of *Otomops*

Of all the species in the genus, *O. martiensseni* has the most widespread distribution, whereas other species have more restricted ranges (as described in section 1.1.5). *Otomops martiensseni* is the most studied member of the genus and as such, more information regarding threats to and plans for its conservation, is available for assessments.

Otomops martiensseni was selected as one of eight potential candidates for the CMS Appendices list because, although there is no direct evidence for species migration, there is marked seasonal absence from certain areas that suggests the occurrence of migration, which should be possible given the wing characteristics of the species (section 1.1.3) (Mutere, 1973; Hutson, 2002).

According to the 2006 IUCN (The World Conservation Union) Red List of Threatened Species, *O. martiensseni* has been classified globally as having a “Vulnerable” status (Mickleburgh *et al*., 2004). A taxon is classified as “Vulnerable” when, although it is not “Critically Endangered” or “Endangered”, it still faces a high risk of extinction in the wild in the medium-term future (Taylor, 2000b; Hutson *et al*., 2001). *Otomops martiensseni* is classified as “Vulnerable” because of a projected and/or suspected reduction in numbers of at least 20% within the next 10 years or three generations (whichever is longer) based on a predicted decline in area of occupancy, extent of occurrence and/or quality of habitat (Hutson *et al*., 2001). This reduction in bat populations is a result of the threats described in section 1.2.1. Bats are poorly known and thus poorly protected, and it is for this reason that it has been recommended that *O. martiensseni* receive improved protection (Gelderblom *et al*., 1995; Taylor, 1999a).

The research and conservation activities applied to *O. martiensseni* may be applicable to the other species, including *O. madagascariensis*, although most species are too poorly known at present to implement any conservation actions (Hutson *et al*., 2001). *Otomops madagascariensis* has, however, found protection through the establishment of protected areas in certain portions of its distribution. According to a study conducted by Goodman *et*

al. (2005), *O. madagascariensis* was found in at least five protected areas throughout Madagascar.

In terms of protection on a local scale, in South Africa, Microchiroptera are listed as Protected Wild Animals under Schedule 2 of the old Cape Province Ordinance No. 19 of 1974, and in 1996, two bat species were granted provincial government protection and listed as Endangered Mammals in KwaZulu-Natal (Schedule 6 of the Natal Provincial Ordinance No. 15 of 1974), one of which was *O. martiensseni*. Those animals under protected status cannot be hunted, killed, captured, sold or bought without a permit (Skinner *et al*., 1977). Additionally, the revised KwaZulu-Natal Ordinance of 1999 acknowledges several bat species as either Specially Protected or Protected (Taylor, 1999a, 2000a, 2000b). East African coastal forests and parts of KwaZulu-Natal have been highlighted as areas in need of additional conservation schemes (Balmford *et al*., 2001). In addition, the protection of *Otomops* falls under the mandate of the South African National Environmental Management: Biodiversity Act No. 10 of 2004 (NEMBA), which aims to manage and conserve the biological diversity in South Africa. *Otomops martiensseni* is in the Threatened or Protected Species (TOPS) list that NEMBA has published since their roosting sites are often in houses which are vulnerable to human disturbance, e.g. fumigation (section 1.2.1). (www.ecoserv.com/news/Terrestrial%20Biodiversity.pdf).

Conservation Acts, Treaties and/or Agreements spark public awareness and with it, the formation of bat groups that offer assistance in enforcing these Agreements (Taylor, 1999a). South Africa has three well-developed local volunteer bat interest groups with good contact with researchers and other conservation bodies (Taylor, 2000b; Hutson *et al*., 2001). These groups were established in KwaZulu-Natal, Gauteng and the Western Cape in 1994, 1995 and 1999 respectively, with the intention of promoting public awareness and knowledge of bats and bat conservation, e.g. the establishment of rehabilitation centres to care for sick, injured or young bats (Taylor, 1999a, 2000a, 2000b). The Bat Interest Group of KwaZulu-Natal (BatsKZN) has chosen *O. martiensseni* as their “flagship species” with respect to carrying out their objectives of promoting public awareness and conservation (Taylor, 1999a, 2000a). Although there is legislation in place in South Africa, elsewhere in southern Africa bats such as *O. martiensseni* are not formally protected, hence the need to establish formal nature conservation agreements (Taylor, 1999a, 2000b).

1.3 Defining Conservation Units

1.3.1 Species

Systematic biology has been defined as the study and description of biological forms to produce a natural system of classification (Stearns and Hoekstra, 2000; Contrafatto and Minelli, 2002; Hausdorf, 2011). Taxonomy involves naming and ranking these biological forms into groups or classes, each individual being part of the ranking hierarchy starting from species to genus and so on. (Van Valen, 1976; Hendry *et al*., 2000; Bradley and Baker, 2001; Contrafatto and Minelli, 2002). "Species" has been defined as the only natural, basic unit of classification, i.e. a group of organisms with structural, functional and developmental similarities that can interbreed to produce fertile offspring but, under natural conditions, do not breed with members of other species (Hale and Margham, 1988; Villee *et al*., 1989; Mayr, 2000a; Noor, 2002). Members of a species share a common evolutionary ancestry and were usually recognized on the basis of morphological characters; however alternatives, such as karyotype, allozymes, behaviour, ecological niche and significant DNA sequence divergence have been used in recent years (Coyne *et al*., 1988; Hale and Margham, 1988; Villee *et al*., 1989; Johnson *et al*., 1999; Bradley and Baker, 2001). Closely related species are grouped together, according to the degree of similarity, in the next higher unit of classification, the genus (Villee *et al*., 1989).

1.3.2 Species concepts

Although there has been general disagreement and debate amongst biologists concerning speciation and whether species are "real" or not, there has been even more debate over the years as to how the term "species" should be defined, hence the development of the different species concepts (Giray, 1976; Van Valen, 1976; Andersson, 1990; Bremer and Eriksson, 1992; Coyne, 1994; Mallet, 1995; Paterson, 1999; Sluys and Hazevoet, 1999; Hendry *et al*., 2000; Bradley and Baker, 2001; Noor, 2002; Isaac *et al*., 2004; Isaac and Purvis, 2004; Sites Jr. and Marshall, 2004; Hausdorf, 2011). These concepts allow species to be defined and these, in turn, have biological, economic and legal consequences, therefore, the species concept applied to each study is of critical importance (Baum, 1992; Templeton *et al*., 2000; Lee, 2002; Isaac *et al*., 2004; Sites Jr. and Marshall, 2004). Species concepts are defined according to their major characteristics and how they are perceived, i.e. as morphological, biological, evolutionary or ecological units (Andersson, 1990; Bremer and Eriksson, 1992; Coyne, 1994; Sluys and Hazevoet, 1999; Miller III, 2001).

1.3.2.1 Morphological species concepts

Species, as morphological units, are grouped together according to similarities in their phenotype or morphological appearance, hence the name: morphological species concept (Mayr, 2000a). Also known as the typological species concept, this concept defines species as a group of organisms whose physical characteristics, colour, size, habitat, etc. separate them from other organisms; hence, the degree of morphological difference determines species status (Ruse, 1969; Giray, 1976; Andersson, 1990; Mallet, 1995; Mayr, 2000a; Lagasche *et al*., 2013).

The phenetic and the paleontological species concepts also fall under the morphological species concept, but are based on mathematically-tested and measurable similarities and differences. Under the phenetic concept, if variation in a set of characters is less within a group than between groups, the entity is recognized as a distinct taxon (Miller III, 2001).

1.3.2.2 Biological species concepts

During the 20th century, new species concepts which integrated biological factors into their definitions, were developed (Giray, 1976; Coyne, 1994; Johnson *et al*., 1999). Since genes determine the morphology of a species, divergence in these genes give rise to morphological variation (Giray, 1976; Coyne *et al*., 1988; Lidén, 1992). It is these gene-based processes upon which the biological species concepts are based (Giray, 1976; Baum, 1992; Johnson *et al*., 1999).

One of the most widely-recognized concepts is the isolation species concept, also known as the biological species concept (BSC) (Van Valen, 1976; Coyne *et al*., 1988; Coyne, 1994; Sluys and Hazevoet, 1999; Noor, 2002; Hausdorf, 2011). Species are seen as groups of interbreeding natural populations that are reproductively isolated from other such groups, i.e. gene pools are held together by gene flow, which only occurs through sexual reproduction (Dobzhansky, 1935; Mayr, 1942; Lagasche *et al*., 2013).

The recognition species concept regards species as the most inclusive population of individual bi-parental organisms sharing a common fertilization system and species therefore arise as incidental effects of adaptive evolution (Paterson, 1980, 1985). Part of this “common fertilization system” is the signals by which conspecific individuals identify each other, i.e. the specific-mate recognition system (SMRS). In this way, reproductive isolation is seen as a

by-product of the SMRS (Paterson, 1980, 1999; Coyne *et al*., 1988; Sluys and Hazevoet, 1999; Mayr, 2000b). Under this concept, species are not defined by reproductive isolation but, much like the BSC, this concept is mainly applicable to extant, bi-parental, sexually producing, animal species thereby excluding other biological criteria when defining species, e.g. asexual reproduction (Coyne *et al*., 1988; Coyne, 1994; Johnson *et al*., 1999; Paterson, 1999; Sluys and Hazevoet, 1999).

The genetic species concept involves the measurement of genetic differences, and defines species as a group of "genetically compatible interbreeding natural populations that is genetically isolated from other such groups" (Baker and Bradley, 2006; Zachos *et al*., 2012). Although there has been a recent increase in the amount of available DNA sequence data, a downfall to this approach is determining the degree of genetic variation required to distinguish between two putative species, i.e. how much differentiation is enough (Bradley and Baker, 2001; Zachos *et al*., 2012)? It also requires knowledge of levels of genetic divergence from closely related species or sister species in order to be implemented hence the employment of genetic species concepts has almost been limited to those groups in which genetic characteristics are relatively easily observed, i.e. recent higher vertebrates (Sluys and Hazevoet, 1999; Bradley and Baker, 2006).

1.3.2.3 Evolutionary species concepts

After the inclusion of biological criteria, concepts of evolutionary biology were then included in species definitions, which made sense since species are "dynamic entities that are constantly changing and diverging over time" (Avise and Wollenberg, 1997; Sluys and Hazevoet, 1999; Mayr, 2000b; Zachos *et al*., 2012).

Under the evolutionary species concept, a species is an entity composed of organisms that maintains its identity from other such lineages and has its own independent evolutionary tendencies and historical fate (Simpson, 1951, 1961; Wiley, 1978; Wiley and Mayden, 2000; Frankham *et al*., 2012). This concept introduces the idea of a lineage present over time and therefore allows for fossils and asexual organisms (Giray, 1976; Johnson *et al*., 1999; Mayr, 2000b). But, even though the concept itself is good in theory, it is not practical since "evolutionary tendencies" and "historical fates" give no characters on which to base a clade (Johnson *et al*., 1999; Mayr, 2000b; Frankham *et al*., 2012). As a result, the phylogenetic species concept was put forward as its alternative.

The phylogenetic species concept (PSC) defines a species as being the smallest diagnosable cluster of individual organisms, within which there is a parental pattern of ancestry and descent (Cracraft, 1983; Lagasche *et al*., 2013). Criticism of the PSC stems mainly from the simplicity of the criterion used for diagnosis, e.g. detailed morphology or molecular techniques can reveal apomorphies for almost every individual and there is no clear rule as to where to separate species in the taxonomic hierarchy (Baum, 1992; Mallet, 1995; Johnson *et al*., 1999; Sluys and Hazevoet, 1999; Hendry *et al*., 2000; Mayr, 2000b; Isaac and Purvis, 2004; Hausdorf, 2011).

1.3.2.4 Ecological species concepts

Although many of the proposed concepts consider biological factors, many do not account for the influence of ecology in defining species (Grant, 1992). Ecological processes have been known to influence gene flow and hence morphology, thereby allowing species to become advantageously adapted to the habitats they occupy and create differences between species (Van Valen, 1976; Andersson, 1990; Bremer and Eriksson, 1992). Each species is said to have its own ecological preferences, i.e. habitat and environment and as a result, geographical distribution and when these parameters are included in species definition, we have the ecological species concepts (Andersson, 1990; Lidén, 1992).

The ecological species concept, according to Turesson (1922) first defines ecotypes, i.e. population sections that are morphologically distinct. These ecotypes are then grouped into ecospecies representing the recognized potential of populations involved, i.e. subspecies and ecospecies are then categorized into coenospecies (species) which are indicative of the full, but undetermined, potential of the species.

Van Valen (1976) defines species as lineages evolving separately from each other that occupy ecological niches (adaptive zones) that are minimally different from other lineages.

The ecogenetic species concept (Levin, 2000) includes reproductive and genetic criteria where species are defined as having a unique way of living in and relating to the environment. They also have a unique genetic system which controls the ability of individuals and populations to interbreed.

Although there have been many concepts developed and used to define species, it is apparent that with each concept comes its own advantages and disadvantages (Sluys and Hazevoet, 1999; Hendry *et al*., 2000; Bradley and Baker, 2001; Noor, 2002). It is for this reason that

species concepts can be situation-specific and not every concept will be appropriate for the type of study being conducted, e.g. a morphological species concept would not be appropriate for a study based primarily on genetic or molecular data (Sluys and Hazevoet, 1999; Lee, 2002; Isaac and Purvis, 2004). There has always been a quest to reach a unified species concept that is applicable to all types of organisms, data and studies since concepts applied in zoology sometimes do not or cannot apply to botany and vice versa (Giray, 1976; Sluys and Hazevoet, 1999). Because of the diversity of organisms and the data used, whether morphological, genetic etc., it appears that this is a near-impossible task, but as long as the correct species concept is used in the correct context and that its implications are understood when applied to the study at hand, then the study should be a successful one (Grant, 1992; Sluys and Hazevoet, 1999; Miller III, 2001). For the purposes of this study, both the genetic species concept and the phylogenetic species concept will be used.

1.3.3 Evolutionary significant units

Conservation biologists have, in recent years, been challenged to devise a means to identify and objectively prioritise units below the species level, hence the development of the evolutionary significant unit (ESU) concept (Ryder, 1986; Hendry *et al*., 2000; Isaac *et al*., 2004; Sites Jr. and Marshall, 2004; Zachos *et al*., 2012).

Although there have been many suggested definitions of ESUs, the first to coin the term was Ryder (1986) who defines ESUs as "subsets of the more inclusive entity species which possess genetic attributes significant for the present and future generations of the species in question". Ryder's (1986) concept is integrative and considers genetic diversity below species level, even though it does not describe many guidelines for operational applications.

According to Waples (1991) *loc. cit.* Fraser and Bernatchez (2001), an ESU is "a population or group that (1) is substantially reproductively isolated from other conspecific population units and (2) represents an important component of the evolutionary legacy of the species". The objectivity of the approach has been questioned as the words "substantially" and "important" imply subjectivity in the process.

Dizon *et al*. (1992) define ESUs as "populations or groups of populations demonstrating significant divergence in allele frequencies", whereas Avise (1994) defines them as "sets of populations derived from consistently congruent gene phylogenies". Moritz (1994) essentially combines the ideas of Dizon *et al*. (1992) and Avise (1994) and defines ESUs as

"populations that (1) are reciprocally-monophyletic for mtDNA alleles and (2) demonstrate significant divergence of allele frequencies at nuclear loci".

Moritz (1994) is more stringent than Waples (1991) or Dizon *et al*. (1992) and provides a qualitative criterion for diagnosing ESUs by applying molecular genetics whilst avoiding the problem of ascertaining how much genetic variation is enough to warrant protection for a population of a species. There are, as with any concept, some disadvantages, e.g. reciprocally-monophyletic relationships may not always infer historical isolation and the very stringency that reciprocal monophyly offers, can be problematic because one differing individual in a new sample can annul a population's reciprocally-monophyletic status. In addition, the potential of species to maximize evolutionary success through the maintenance of adaptive diversity is not emphasized and the dependence on mtDNA groupings makes the concept of limited use for phylogeographical studies in plants. No one method exists that can best construct the most-likely phylogeny in all situations (Waples 1995 *loc. cit.* Fraser and Bernatchez, 2001).

Vogler and DeSalle (1994) use the PSC to define conservation units where "groups that are diagnosed by characters that cluster individuals or populations, to the exclusion of other such clusters", are considered to be ESUs. This diagnosis by characters is conceptually appealing because it avoids the problem of universally applying criteria such as reproductive isolation or phenetic similarity and is also stringent, making it testable, however it should be noted that this concept is prone to the same disadvantages experienced when defining ESUs by the Moritz (1994) criteria. All diagnosable evolutionary lineages, under this concept, can be elevated to species level and therefore ESUs that are not part of the formal nomenclature need not be acknowledged, although this can underestimate the number of species (Johnson *et al*., 1999).

Conversely, it has been suggested that this concept can over-split taxa and promote over-protection (Moritz, 1994). Characters used in analysis can also be problematic in cases where they are difficult to score, e.g. ecological or behavioural. In addition, the PSC method lacks flexibility because, like Moritz (1994), the advantage of stringency is lost if an individual in a new sample is an anomaly for the character being scored. In situations where only small sample sizes can be obtained (e.g. endangered species), characters may, by chance, appear to be diagnostic and therefore may lead to incorrect management decisions.

By contrast, Crandall *et al*. (2000) abandon the term ESU for a more holistic concept of species, consisting of populations with varying levels of gene flow evolving through drift and

selection. This approach has been criticized for forcing the continuous distribution of genetic diversity into two categories, i.e. ESU or not, and for its limited usefulness where already established species are likely to be identified (Fraser and Bernatchez, 2001).

In the adaptive evolutionary conservation (AEC) model, Fraser and Bernatchez (2001) define the ESU as a lineage demonstrating highly restricted gene flow from other such lineages within the higher organizational level of the species, i.e. each lineage is isolated through reduced (or absent) gene flow, thereby having limited impact on the evolution, genetic variance and demography of other such lineages. The AEC concept should enable conservationists to classify biologically significant ESUs, integrate exceptions as they occur and prove useful when the problem of limited resources affects management decisions.

ESUs have important legal and biological ramifications for those involved in conservation management and legislation, e.g. in the USA Endangered Species Act (ESA) and the Australian Endangered Species Act and equivalent legislation in other countries (Hendry *et al*., 2000). However how the ESU should be defined is still a matter for debate. For the purposes of this study, the AEC concept will be used for defining ESUs.

1.3.4 Management units

The management unit (MU) closely resembles the "stock" definition of Dizon *et al*. (1992) and was created to be a conservation unit below that of the ESU. These units have diverged in allele frequencies but do not show reciprocal monophyly in either nuclear or mitochondrial alleles. As such, these populations are linked by low levels of gene flow and are hence important when regarding conservation issues. The MU focuses on current population structuring, allele frequencies and short-term management planning rather than historical factors and long-term management, which are the focus of ESUs.

1.4 Methods in investigating taxonomic relationships

The mitochondrial genome is maternally inherited and has a higher copy number than the biparentally inherited nuclear genome in most cells and is therefore easier to work with. In this study, two mitochondrial regions have been sequenced, (cytochrome *b* gene and the D-loop). The mitochondrial genome in animals has been well characterized, and primer pairs for many conserved regions have been designed and constructed. It has been frequently used

for comparisons at the genus, species and subspecies level, i.e. the level that is required for this study (Table 1).

1.4.1 Review of studies incorporating use of mitochondrial DNA

Mitochondrial regions used in other phylogenetic studies included the ND1, ND2, 12S rRNA, $tRNA^{Val}$, 16S rRNA, cytochrome oxidase II and cytochrome *b* genes and the D-loop. In certain cases, these sequencing data were coupled with morphological data, echolocation data, restriction site data, sex chromosome data, allozyme data, chromosome banding data and/or natural history data to be used in phylogenetic, phenetic, population genetic, phylogeographic, evolutionary and coalescent theory analyses to test different hypotheses. Data can also be used to identify new species and subspecies, confirm the presence of species in new areas (first records), resolve taxonomic relationships within and between species and challenge previous classifications, date the divergence of certain lineages, determine male and female dispersal patterns, ascertain the presence of barriers to gene flow, determine the presence of cryptic or sibling species and determine lineages and haplotype networks.

Jacobs *et al*. (2004) used the cytochrome *b* gene to investigate the status of light and dark-winged forms of *Chaerephon pumilus* in southern Africa and found that the two forms are not distinct. Similarly, Benda *et al*. (2004) used both cytochrome *b* and morphological data to determine the systematic status of African populations of the *Pipistrellus pipistrellus* complex by comparing them to Eurasian specimens. Although differences between the northwest African and Eurasian complexes could not allow for separation at the species level (*P. pipistrellus* – Eurasian complex, 3 – 5% sequence difference), it was concluded that northwest African *P. pipistrellus* was a possible subspecies. Jacobs *et al*. (2006) used genetic, morphological and echolocation data to show the existence of a cryptic species within *Scotophilus dinganii*. The cytochrome *b* genetic difference between the two forms was 3.3%, and phylogenetic analysis indicates that the two types are reciprocally-monophyletic, suggesting that they are sibling species.

Table 1. List of some articles that have used mitochondrial DNA sequencing techniques in phylogenetic/phylogeographic bat studies.

Author(s)*	Journal article title	Technique(s)
Agirre-Mendi *et al*. (2004)	Presence of *Myotis alcathoe* Helversen & Heller, 2001 (Chiroptera: Vespertilionidae) in the Iberian Peninsula.	Mitochondrial ND1 sequencing
Benda *et al*. (2003)	First record of *Myotis alcathoe* (Chiroptera: Vespertilionidae) in Slovakia.	Mitochondrial ND1 sequencing; morphological data
Benda *et al*. (2004)	Systematic status of African populations of *Pipistrellus pipistrellus* complex (Chiroptera: Vespertilionidae), with a description of a new species from Cyrenaica, Libya.	Cytochrome *b* sequencing; morphological data
Campbell *et al*. (2004)	Phylogeny and phylogeography of Old World fruit bats in the *Cynopterus brachyotis* complex.	Mitochondrial control region sequencing; cytochrome *b* sequencing
Castella *et al*. (2000)	Is the Gibraltar Strait a barrier to gene flow for the bat *Myotis myotis* (Chiroptera: Vespertilionidae)?	Cytochrome *b* sequencing; microsatellite genotyping
Castella *et al*. (2001)	Contrasted patterns of mitochondrial and nuclear structure among nursery colonies of the bat *Myotis myotis*.	Mitochondrial HVII sequencing; nuclear DNA microsatellite genotyping
Cooper *et al*. (2001)	Assessment of species boundaries in Australian *Myotis* (Chiroptera: Vespertilionidae) using mitochondrial DNA.	Mitochondrial ND2 sequencing; Cytochrome *b* sequencing
Dávalos and Jansa (2004)	Phylogeny of the *Lonchophyllini* (Chiroptera: Phyllostomidae).	Cytochrome *b* sequencing; morphological data; sex chromosome data; restriction site data

Table 1 continued.

Author(s)*	Journal article title	Technique(s)
Goodman *et al*. (2006)	A new species of *Emballonura* (Chiroptera: Emballonuridae) from the dry regions of Madagascar.	D-loop sequencing; cytochrome *b* sequencing; morphological data
Hoffman and Baker (2001)	Systematics of bats of the genus *Glossophaga* (Chiroptera: Phyllostomidae) and phylogeography in *G. sorcina* based on the cytochrome-*b* gene.	Cytochrome *b* sequencing
Hoffman *et al*. (2003)	MtDNA perspective of chromosomal diversification and hybridisation in Peters' tent-making bat (*Uroderma bilobatum*: Phyllostomidae).	Cytochrome *b* sequencing
Hoofer and Van Den Bussche (2001)	Phylogenetic relationships of Plecotine bats and allies based on mitochondrial ribosomal sequences.	Mitochondrial 12S rRNA, $tRNA^{Val}$ and 16S rRNA gene sequencing
Hoofer and Van Den Bussche (2003)	Molecular phylogenetics of the chiropteran family Vespertilionidae.	Mitochondrial 12S rRNA, $tRNA^{Val}$ and 16S rRNA gene sequencing
Hoofer *et al*. (2003)	Molecular phylogenetics and taxonomic review of Noctilionoid and Vespertilionoid bats (Chiroptera: Yangochiroptera).	RAG-2 nuclear gene sequencing; Mitochondrial 12S rRNA, $tRNA^{Val}$ and 16S rRNA gene sequencing
Hulva and Horáček (2002)	*Craseonycteris thonglongyai* (Chiroptera: Craseonycteridae) is a rhinolophoid: molecular evidence from cytochrome *b*.	Cytochrome *b* sequencing
Jacobs *et al*. (2004)	Genetic similarity amongst phenotypically diverse little free-tailed bats, *Chaerephon pumilus*.	Cytochrome *b* sequencing

Table 1 continued.

Author(s)*	Journal article title	Technique(s)
Jacobs *et al*. (2006)	Cryptic species in an insectivorous bat, *Scotophilus dinganii*.	Cytochrome *b* sequencing; echolocation data; morphological data
Juste *et al*. (2003)	Phylogeography of Barbastelle bats (*Barbastella barbastellus*) in the western Mediterranean and the Canary Islands.	Cytochrome *b* sequencing
Kerth *et al*. (2000)	Mitochondrial DNA (mtDNA) reveals that female Bechstein's bats live in closed societies.	D-loop R1 repeat sequencing (X2); D-loop microsatellite sequencing
Kerth *et al*. (2002a)	Extreme sex-biased dispersal in the communally breeding, nonmigratory Bechstein's bat (*Myotis bechsteinii*).	Mitochondrial DNA and nuclear DNA microsatellite genotyping
Kiefer *et al*. (2002)	Conflicting molecular phylogenies of European long-eared bats (*Plecotus*) can be explained by cryptic diversity.	Mitochondrial 16S rRNA sequencing; ND1 sequencing; D-loop sequencing
Lee Jr. *et al*. (2002)	Molecular phylogenetics and taxonomic revision of the genus *Tonatia* (Chiroptera: Phyllostomidae).	Mitochondrial 12S rRNA, tRNA^{Val} and 16S rRNA gene sequencing
Lim *et al*. (2004)	Molecular differentiation of large species of fruit-eating bats (*Artibeus*) and phylogenetic relationships based on the cytochrome *b* gene.	Cytochrome *b* sequencing
Lloyd (2003)	Intraspecific phylogeny of the New Zealand short-tailed bat *Mystacina tuberculata* inferred from multiple mitochondrial gene sequences.	Mitochondrial control region sequencing; 12S rRNA sequencing; 16S rRNA sequencing; ND2 sequencing
Mayer and von Helversen (2001)	Cryptic diversity in European bats.	Mitochondrial ND1 sequencing

Table 1 continued.

Author(s)*	Journal article title	Technique(s)
Mayer *et al*. (2007)	Molecular species identification boosts bat diversity.	Mitochondrial ND1 sequencing
Miller-Butterworth *et al*. (2003)	Strong population substructure is correlated with morphology and ecology in a migratory bat.	Mitochondrial control region sequencing; microsatellite genotyping
Mucedda *et al*. (2002)	A new species of long-eared bat (Chiroptera: Vespertilionidae) from Sardinia (Italy).	Mitochondrial 16S rRNA sequencing
Newton *et al*. (2003)	Genetic population structure and mobility of two nectar-feeding bats from Venezuela deserts: inferences from mitochondrial DNA.	Mitochondrial control region sequencing
Piaggio *et al*. (2002)	Systematics of *Myotis occultus* (Chiroptera: Vespertilionidae) inferred from sequences of two mitochondrial genes.	Cytochrome *b* sequencing; cytochrome oxidase II sequencing
Porter *et al*. (2003)	Systematics of round-eared bats (*Tonatia* and *Lophostoma*) based on nuclear and mitochondrial DNA sequences.	RAG-2 nuclear gene sequencing; mitochondrial 12S rRNA, $tRNA^{Val}$ and 16S rRNA gene sequencing
Porter and Baker (2004)	Systematics of *Vampyressa* and related genera of Phyllostomid bats as determined by cytochrome-*b* sequences.	Cytochrome *b* sequencing
Ruedi and Castella (2003)	Genetic consequences of the Ice Ages on nurseries of the bat *Myotis myotis*: a mitochondrial and nuclear survey.	D-loop hypervariable domain II (HVII) sequencing; microsatellite genotyping
Russell *et al*. (2005)	Genetic variation and migration in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*).	D-loop sequencing; allozyme data; banding data; natural history data

Table 1 continued.

Author(s)*	Journal article title	Technique(s)
Russell *et al*. (2007)	Working at the interface of the phylogenetics and population genetics: a biogeographical analysis of *Triaenops* spp. (Chiroptera: Hipposideridae).	Cytochrome *b* sequencing
Salgueiro *et al*. (2004)	Mitochondrial DNA variation and population structure of the island endemic Azorean bat (*Nyctalus azoreum*).	D-loop hypervariable domain II (HVII) sequencing
Stadelmann *et al*. (2004a)	Molecular systematics of the fishing bat *Myotis (Pizonyx) vivesi*.	Cytochrome *b* sequencing
Stadelmann *et al*. (2004b)	Phylogeny of African *Myotis* bats (Chiroptera, Vespertilionidae) inferred from cytochrome *b* sequences.	Cytochrome *b* sequencing
Van Den Bussche and Hoofer (2000)	Further evidence for inclusion of the New Zealand short-tailed bat (*Mystacina tuberculata*) within Noctilionoidea.	Mitochondrial 12S rRNA, tRNA^{Val} and 16S rRNA gene sequencing
Van Den Bussche and Hoofer (2001)	Evaluating monophyly of Nataloidea (Chiroptera) with mitochondrial DNA sequences.	Mitochondrial 12S rRNA, tRNA^{Val} and 16S rRNA gene sequencing
Van Den Bussche *et al*. (2002)	Phylogenetic relationships of Mormoopid bats using mitochondrial gene sequences and morphology.	Mitochondrial 12S rRNA, tRNA^{Val} and 16S rRNA gene sequencing
Van Den Bussche and Weyandt (2003)	Mitochondrial and nuclear DNA sequence data provide resolution to sister-group relationships within *Pteronotus* (Chiroptera: Mormoopidae).	Mitochondrial 12S rRNA, tRNA^{Val} and 16S rRNA gene sequencing; cytochrome *b* sequencing; RAG-2 nuclear gene sequencing

* Full author listings in section 5.

A number of other authors have used mitochondrial DNA, either alone or in combination with other data, to resolve relationships and classifications. Stadelmann *et al*. (2004a) reconstructed the phylogenetic origins of *Myotis vivesi* relative to other *Myotis* in an attempt to resolve the contentious phylogenetic position of *M. vivesi*. Hoffmann and Baker (2001) assessed systematic relationships within the five recognized species of *Glossophaga*. Piaggio *et al*. (2002) used cytochrome *b* and cytochrome oxidase II to re-examine the systematic relationship between *M. lucifugus carissima* and *M. occultus*. Hoofer *et al*. (2003) used both nuclear and mitochondrial DNA to test different hypotheses concerning the relationships of five families in Yangochiroptera. Van Den Bussche *et al*. (2002) tested noctilionoid interfamilial and mormoopid intrafamilial relationships using a combination of DNA and morphological data. Porter *et al*. (2003) examined the systematics of the round-eared bats, *Tonatia* and *Lophostoma*. Hoofer and Van Den Bussche (2001) used 12S rRNA, $tRNA^{Val}$ and 16S rRNA gene sequencing to assess the taxonomic position of *Otonycteris*. Lee Jr. *et al*. (2002) used mitochondrial DNA sequences from members of the phyllostomid genera to help resolve the taxonomy of *Tonatia*, while Van Den Bussche and Hoofer (2000) tested the hypothesis that *Mystacina tuberculata* is more closely related to Noctilionoidea than Molossidae. Similarly, Lloyd (2003) used four different mitochondrial genes/regions to establish an intraspecific phylogeny for the New Zealand short-tailed bat, *Mystacina tuberculata* and Dávalos and Jansa (2004) used cytochrome *b* to help resolve relationships among the lonchophylline taxa. Campbell *et al*. (2004) used sequence data to infer phylogenetic relationships among the three most broadly distributed members of the genus *Cynopterus*, as well as to determine whether *C. brachyotis* represents a single widespread species or a complex of distinct lineages. Finally, Mayer and von Helversen (2001) used NADH dehydrogenase 1 (ND1) to estimate the amount of cryptic diversity among European bats. The data was in agreement with current classifications within the family and also helped elucidate the presence of new species, divergent taxa, subspecies, morphotypes and cryptic species.

Mitochondrial sequence data can also be used to investigate evolution via molecular dating, as done by Stadelmann *et al*. (2004b). Molecular dating of cytochrome *b* data revealed that the Ethiopian clade of *Myotis* diverged relatively early from other Old World *Myotis* during the *Myotis* radiation. According to Stadelmann *et al*. (2004b), this is indicative of other evolutionary processes being responsible for the poor species diversity of *Myotis* in Africa. Similarly, Van Den Bussche and Hoofer (2001) investigated four families of bat (Myzopodidae, Furipteridae, Natalidae and Thyropteridae) to ascertain whether they share a most recent common ancestor and should be grouped into the superfamily, Nataloidea. Analysis showed that monophyly was not supported and that Furipteridae, Natalidae and

Thyropteridae are most closely related to Noctilionoidea, while Myzopodidae is a basal microchiropteran lineage.

Juste *et al*. (2003) used the cytochrome *b* and control regions to answer a number of different questions regarding genetic discontinuity, barriers to gene flow and the existence of a genetically-distinct subspecies in western Palaearctic populations of *Barbastella barbastellus*. Data revealed a shallow genetic structuring of Iberian populations, found that the Gibraltar Strait does not hinder gene flow and showed the Canary Island subspecies to be endemic.

Sequence data can also be used to elucidate the more complex association of migration and genetic structure and its influence on populations and systematic classification. Russell *et al*., (2005) used a variety of data to evaluate hypotheses regarding the relationship between migration and genetic structure in *Tadarida brasiliensis* populations. An analysis of molecular variance showed no significant genetic structuring of behaviourally distinct migratory groups, and demographic analyses were consistent with population growth, except that expansion event timing differed between migratory and non-migratory populations. Porter and Baker (2004) also used mitochondrial data to examine geographic relationships within the *Vampyressa* species, while Cooper *et al*. (2001) used cytochrome *b* and NADH dehydrogenase 2 (ND2) to test the proposal that three species of large-footed *Myotis* occur in Australia. Data, however, revealed that only one species occurs in Australia, *M. macropus*, and that this is taxonomically distinct from *M. adversus* of Indonesia. Castella *et al*. (2001) used mitochondrial and nuclear data to investigate the dispersal behaviour of the bat *M. myotis* in central Europe; data revealed that local haplotypic variability is largely influenced by colonisation, and that although females are faithful to their natal colony, movement of males and females does occur outside the breeding period.

Goodman *et al*. (2006) used both morphological and genetic data to describe a new species of *Emballonura* from Madagascar. Quantitative and qualitative analyses were initially done to describe the new species, and genetic analyses then done to assess whether they corroborated the taxonomic conclusions drawn from the initial analyses. Benda *et al*. (2003), like Goodman *et al*. (2006), used morphological and molecular data for the description of a first record of *Myotis alcathoe* in Slovakia, where the species was first identified based on physical characteristics and confirmed by sequencing part of the ND1 gene.

1.5 Methods in genetic data analysis

1.5.1 Genetic distance models

In order to determine evolutionary distances between pairs of sequences, the number of nucleotide (or amino acid) substitutions occurring between them is calculated (Nei and Kumar, 2000). Evolutionary distances are fundamental for the study of molecular evolution and are useful for phylogenetic reconstructions and the estimation of divergence times (Huelsenbeck and Rannala, 1997; Nei and Kumar, 2000).

Two simple methods of assessing distance involve calculating the number of sites at which the two compared sequences differ or the proportion (p) of nucleotide sites at which two sequences differ (Nei, 1987; Nei and Kumar, 2000). These do not allow for corrections for multiple substitutions at the same site, substitution rate biases or differences in evolutionary rates among sites (Nei, 1987).

The Jukes-Cantor (1969) model produces a maximum-likelihood estimate of the number of nucleotide substitutions between two sequences by assuming an equal rate of nucleotide substitution across all four nucleotides and equal nucleotide frequencies (Li and Graur, 1991; Hillis *et al*., 1994; Nei and Kumar, 2000; Hall, 2001). It does not correct for a higher transitional as opposed to transversional, substitution rate (Li and Graur, 1991). There is a variation of this model, the Jukes-Cantor Gamma distance, where rate variation among sites is modelled using the Gamma distribution (Nei and Kumar, 2000).

Kimura's Two Parameter model (1980) was a widely used model due to the lack of other models at the time. It corrects for multiple hits, taking into account transitional and transversional substitution rates while assuming that nucleotide frequencies are equal and that rates of substitution are unvaried among sites (Li and Graur, 1991; Hillis *et al*., 1994; Nei and Kumar, 2000; Hall, 2001).

When nucleotide frequencies are unequal, as is most often the case, the Tajima-Nei distance (Tajima and Nei, 1984) can give a better estimate of nucleotide substitution numbers than the Jukes-Cantor distance, although this assumes equal substitution rates among sites and between transitional and transversional substitutions (Nei and Kumar, 2000). Other variations of this model include: Tajima-Nei distance (Gamma rates); the Tajima-Nei Distance (Heterogeneous patterns), where the assumption of substitution pattern homogeneity is

relaxed and used when nucleotide frequencies differ between sequences; and Tajima-Nei Distance (Gamma Rates and Heterogeneous patterns), which combines the two afore-mentioned models.

Tamura's 3-parameter model (1992) corrects for multiple hits, taking into account differences in transitional and transversional rates and G+C-content bias and assumes an equality of substitution rates among sites (Nei and Kumar, 2000). Other variations include Tamura 3-parameter (Gamma), Tamura 3 parameter (Heterogeneous patterns), and Tamura 3 parameter (Gamma rates and Heterogeneous patterns).

The Tamura-Nei (1993) is another model that assumes equality of substitution rates among sites but corrects for multiple hits and takes into account differences in substitution rate between nucleotides and the inequality of nucleotide frequencies (Nei and Kumar, 2000). It distinguishes between transitional substitution rates between purines and transversional substitution rates between pyrimidines. Other versions of this model include the Tamura-Nei Gamma distance, Tamura-Nei distance (Heterogeneous Patterns) and the Tajima-Nei Distance (Gamma Rates and Heterogeneous patterns) (Nei and Kumar, 2000).

Hasegawa, Kishino and Yano introduced the HKY85 model in 1985 which stipulates that pyrimidine and purine transitions have the same rate. This model is considered to be a hybrid of Kimura's 2-parameter and the equal input model, and takes into account both the transitional/transversional and GC content biases (Nei and Kumar, 2000).

The General Time Reversible (GTR) model, by comparison, considers two independent rate parameters, i.e. the ratio of transitions and transversions, and ratio of the two types of transitions. There are six different rates available and time reversible models assume that the overall instantaneous rate of change from base *a* to base *b* is the same as base *b* to base *a* (Hall, 2001).

When investigating closely-related sequences, simpler distance methods, such as Jukes-Cantor and Kimura's 2-parameter, can be used because of the smaller variance (Nei and Kumar, 2000). Simpler distance models can also be used to infer phylogenetic trees since it is not guaranteed that the more sophisticated distances are more efficient in obtaining the correct topology than simpler ones (Nei and Kumar, 2000).

In order to determine which model best suits a dataset, the program MrModeltest version 2 (Nylander, 2004) can be used. MrModeltest v2 (Nylander, 2004) tests the likelihood scores

generated through PAUP 4.0b10 (Swofford, 1993) in order to determine which model of evolution, of the 24 available, is most appropriate for the data, and may be used in conjunction with Bayesian analysis.

1.5.2 Phylogenetic data

Relationships between organisms can be deduced by comparing homologous sites (Li and Graur, 1991; Nei and Kumar, 2000; Stearns and Hoekstra, 2000; Vandamme, 2003). Orthologous genes (homologous genes in different species that have started a separate evolution due to speciation) are always preferred in studies because they can be found in different species and traced back to a common ancestor (Li and Graur, 1991; Nei and Kumar, 2000). Orthologous genes can therefore provide information regarding speciation events.

Other factors that need to be taken into consideration are mistaken homology as a result of convergent or parallel evolution, sequence reversals, multiple hits and parallel substitution (Vandamme, 2003). These events result in homoplasy, which disturbs the linear relationship between time of evolution and sequence divergence (Vandamme, 2003).

Sequences are aligned using appropriate software packages to form columns of homologous sites in the alignment and manual editing is done to obtain the best alignment, i.e. indels and sequence ends are removed to eliminate ambiguity and give each sequence equal length (Li and Graur, 1991; Nei and Kumar, 2000; Vandamme, 2003). Obtaining a good initial alignment is one of the most important steps when constructing a phylogenetic tree.

To ensure that no bias or errors occur, it is standard procedure to compare sequence data from the study against data from another source, i.e. reference sequences. This need and the availability of DNA sequences has prompted the creation of online databases such as the NCBI, the National Library of Medicine (NLM) and the European Molecular Biology Organization (EMBO) (Bisby, 2000; Stearns and Hoekstra, 2000; Bottu and Ranst, 2003; Vandamme, 2003). For this particular study, reference sequences were obtained from the NCBI.

Choosing an appropriate outgroup may prove to be difficult since a distantly-related outgroup may acquire multiple substitutions at the same sites, whereas a closely-related outgroup might not be an outgroup at all, thereby affecting the correctness of the tree (Li and Graur, 1991; Vandamme, 2003). It is standard practice that more than one outgroup should be used to improve the estimate of tree topology (Vandamme, 2003).

The date of origin of a species can be resolved using the sequence variability of different alleles of a gene, where the coalescence time depends on the extinction of said alleles (Vandamme, 2003). Coalescence time is defined as the time since the most recent common ancestor and is estimated on the basis that sequence divergence increases over time, and time runs in one evolutionary direction (Page, 1996; Tavaré, 1997; Stearns and Hoekstra, 2000; Vandamme, 2003). Statistical methods such as maximum likelihood and the Bayesian model take into account the rate of variation across lineages in order to calculate divergence times (Felsenstein, 2001; Drummond *et al*., 2006). Divergence times, calculated by a molecular clock, are based on the assumption that divergence accumulates linearly over time (Li and Graur, 1991; Huelsenbeck and Rannala, 1997; Nei and Kumar, 2000; Stearns and Hoekstra, 2000; Vandamme, 2003). Relaxed molecular clocks, as implemented via Bayesian modelling, explore a weighted range of tree topologies whilst estimating the parameters of the chosen substitution model (Felsenstein, 2001; Drummond *et al*., 2006). It should be noted, however, that evolutionary rate is dependent on many factors including metabolic rate, generation time, bottleneck events and selective pressure; therefore, it has been argued that it is impossible for an absolute molecular clock to exist (Li and Graur, 1991; Huelsenbeck and Rannala, 1997; Stearns and Hoekstra, 2000; Vandamme, 2003; Ruedi and McCracken, 2009).

1.5.3 Methods of phylogenetic tree construction

Methods for constructing phylogenetic trees from molecular data can be grouped according to two criteria: whether discrete character-states or a distance matrix of pairwise dissimilarities are used; and whether OTUs are clustered step-wise, resulting in only one best tree or whether all theoretically possible trees are considered (Li and Graur, 1991; Karp *et al*., 1997; Vandamme, 2003; Ruedi and McCracken, 2009). Although reconstructing phylogeny may seem simple, it is rarely possible to verify that the right conclusion has been reached, since the tree is inferred (Vandamme, 2003).

Character-state methods use any set of discrete characters that are analysed separately and usually independently of each other, e.g. with sequence data, each nucleotide position in the aligned sequences is a character (Vandamme, 2003). Because character-state methods retain the original character status of the taxa, they can be used to reconstruct the character state of ancestral nodes (Vandamme, 2003). By comparison, distance-matrix methods calculate a measure of dissimilarity of each pair of OTUs to produce a pairwise distance matrix from which phylogenetic relationships are then estimated (Vandamme, 2003).

Distance-matrix methods yield consistent results, multiple hits can be scored and the methods are less computer-intensive, making it easier to compare many taxa (Vandamme, 2003). However, character states of ancestral nodes cannot be reconstructed with distance-matrix methods since the original character states of the taxa is discarded (Vandamme, 2003).
Unlike exhaustive-search methods, clustering is usually fast even when the number of OTUs is large (Nei and Kumar, 2000; Vandamme, 2003). Although only one tree is produced, statistical methods can estimate the confidence that this tree represents the true phylogeny. Most distance-matrix methods use step-wise clustering to generate the best tree, whereas most character-state methods implement methods such as heuristic and exhaustive-searches and quartet puzzling (Vandamme, 2003; Felsenstein, 2004).

Exhaustive search methods examine the theoretically possible tree topologies for a given number of taxa and then use certain criteria to choose the best one (Karp *et al*., 1997; Nei and Kumar, 2000; Vandamme, 2003). In maximum-likelihood methods the likelihood for any possible combination of tree topology and branch lengths is calculated and those topologies and branch lengths that maximize this likelihood are the maximum-likelihood estimates (Whelan *et al*., 2001). Based on the given data and a specific evolutionary model, a large number of trees is produced and the probability that each is representative of the true phylogeny is estimated (Nei and Kumar, 2000; Vandamme, 2003). The disadvantage with this method is that as the number of taxa increases, so too does the number of trees and therefore the computing time (Nei and Kumar, 2000; Vandamme, 2003; Ruedi and McCracken, 2009).

Bayesian analysis is a relatively new strategy and, although similar to maximum likelihood, it constructs the best set of trees and the entire probability distribution of likelihoods on the basis that the data and evolutionary models are specified (Nei and Kumar, 2000; Hall, 2001; Holder and Lewis, 2003). Bayesian analysis is implemented with a program, MrBayes (Huelsenbeck and Ronquist, 2001) which uses the Markov Chain Monte Carlo method to converge on a set of likely trees and thus has a better perspective of likelihood (Hall, 2001; Holder and Lewis, 2003). This program is less computer-intensive than other likelihood methods and can be run in a lesser amount of time (Holder and Lewis, 2003).

Most of the algorithms discussed here produce strictly bifurcating trees, i.e. the internal node is connected to only three other branches, thereby assuming that, during the course of evolution, any ancestral nodes can only give rise to two separate lineages (Nei and Kumar, 2000; Vandamme, 2003). There are, of course, exceptions, and such is the case where explosive evolutionary radiation takes place, yielding a non-strictly bifurcating/multifurcating

tree when analysed (Nei and Kumar, 2000; Ruedi and McCracken, 2009). For those relationships that are not strictly bifurcating, it has been suggested that networks, which can accommodate reticulate evolution and polytomies, best represent these relationships (Nei and Kumar, 2000; Ruedi and McCracken, 2009).

1.5.4 Estimating the reliability of phylogenetic trees

Three commonly-used methods of assessing the reliability of a tree node are bootstrap analysis, jack-knifing and the decay index/Bremer support (Stearns and Hoekstra, 2000; DeBry, 2001; Van de Peer, 2003). Reliability estimation in Bayesian analysis, however, is given in the form of posterior probabilities.

Felsenstein's (1985) bootstrap analysis is one of the most commonly-used tests for checking the reliability of an inferred node (Nei and Kumar, 2000; Stearns and Hoekstra, 2000). Sites in a sequence are resampled and trees reconstructed; this is repeated a specified number of times. The percentage of times that each node is present is noted; this is the bootstrap value (Nei and Kumar, 2000; Stearns and Hoekstra, 2000). As a general rule, bootstrap values of 95% or higher are considered to indicate reliability, whilst those with support of 70% or lower should be viewed with caution (Nei and Kumar, 2000; Stearns and Hoekstra, 2000).

Jack-knifing evaluates the reliability of specific clades in the phylogenetic tree by randomly deleting a percentage of the sites, as determined by the user, from the original data set, hence the alternative name, delete-half (in the case of 50% deletion) or delete-fraction jack-knifing (Van de Peer, 2003). The resulting sequence, from which trees are constructed, is half the length of the original; this procedure is continually repeated until an estimate of reliability can be made, i.e. the frequencies of nodes are counted from the reconstructed trees (Van de Peer, 2003).

The decay index gives a measure support at a node of interest in a phylogenetic tree and alludes to insufficiently-supported nodes of a tree (Bremer, 1988, 1994; O'Grady *et al*., 2001). It reports the number of extra steps needed to lose a clade that is found in the most-parsimonious tree (DeBry, 2001). Any clade not found in the strict consensus of all most-parsimonious trees has a Bremer support value of 0 and clades not found in the strict consensus of all trees one step longer than the most-parsimonious trees has a Bremer support value of 1, and so on until a shortest tree that does not contain any clade is found.

Bayesian inference is based on the concept of posterior probabilities, i.e. probabilities that are estimated, based on a model, after learning something about the data (Hall, 2001). In Bayesian analysis (section 1.5.3), instead of a single tree, a set of trees of roughly equal likelihoods (within 95% confidence of each other) is produced. From these trees, each clade is assigned a frequency, and this frequency is virtually identical to the probability of that clade. Thus, the probabilities of each clade are an estimate of the reliability of the tree structure.

1.5.5 Phylogeography

In 1987, Avise and colleagues introduced the concept of phylogeography to the field of evolutionary genetics and, over the past three decades, its use in current literature has grown. Phylogeography, as the name implies, is concerned with phylogenetic components of the spatial distributions of gene lineages, i.e. time and space are the axes of phylogeography onto which gene genealogies are mapped (Avise, 2000; Ruedi and McCracken, 2009). Phylogeographic investigations encompass many fields of study, since the analysis and interpretation of lineage distributions usually requires extensive input from molecular genetics, population genetics, ethology, demography, phylogenetic biology, palaeontology, geology and historical geography (Avise, 2000).

Of all phylogeographic studies done, a large majority of these have utilized animal mtDNA, since animal mtDNA is maternally-inherited, evolves rapidly and demonstrates non-recombinant inheritance (Avise, 2000). These advantageous characteristics of mtDNA provide haplotype information that can be sorted phylogenetically within a species, yielding an intraspecific phylogeny. The challenge is to interpret the status of gene trees as meaningful components of extended organismal pedigrees.

Animal species living in spatially-structured demes may exchange genes with neighbouring populations, which has a homogenizing effect on the population and may thus show insignificant genetic structuring (Ruedi and McCracken, 2009). If there is no gene flow, however, then the converse is true and there will be a high level of genetic structure in the population. Heterogeneity among populations is created by various methods, including mutation and the loss of genetic diversity from generation to generation, i.e. genetic drift (Ruedi and McCracken, 2009).

The most common phylogeographic approach is to construct a haplotype network onto which geographical locations are overlayed (Emerson and Hewitt, 2005). When the genealogical

network is combined with population frequency and geographic distribution, inferences on the evolutionary history of taxa can be made (Emerson and Hewitt, 2005). Genetic data can be used to estimate the demographic history of a population e.g. historical bottlenecks or expansions; ancestral population size; location of refugia; approximate divergence dates and extent of migration, gene flow and fragmentation (Emerson and Hewitt, 2005).

Phylogeographical patterns were placed into five categories by Avise (2000). Category I is characterized by deep gene trees in distinct geographic areas, and applies to situations where major haplotypes are found in distinct geographic areas (Avise, 2000; Ruedi and McCracken, 2009). In category II, deep genes trees coexist in the same local population. This can occur in various situations, one of which is where conspecific populations have accumulated differences while separated, but have recently come into contact again. In category III, shallow gene trees are found in distinct geographic areas which is typical of populations that have recently expanded from a common area and have low subsequent gene flow. Category IV comprises shallow gene trees coexisting in same local population and is a common pattern seen in local populations that are linked by high levels of gene flow. Category V is intermediate between III and IV, where there is a mixture of common, widespread and ancestral haplotypes existing with rare and localized variants. These categories are intended to aid in classifying the patterns found among populations that are presumed to be conspecific. Avise has also modified these categories to suit those situations where there is strong phenotypic divergence. Once phylogenetic trees and haplotype networks have been constructed, these categories can be applied to the patterns found and insight given into the history of the organism in terms of time and space.

1.5.6 Methods used in this study

In this study, two complementary methods were used. These were neighbour-joining, a phenetic step-wise clustering method in which the reliability of nodes on the tree was estimated by bootstrap resampling analysis, and Bayesian analysis, a character-state exhaustive search method in which the reliability of nodes on the tree was estimated as posterior probabilities. In addition, haplotype networks were created in order to display polytomies and reticulations.

1.6 Rationale, aims and objectives

Bats are the second most diverse order of mammals worldwide and are considered to be key components of biological diversity, playing fundamental roles in both ecology and the economy (Taylor, 1999a, 2000b; Hutson, 2002; Mickleburgh *et al*., 2002). In certain areas, bats are considered keystone species in pollination and seed dispersal, whereas other species may be important for insect pest control, such as *Otomops* individuals which provide a service to farmers by destroying insects that eat crops (Fenton, 1997; Taylor, 1999a; Burland and Worthington Wilmer, 2001; Andriafidison *et al*., 2006). A great number of bat species remain poorly studied and are misunderstood in many parts of the world, resulting in a lack of general information about them and a worldwide decline in numbers over the years (Taylor, 2000b; Mickleburgh *et al*., 2002). In addition, currently-accepted classifications might be erroneous if they are based on meagre data (Warner *et al*., 1974; Avise, 1989). This leads to controversy and debate over the validity of classifications, as has been the case for the genus *Otomops*.

Classification of *Otomops* species in Africa and Madagascar has long been debated, with different species or possible subspecies said to exist. *Otomops martiensseni* and *O. madagascariensis* are thought to be the two extant species on mainland Africa and Madagascar, respectively (Peterson *et al.,* 1995; Al-Jumaily, 1999; Taylor, 2005). *Otomops icarus* from Durban, South Africa is considered to be a synonym of *O. martiensseni*, although some still view it as being a separate species (Gelderblom *et al*., 1995; Al-Jumaily, 1999).

Otomops martiensseni has been classified as a "Vulnerable" species, but, without proper data, it is impossible to determine whether this situation has changed at all (Isaac *et al*., 2004). It has been suggested that *O. martiensseni* in KwaZulu-Natal may, instead, be a threatened species with stabilizing or increased abundance, and hence a candidate for inclusion on a "blue" data list (Meester *et al*., 1986; Gigon *et al*., 2000; Fenton *et al*., 2002). But this classification is uncertain without appropriate data

The aim of this project was to investigate the genetic diversity of *Otomops* in Africa and Madagascar. The information obtained from this study will be used to appropriately classify Afrotropical and Malagasy *Otomops* and, therefore, provide the foundation to address their conservation status.

The specific objectives of this project were:

(1) To assess genetic diversity using two contrasting, i.e. slower-evolving and faster-evolving mitochondrial molecular markers

(a) The complete cytochrome *b* gene

(b) The D-loop region.

(2) To infer phylogenetic patterns using appropriate phenetic and cladistic (i.e. Bayesian inference) methods.

(3) To establish the number of haplotypes present, to analyse the relationships among these haplotypes, and to look for phylogeographic patterns in the distribution of haplotypes.

(4) To determine the number of *Otomops* species presently in existence in Africa and Madagascar and suggest amendments to current classifications, if required.

(5) To assess and make recommendations regarding the need for conservation of *Otomops* species in Africa and Madagascar.

2. MATERIALS AND METHODS

2.1 Sample collection

The genetic variation in *Otomops martiensseni* and *O. madagascariensis* was investigated using mitochondrial cytochrome *b* gene sequencing (n=48) and D-loop sequencing (n=50). For cytochrome *b* and D-loop sequencing, samples were obtained from several localities/colonies, including Durban (South Africa), Burundi, Kenya, Ethiopia, Zimbabwe, Yemen, Ivory Coast, Tanzania and Madagascar. Dr Peter Taylor from the Durban Natural Science Museum, South Africa, and students and associates from the Bat Interest Group of KZN and UKZN, provided the South African and Kenyan (via the National Museum of Kenya) samples. Dr Petr Benda of the Department of Zoology in the Narodni Museum, Czech Republic, provided the Ethiopian samples, while Dr Jakob Fahr and the Senckenberg Museum in Germany provided the samples from Yemen, Ivory Coast and Tanzania. The Royal Ontario Museum provided the sample from Zimbabwe, while the Field Museum of National History in Chicago, USA provided the samples from Burundi and Madagascar, courtesy of Dr Steven Goodman.

Samples were obtained either via museum collections or through live capture, using mist nets or other devices. Collection was conducted under the Ezemvelo KZN Wildlife and 'ToPS' permit (OP 853/2009). In the case of museum collection samples, tissues were collected from the liver, heart, kidney, lungs or thoracic muscle and preserved in ethanol (80% and higher), formalin or lysis buffer (Appendix 1). Samples preserved in formalin were washed thoroughly with the buffer recommended in the QIAGEN DNeasy® Tissue Kit handbook (QIAGEN Inc.). Where bats were released after capture, tissue was taken in the form of wing punches that were then preserved in 90% ethanol. Wing punches were taken using a 3mm biopsy punch. Sample details are summarized in Table 2. It is of interest to note that the sample taken from Tanzania was found close to the holotype locality of the species, *O. martiensseni*.

Table 2. Locality, specimen details and Genbank accession numbers of sampled individuals of *Otomops*.

Species	Geographic origin	Co-ordinates	Tissue type	Museum #	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops martiensseni sensu lato	RSA: Ballito, 20km north Durban	29.533°S 31.211°E	Wing punch	N/A	N/A	EF216411	
			Wing punch	N/A	N/A	EF216412	
	RSA: 473 Silverglen Dr., Chatsworth, Durban	29.928°S 30.90°E	Wing punch	N/A	N/A	EF216406	
			Wing punch	N/A	N/A	EF216407	EF216451
			Wing punch	N/A	N/A	EF216408	
			Wing punch	N/A	N/A	EF216409	EF216452
			Wing punch	N/A	N/A	EF216410	EF216453
	RSA: 31 Ann Arbour Rd, Scottburgh, 25km south Durban	30.300°S 30.745°E	Wing punch	N/A	F	EF216418	
			Wing punch	N/A	M	EF216419	
	RSA: Ocean View Farm, Park Rynie, 30km south Durban	30.339°S 30.731°E	Thoracic muscle	DM8031	F	EF216426	EF216446
			Thoracic muscle	DM8032	F	EF216427	
	RSA: Brynderyn Flats. Morningside, Durban	29.864°S 31.040°E	Wing punch	DM7909	M	EF216424	EF216444

Table 2 continued.

Species	Geographic origin	Co-ordinates	Tissue type	Museum #	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops martiensseni sensu lato	RSA: Kingsway School, Amanzimtoti, Durban	30.039°S 30.894°E	Wing punch	DM7914	M	EF216425	EF216445
	RSA: 8 Buys Rd, Pinetown, Durban	29.757°S 30.639°E	Wing punch	DM8421	F	EF216413	EF216447
			Wing punch	N/A	F	EF216414	EF216448
			Wing punch	N/A	F	EF216415	EF216449
			Wing punch	N/A	F	EF216416	EF216450
			Wing punch	N/A	M	EF216417	
	Ethiopia: S of Omar Caves, 40 km west Ginir, Bale Province	6.9°N 40.859°E	Wing punch	NMP91203	F	EF216429	EF216461
			Foetal tissue	NMP91203	N/A	EF216430	EF216462
			Wing punch + muscle	Pb2512	F	EF216431	EF216463
			Foetal tissue	Pb2512x	N/A	EF216432	EF216464
			Wing punch + muscle	NMP91202	F	EF216433	EF216465

Table 2 continued.

Species	Geographic origin	Co-ordinates	Tissue type	Museum #	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops martiensseni sensu lato	Ethiopia: S of Omar Caves, 40 km west Ginir, Bale Province	6.9°N 40.859°E	Foetal tissue	NMP91202	N/A	EF216434	EF216466
			Wing punch	NMP91201	F	EF216435	EF216467
			Foetal tissue	NMP91201	N/A	EF216436	EF216468
	Kenya: Ithundu Caves, Chyulu Hills, Kiboko, Makuena District	2.358°S 37.717°E	Liver/ kidney	NMK15462	M	EF216428	EF216455
			Liver/ kidney	NMK15461	F	EF216438	
			Liver/ kidney	NMK15464	F	EF216439	EF216456
			Liver/ kidney	NMK15463	M	EF216440	EF216457
			Liver/ kidney	NMK15459	M	EF216441	EF216458
			Liver/ kidney	NMK15465	M		EF216459
			Liver/ kidney	NMK15460	F	EF216442	EF216460
	Burundi: 2.3 km N, 0.7 km W Teza, Kibira NP	3.200°S, 29.550°E	Unknown	FMNH137633	M	EF216423	EF216443
	Zimbabwe: Hostes Nicholle Institute, Sengwa	18.167°S 28.217°E	Thoracic muscle	ROM83979	M	EF216421	

Table 2 continued.

Species	Geographic origin	Co-ordinates	Tissue type	Museum #	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops martiensseni sensu lato	Yemen: Hud Sawa Cave, Al Mawhit	15.483°N 43.533°E	Muscle + skin	SMF87650	M	EF216437	EF216469
	Ivory Coast: Comoé NP	8.733°N, 3.3848°W	Heart + lung	SMF92049	M	EF216420	EF216454
	Tanzania: Tongwe F.R, Tanga, Muheza District	5.317°S, 38.733°E	Heart + liver	SMF79542	M	EF216422	
Otomops madagascariensis	M: Parc National de Bemaraha, Province de Mahajanga,	18.695°S 44.717°E	Thoracic muscle	FMNH169667	M	EF216373	EF216384
			Thoracic muscle	FMNH169694	M	EF216374	EF216385
			Thoracic muscle	FMNH169695	M	EF216375	EF216386
	M: Parc National de Isalo, Province de Fianarantsoa	22.540°S 45.38°E	Thoracic muscle	FMNH166073	F	EF216372	EF216383
			Thoracic muscle	UABDA – SMG10996	F		EF216401
	M: Grotte d’Ambanila, Province de Toliara	23.548°S 43.747°E	Thoracic muscle	FMNH172940	M	EF216377	EF216388
			Thoracic muscle	FMNH172934	M	EF216378	EF216389

Table 2 continued.

Species	Geographic origin	Co-ordinates	Tissue type	Museum #	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops madagascariensis	M: Grotte d'Ambanila, Province de Toliara	23.548°S 43.747°E	Thoracic muscle	FMNH172936	M		EF216394
			Thoracic muscle	FMNH172942	M		EF216395
	M: Grotte d'Bishihiko, Province de Toliara	23.54°S 43.77°E	Thoracic muscle	FMNH172944	F	EF216379	EF216390
			Thoracic muscle	FMNH172948	M	EF216380	EF216391
			Thoracic muscle	FMNH172951	M		EF216392
			Thoracic muscle	FMNH172953	F		EF216393
			Thoracic muscle	FMNH172947	M		EF216396
	M: Réserve Spéciale d'Ankarana, Province d'Antsiranana	12.942°S 49.055°E	Thoracic muscle	FMNH176354	M	EF216381	EF216397
			Thoracic muscle	FMNH176355	M		EF216398
			Thoracic muscle	FMNH176356	M		EF216399
			Thoracic muscle	FMNH176357	M	EF216382	EF216400
	M: Réserve Spéciale d'Analamerana, Province d'Antsiranana	12.712°S 49.474°E	Thoracic muscle	FMNH172938	M	EF216376	EF216387
			Thoracic muscle	FMNH178849	F		EF216402

Table 2 continued.

Species	Geographic origin	Co-ordinates	Tissue type	Museum #	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops madagascariensis	M: Réserve Spéciale d'Analamerana, Province d'Antsiranana	12.712°S 49.474°E	Thoracic muscle	FMNH178850	F		EF216403
			Thoracic muscle	FMNH178851	F		EF216404
			Thoracic muscle	FMNH178852	M		EF216405
Otomops wroughtoni	Cambodia: Chhep District, Preah Vihear Province	13.59°N 105°16°E	Unknown	HZM3.33440	M		
Otomops cf. *formosus*	Philippines: Barangay Balbalasang, Kalinga Province, Luzon Is	17.458°S 121.0683°E	Unknown	FMNH67240	M		

RSA: Republic of South Africa; M: Madagascar; DM: Durban Natural Science Museum; NMP: National Museum of the Czech Republic, Prague; NMK: National Museum of Kenya, Nairobi; FMNH: Field Museum of Natural History, Chicago; ROM: Royal Ontario Museum, Toronto; SMF: Senckenberg Museum, Frankfurt; UADBA: Université d'Antananarivo, Département de Biologie Animale, Antananarivo; HZM: Harrison Zoological Museum, Sevenoaks.

2.2 DNA isolation

Otomops DNA was isolated from tissues preserved in ethanol (denatured or 80% and higher), formalin or EDTA using the QIAGEN DNeasy® Tissue Kit (QIAGEN Inc.). Isolation was carried out according to the methods outlined in the handbook provided. A maximum of 25 mg of tissue was used, as overloading may have resulted in a decreased yield of DNA. The tissue type was also first determined because certain tissues, e.g. spleen, require a lower sample weight to be used for the procedure. Most tissue samples comprised wing membrane, muscle, liver or kidney, although foetal material was also used for certain juvenile samples. For optimal recovery and stability, DNA was eluted using the buffer provided and stored in Eppendorf tubes. DNA was stored in a –20 °C freezer to ensure prolonged integrity, whilst working stocks were kept at 4 °C.

2.3 DNA Quantification

2.3.1 Evaluation of DNA integrity

The integrity of the DNA was assessed visually via agarose gel electrophoresis. Thus it was possible to check that the DNA was of a high molecular weight which is necessary for the polymerase chain reaction (PCR). A 1% (w/v) agarose gel was made using 0.5x TBE, to which ethidium bromide (EtBr) (0.05mg/ml) was added (Appendix 1). Approximately 5 µl of each DNA sample was mixed with 3 µl of marker dye, i.e. 6x Orange Loading Dye Solution (Fermentas Life Sciences) or bromophenol blue (Appendix 1) before being loaded and run in 0.5x TBE buffer containing EtBr (0.05 mg/ml). Samples were compared to markers of known molecular weight (MW), i.e. Molecular Weight Marker III (Roche Molecular Biochemicals) and O'GeneRuler™ 100 bp DNA Ladder (Fermentas Life Sciences). Electrophoresis was conducted at 70 Volts for 2 hours. Ethidium bromide stained bands were visualized by trans-illumination with short wave UV light on a Uvitec transilluminator. The image was captured using an Uvitec camera and saved to disk using the Uvisave facility. Images of the gel were printed using a Mitsubishi Video Copy Processor P66E (Mitsubishi).

2.3.2 Measurement of DNA concentration

Fluorometry was used to measure the concentration of double-stranded DNA in a sample. Hoechst 33258, a fluorescent DNA-binding dye (Appendix 2), was added to the DNA sample. The fluorescence was then measured against that of a standard, i.e. a known concentration of

calf thymus DNA (Mathews *et al*., 2000). From this, it was possible to determine the DNA concentration.

Fluorescence was measured using a Hoefer DyNA Quant™ 200 Fluorometer (Amersham Biosciences) according to manufacturer's instructions, using the appropriate low range assay solution (Appendix 2) and calf thymus DNA standard (Appendix 2). Concentrations were measured in this way after both DNA isolation and gel extraction.

In addition to fluorometry, agarose gel electrophoresis was used to assess DNA concentrations. Agarose gel electrophoresis was carried out as described in section 2.3.1 and the fluorescence of the sample DNA then compared to the fluorescence intensities of known quantities of DNA in the individual bands of the marker.

2.4 Polymerase Chain Reaction (PCR) amplification and sequencing

2.4.1 Mitochondrial cytochrome *b* gene amplification

Due to the relatively long length of the cytochrome *b* gene (1140 bp), it was PCR-amplified as two overlapping fragments (Saiki *et al*., 1988) (Fig. 2). These fragments were amplified by two primer pairs (Irwin *et al*., 1991): L 14723 (5'- ACC AAT GCA ATG AAA AAT CAT CGT T -3') and H 15553 (5'- TAG GCA AAT AGG AAA TAT CAT TCT GGT -3') for the 5' portion of the sequence; and L 15146 (5'- CAT GAG GAC AAA TAT CAT TCT GAG -3') and H 15915 (5'- TCT CCA TTT CTG GTT TAC AAG AC -3') for the 3' portion. Although the initial amplifications gave the desired fragments, in order to get unambiguous sequence data, primers L14723 and L46RC (5'- CTC AGA AAG ATA TTT GTC CTC ATG -3') were used to provide additional data on the first 400 bp of sequence and primers H53RC (5'- ACC AGA ATG ATA TTT CCT ATT TGC CTA -3') and H15915 on the last 400 bp of sequence. Amplifications were performed in 25 µl reactions containing 30 – 60 ng template DNA (totalling 9 µl when mixed with sterile water), 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Roche Diagnostics), 0.2 µl 5 U/µl *Taq* polymerase (Super-Therm) and 4 µl of 6 µM primer dilution (forward and reverse) per reaction. The thermal cycling parameters used were as follows: 94 °C for 4 min; followed by 36 cycles of (94 °C for 40 s, 50 °C for 45 s and 72 °C for 40 s); followed by 72 °C for 10 min.

2.4.2 Mitochondrial D-loop region amplification

The D-loop region was PCR-amplified as a single fragment using primers P (forward) (5'-TCC TAC CAT CAG CAC CCA AAG C -3') and E (reverse) (5'- CCT GAA GTA GGA ACC AGA TG -3') (Wilkinson and Chapman, 1991) (Fig. 2). Amplifications were performed in 25 µl reactions as described in section 2.4.1. The thermal cycling parameters were as follows: 95° C for 2 min; followed by 40 cycles of (95° C for 60 s, 55° C for 90 s and 72° C for 120 s); followed by 72° C for 7 min.

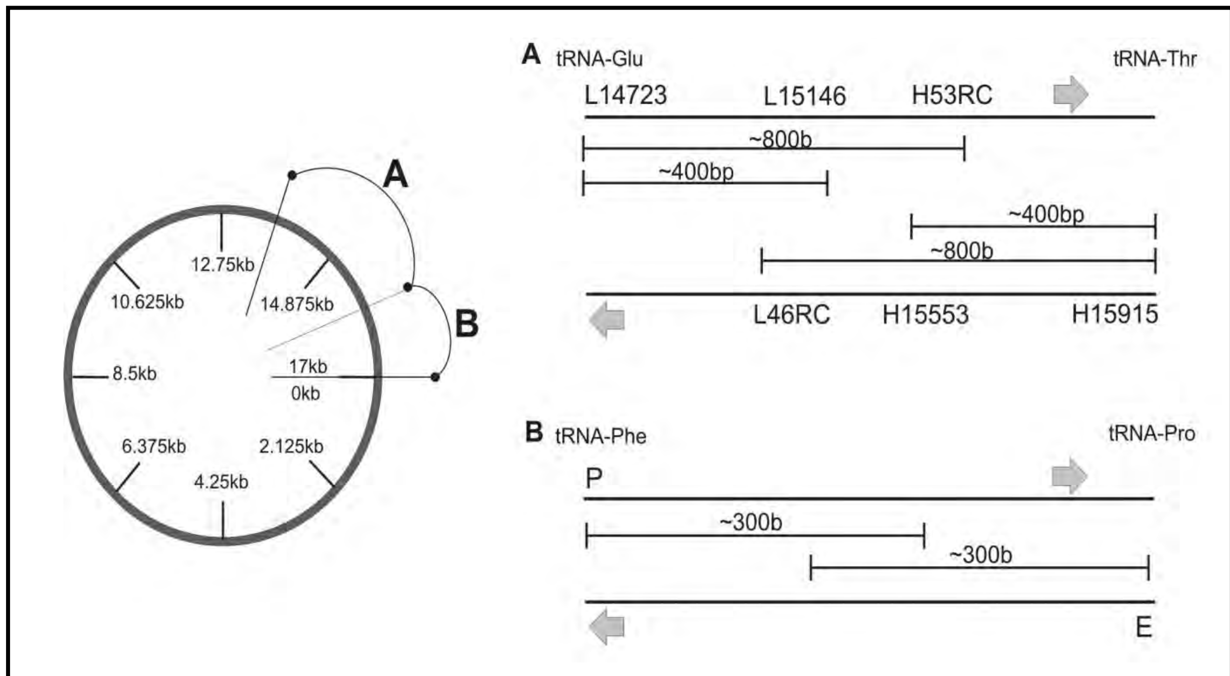

Figure 2. Representation of the circular structure of vertebrate mitochondrial DNA and the relative positions of the cytochrome *b* and D-loop regions. A: Representation of primer positions used in cytochrome *b* region amplification. B: Representation of primer positions used in D-loop region amplification. Large arrows indicate the direction in which amplification occurred using each of the primers.

2.4.3 DNA recovery and concentration measurement

Amplified fragments were separated via electrophoresis (as described in section 2.3.1) on 1.7% (w/v) agarose gels. Due to the frequent presence of multiple bands, the appropriate bands, as determined by molecular weight (cytochrome *b*: ~800 bp; D-loop: ~300 bp) relative to the marker, were excised from the gel using a sharp, sterile scalpel on a UV transilluminator, and purified using the QIAquick® Gel Extraction Kit (QIAGEN Inc.)

according to manufacturers instructions. The band was sequenced to confirm its identify before the other fragments were quantified and concentrations checked (as described in section 2.3.2) and sequenced.

2.4.4 DNA sequencing

Otomops material was sequenced directly from purified PCR products using the primers used for the initial amplifications. Sequencing was carried out using the Big-Dye™ Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems) in a 10 µl reaction containing 3 µl of ready reaction mix, 7 pmol of primer and 50 – 100 ng of purified PCR fragment. The following thermal cycling parameters were used: 25 cycles of 10 s at 94° C, 5 s at 50°C, and 40 s at 72 °C. The sequencing amplification products were cleaned of unincorporated nucleotides by precipitation and resuspension of amplification products. 100 pmol ml^{-1} of PCR product was analysed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, U.S.A.) and/or a CEQ2000 (Beckman). Reactions were carried out in the facilities of the South African Sugar Research Institute (SASRI) Biotechnology Unit, Mount Edgecombe, KwaZulu-Natal, South Africa and/or Inqaba Biotec, Pretoria, Gauteng, South Africa. All fragments were sequenced in both directions. Sequences were deposited in Genbank (Accession numbers: EF216372 – EF216469).

2.5 General data analyses

2.5.1 Construction of consensus sequences

A consensus sequence for each sample was constructed by checking forward and reverse electropherograms against each other for homology and, where discrepancies arose, making appropriate changes to this sequence. Sequences were aligned with the BioEdit Sequence Alignment Editor (Version 5.0.9 for Windows 95/98/NT) (Hall, 1999) using the Clustal W option (Thompson *et al*., 1994). The alignment was also corrected by visual inspection. All cytochrome *b* and D-loop sequences were trimmed to 1004 and 290 nucleotides, respectively, to facilitate comparisons between individual samples. Included in both cytochrome *b* and D-loop alignments were sequences provided by Dr Wieslaw Bogdanowicz, *Otomops formosus* and *O. wroughtoni* from Asia, and *Otomops martiensseni* sequences obtained from the National Center for Biotechnology Information (NCBI) GenBank (Table 2). Other outgroups used in alignments included *Pipistrellus abramus*, *Mops midas* and *Tadarida fulminans* (cytochrome *b*) and *Mormopterus jugularis* (D-loop). *Otomops formosus* and *O. wroughtoni*

were included in alignments to show the position of the African and Malagasy species relative to the Asian species, whereas outgroups such as *Mops* and *Pipistrellus* were included in the alignments because they are representative of related molossid and vesper bats and thus ensure that the results are not skewed (Jones *et al*. 2002).

2.5.2 Data saturation

The program DAMBE (Data Analysis in Molecular Biology and Evolution) version 4.5.34 (Xia, 2000) was used to determine the degree of saturation of both the cytochrome *b* and D-loop data sets used in analysis. Saturated data may not be suitable for analyses because it underestimates the accumulation of mutations over time. For both analyses, the F84 model was used, as it was the most appropriate of the available models. The Xia *et al*. (2003) test was also used to measure substitution saturation for nucleotide sequences by calculating the index of substitution saturation (Iss) and comparing this to the Iss critical value assuming a symmetrical topology (Iss.cSym). The significance of these probabilities was calculated and used to infer the degree of saturation in the data and whether it was appropriate for use in phylogenetic and phylogeographic analysis.

2.5.3 Data statistics

Descriptive analyses on each of the data sets were carried out using PAUP 4.0b10 (Swofford, 1993). These analyses included calculation of conserved, variable, parsimony informative and singleton sites. Nucleotide composition frequencies for individuals and groups, and nucleotide pair frequencies for groups were also calculated, as well as the number of identical pairs, transitions and transversions among the sequences and the ratio of transitions to transversions.

2.6 Phenetic analyses

2.6.1 Molecular model

Before analyses were carried out, MrModeltest v.2 (Nylander, 2004) was used to determine which substitution model would work best for the cytochrome *b* and D-loop data. It was found that the sequences best fit a General Time Reversible + Invariant site + Gamma (GTR+I+G) model (refer to section 1.5.1) and analyses were subsequently performed using the assumptions of this model.

2.6.2 Genetic distances

All genetic distances were calculated using the GTR+I+G model in PAUP 4.0b10 (Swofford, 1993). Individual pairwise distances were calculated for each data set, as well as net between-group distances for defined groups. Groups were defined according to larger geographical regions.

2.6.3 Neighbour-joining analysis

Sequence data from cytochrome *b* and D-loop regions were used to construct phylogenetic trees to represent relationships within and between the various *Otomops* samples and outgroups. Trees were generated in both PAUP 4.0b10 (Swofford, 1993) and MEGA 3.1 (Kumar *et al*., 2004) using the GTR+I+G and Kimura-2-parameter models, respectively. Where trees showed congruence in topology and bootstrap values, those trees generated and edited in MEGA 3.0 (Kumar *et al*. 2004) are used for presentation purposes. Reliability of nodes was estimated using bootstrap resampling analysis (500 replications in PAUP) (Felsenstein, 1985).

2.6.4 Analysis of molecular variance (AMOVA)

An analysis of molecular variance was executed using the program Arlequin 3.01 (Excoffier *et al*., 2005) in order to test for significant molecular variance between the groups defined by phylogeographic analysis. Individuals were separated into four groups for analysis, i.e. south/west Africa (South Africa, Zimbabwe, Burundi and Tanzania, excluding Ivory Coast), Ivory Coast, north/east Africa (Kenya, Ethiopia and Yemen) and Madagascar according to the clusters identified in phylogenetic analyses.

Fixation indices were calculated (for individuals, populations and groups) and their significance tested using a non-parametric permutation approach described in Excoffier *et al*. (1992), consisting of permuting haplotypes, individuals or populations, among individuals, populations, or groups of populations. After each permutation round, all statistics were recomputed to obtain their null distribution.

2.7 Phylogenetic analyses

2.7.1 Bayesian analysis

A Bayesian likelihood analysis was performed using MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001) using the cytochrome *b* and D-loop samples. Bayesian analysis was performed under the GTR+I+G model for both cytochrome *b* and D-loop data sets. Four Markov chains were run for 15 million generations each, sampling every 100 generations to ensure that the resulting tree was well-resolved, and the first 500000 trees discarded as burn-in. The burn-in value was determined via visual inspection of probabilities, determined when they had reached a steady state in the analysis. The priors for the five active parameters were as follows: transition/transversion ratio = Beta (1.00, 1.00); state frequency = dirichlet (1, 1, 1, 1); proportion of invariant sites = uniform (0.00, 1.00); topology = all topologies equally probable a priori; and branchlengths = unconstrained:exponential (10.0). From the remaining trees, a 50% majority rule consensus tree was constructed. Bayesian analysis was conducted in lieu of a Maximum Parsimony tree since Bayesian analysis is a modified likelihood based approach.

2.8 Phylogeographical analyses

2.8.1 Population genetic analysis

Haplotype analysis was performed using the program DnaSP (DNA Sequence Polymorphism) version 4.10.9 (Rozas *et al.*, 2003) to determine the number of haplotypes for each data set. Analyses were performed separately for each of the three genetically-defined species-groups. Results from D-loop haplotype (h) and nucleotide (π) diversity values, neutrality tests (Fu's (1997) F_S and Fu & Li's (1993) D^* and F^*) and mismatch distribution analysis were used to estimate whether each population-group was stationary or had undergone a historical population expansion. High h with low π, a unimodal pairwise difference distribution, significant F_S but non-significant D^* and F^*, and a high ratio of number of variable sites (S) to average number of pairwise differences (d), are indicators of a historical population expansion event (Peck and Congdon, 2004; Hull and Girman, 2005; Russell *et al.*, 2005). Time since expansion was calculated for each expanding population based on the formula outlined by Rogers and Harpending (1992), i.e. $\tau = 2u$ t. Tau (τ) was calculated using DnaSp version 4.10.9, u was the product of the mutation rate (μ: mutations per site per generation) and sequence length and t is time in generations since expansion. Mutations per site per

generation had a lower limit of 1.73×10^{-7} and an upper limit of 3.3×10^{-7} for D-loop (Rogers and Harpending, 1992). Generation time was estimated at approximately two years for *Otomops* (Lamb *et al*., 2008).

2.8.2 Haplotype networks

Haplotype networks were created using TCS version 1.21 (Clement *et al*., 2000), which implements the estimation of gene genealogies from DNA sequences as described by Templeton *et al*. (1992) to create a statistical parsimony network.

3. RESULTS

3.1 DNA isolation and quantification

Isolation with the QIAGEN DNeasy® Tissue Kit (QIAGEN Inc.) yielded usable quantities of high molecular weight DNA. Each 50 µl elution yielded DNA with concentrations ranging from 2 – <200 ng/µl. The presence of a single discrete band of high molecular weight, as estimated from its electrophoretic mobility, combined with the lack of a lower-molecular weight smear, was taken to indicate that the DNA was of high integrity and suitable as a template for PCR amplification (Fig. 3.1).

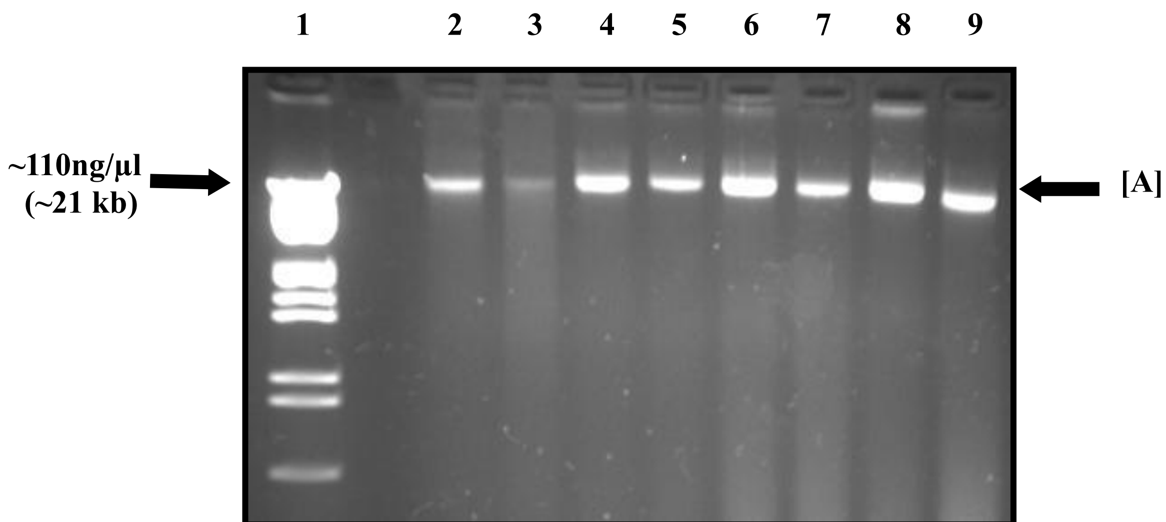

Figure 3.1. Agarose gel electrophoretic separation of isolated DNA. Lane 1 contains Molecular Weight Marker III (Roche Molecular Biochemicals). Lanes 2 and 3 contain DNA isolated from *O. martiensseni* from Ivory Coast and lanes 4 – 9 contain DNA isolated from *O. madagascariensis* from Madagascar. [A: high molecular weight band].

3.2 Polymerase Chain Reaction (PCR) amplification and sequencing

3.2.1 Mitochondrial cytochrome *b* gene amplification

PCR amplification of the ~1140 bp of the cytochrome *b* region was successfully carried out using primer sets L 14723 and H 15553; L 15146 and H 15915; L14723 and L46RC and H53RC and H15915 (Irwin *et al.*, 1991) (Figs. 3.2, 3.3, 3.4, 3.5).

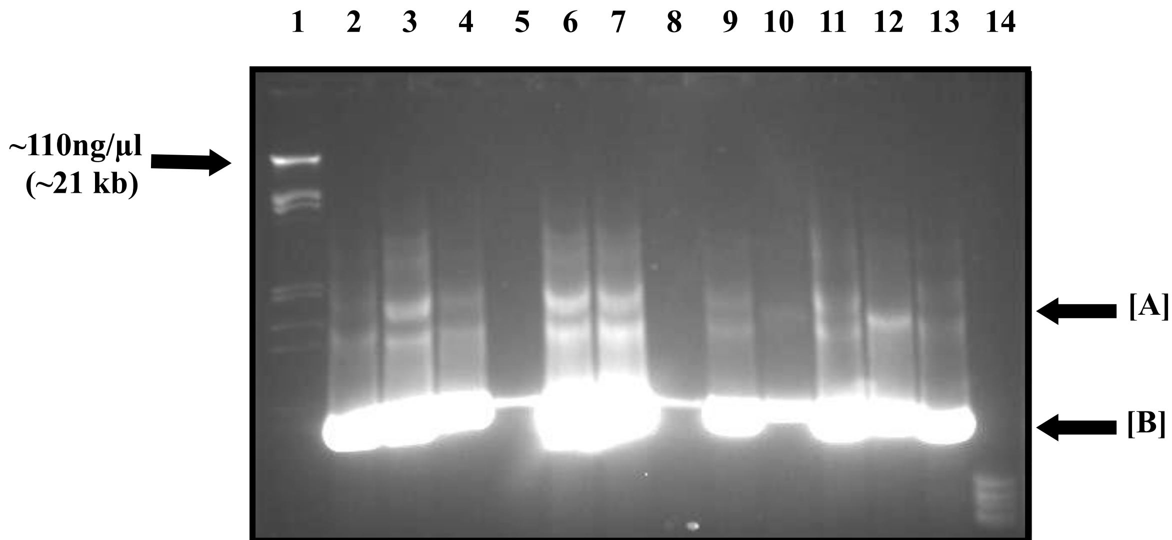

Figure 3.2. Agarose gel electrophoretic separation of PCR amplification products primed using L 14723 and H 15553. Lanes 1 and 14: Molecular Weight Marker III and Molecular Weight Marker V (Roche Molecular Biochemicals), respectively. Lanes 2 and 3: Ethiopia; lanes 4 and 5: Ivory Coast; lanes 6 and 7: Madagascar; lanes 8, 9, 12 and 13: South Africa; lanes 10 and 11: Kenya. [A: non-target fragments] [B: target fragments].

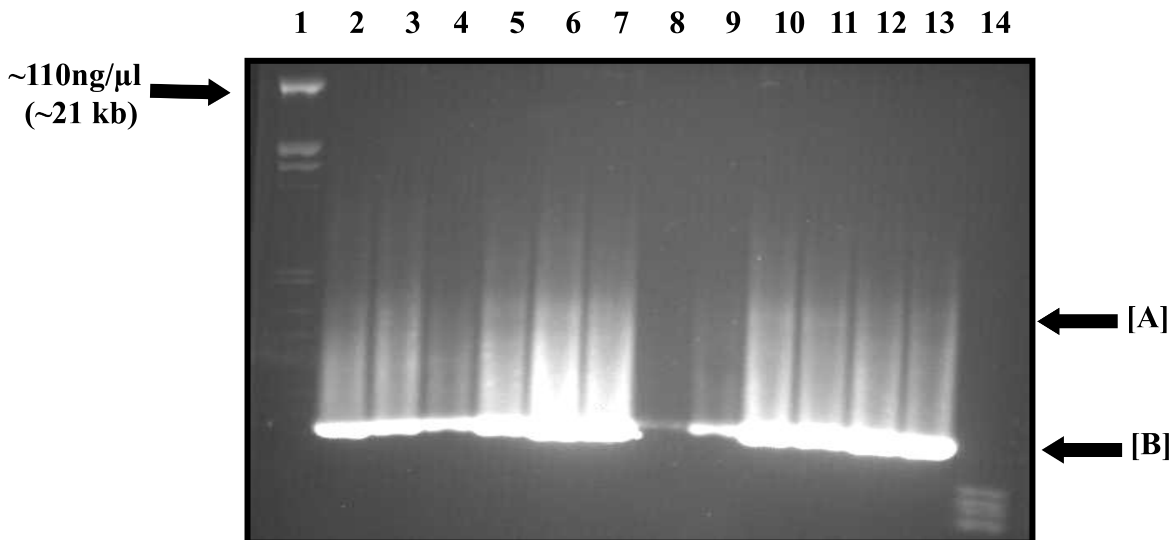

Figure 3.3. Agarose gel electrophoretic separation of PCR amplification products primed using L 15146 and H 15915. Lanes 1 and 14: Molecular Weight Marker III and Molecular Weight Marker V (Roche Molecular Biochemicals), respectively. Lanes 2 and 3: Ethiopia; lanes 4 and 5: Ivory Coast; lanes 6 and 7: Madagascar; lanes 8, 9, 12 and 13: South Africa; lanes 10 and 11: Kenya.

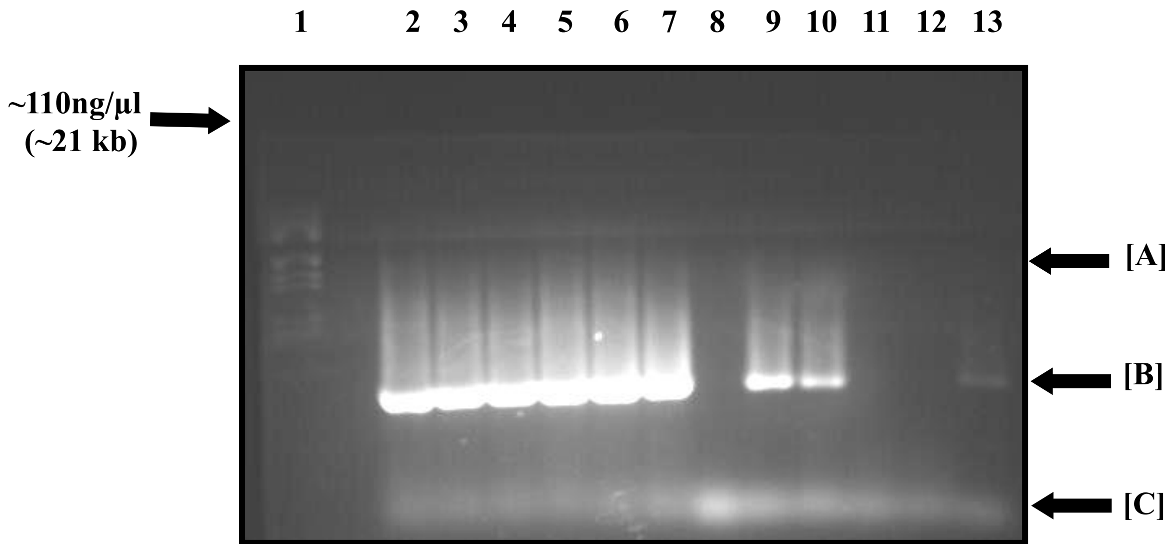

Figure 3.4. Agarose gel electrophoretic separation of PCR amplification products primed using L 14723 and L 46RC. Lane 1: Molecular Weight Marker III (Roche Molecular Biochemicals). Lanes 2 – 7: Madagascar; lanes 8 – 13: South Africa. Lanes 8, 11 and 12 contain failed reactions [A: non-target fragments] [B: target fragments] [C: unincorporated nucleotides].

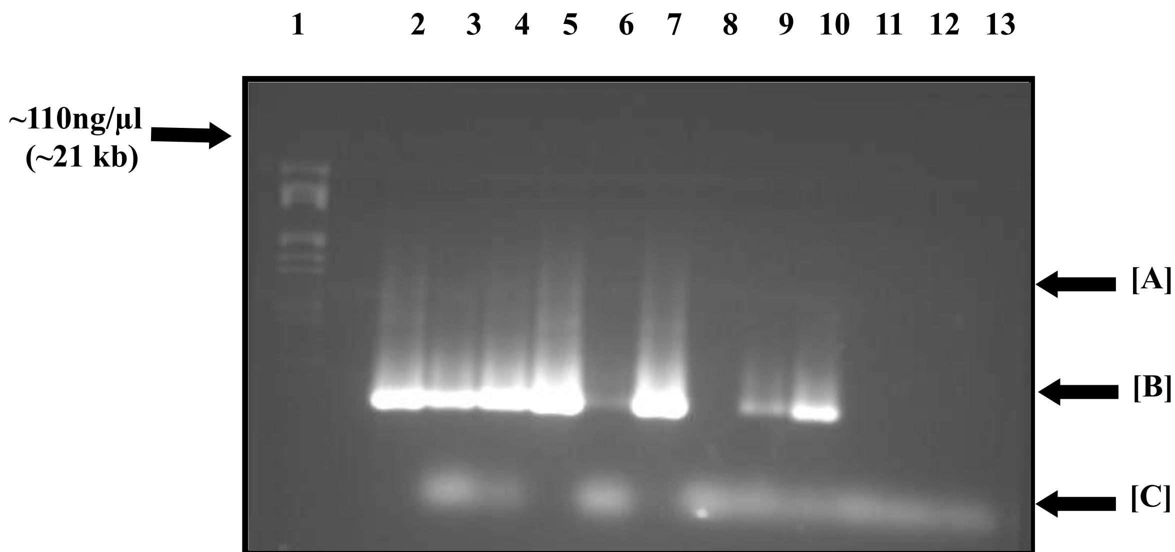

Figure 3.5. Agarose gel electrophoretic separation of PCR amplification products primed using H 53RC and H 15915. Lane 1: Molecular Weight Marker III (Roche Molecular Biochemicals). Lanes 2 – 7: Madagascar; lanes 8 – 13: South Africa. Lanes 8 and 11 – 13 contain failed reactions [A: non-target fragments] [B: target fragments] [C: unincorporated nucleotides].

3.2.2 Mitochondrial D-loop region amplification

PCR amplification of ~300 bp of the D-loop region was successfully completed using the primer set P and E (Wilkinson and Chapman, 1991) (Fig. 3.6).

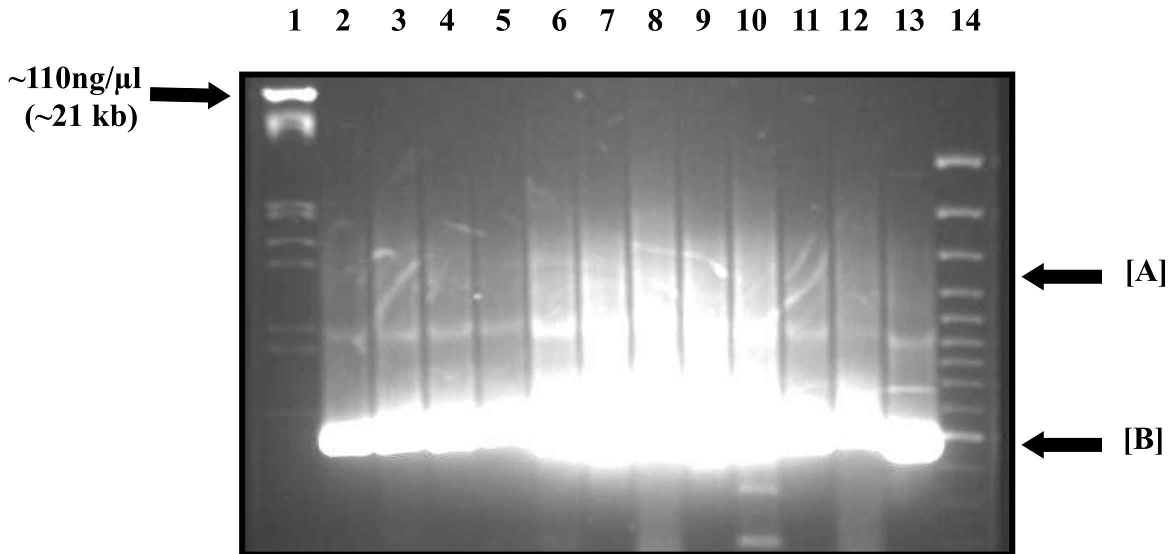

Figure 3.6. Agarose gel electrophoretic separation of PCR amplification products primed using P and E. Lanes 1 and 14: Molecular Weight Marker III (Roche Molecular Biochemicals) and O'GeneRuler™ 100bp DNA Ladder (Fermentas Life Sciences). Lanes 2 – 13: Madagascar. [A: non-target fragments] [B: target fragments].

Variation in concentrations of template DNA between 30 and 60 ng per reaction did not appear to influence target amplification. Not all PCR reactions were successful, as seen in Figs. 3.4 and 3.5, lanes 8 and 11 – 13. This was thought to be due to degradation of DNA or imperfect binding of primers to the template DNA. In many reactions non-target regions co-amplified with the target fragment. For this reason, the desired band was excised from the gel and purified to remove unwanted product.

3.2.3 DNA recovery and concentration measurement

After excision of product bands from the gel, DNA was recovered using a QIAquick® Gel Extraction Kit (QIAGEN Inc.). Purified DNA was subjected to agarose gel electrophoresis in order to check for the presence of a single band (Fig. 3.7). Samples invariably comprised a single band with little evidence of co-amplification products. DNA concentrations ranged from 1.6 – 150 ng/µl with a total volume of 28 µl per sample available for sequencing.

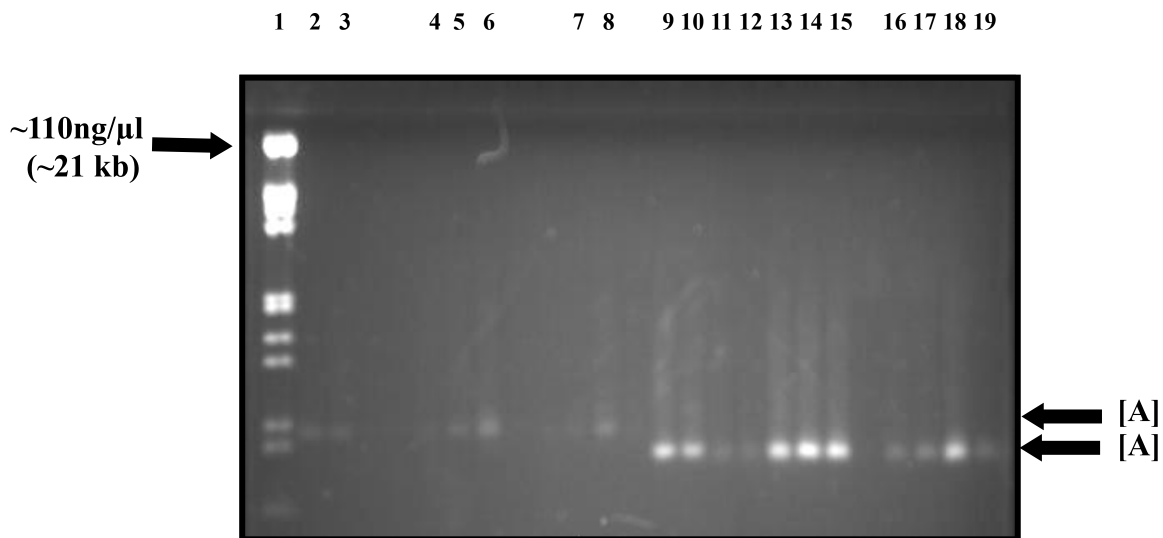

Figure 3.7. Agarose gel electrophoretic separation of PCR products after gel extraction. Lane 1: Molecular Weight Marker III (Roche Molecular Biochemicals). Lanes 2 – 8: cytochrome *b* primer set L 14723 and H 15553 and lanes 9 – 19: cytochrome *b* primer set L 15146 and H 15915. Lanes 2 – 3, 9 – 10: Madagascar; lanes 4 – 6, 11 – 15: South Africa; lanes 7 and 17: Yemen; lanes 8 and 18: Ivory Coast; lane 16: Zimbabwe; lane 19: Tanzania. [A: purified PCR product].

3.3 Analysis of cytochrome b sequence data

3.3.1 Data saturation

The proportion of transitions and transversions in the cytochrome *b* data set were plotted against divergence to assess whether or not the data was saturated (Fig. 3.8).

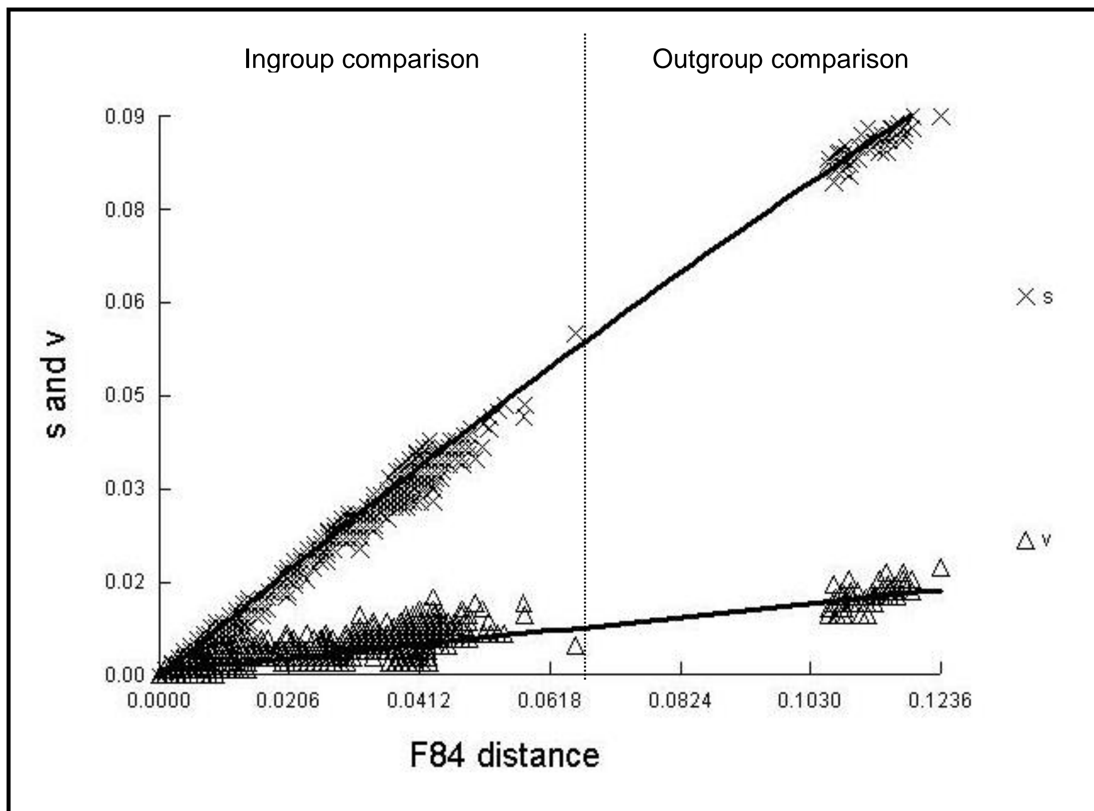

Figure 3.8. Transitions and transversions versus genetic distance for the cytochrome *b* sequences; s = transitions and v = transversions. Solid lines represent the least squares best-fit line. *O. wroughtoni* and *O. formosus* were outgroups in analysis.

Both transitions and transversions appear to follow an approximate linear model (Fig. 3.8), indicating little saturation in the data.

The Xia *et al*. (2003) test measures substitution saturation and, in this case, revealed that the index of substitution saturation (Iss = 0.040) is significantly lower than the critical value (Iss.cSym = 0.748 when assuming a symmetrical topology; Iss.cAsym = 0.447 when assuming an assymetrical topology) with a probability of <0.001, indicating that there is little saturation in the data.

3.3.2 Statistical analyses of sequence data

Groupings comprised *Otomops* from south/west Africa (excluding Ivory Coast), Ivory Coast, north/east Africa, Madagascar and the outgroups, *O. wroughtoni*, *O. formosus*, *M. midas*, *T. fulminans* and *P. abramus* (Table 3.1).

Table 3.1. Sample groupings used in cytochrome *b* analyses.

Group	Locality	Samples	GenBank #
[1] *Otomops* south/west Africa	Silverglen, RSA	Silverglen1D	EF216406
	Silverglen, RSA	Silverglen2D	EF216407
	Silverglen, RSA	Silverglen3D	EF216408
	Silverglen, RSA	Silverglen4D	EF216409
	Silverglen, RSA	Silverglen5D	EF216410
	Ballito, RSA	Ballito6D	EF216411
	Ballito, RSA	Ballito7D	EF216412
	Pinetown, RSA	Pinetown1D	EF216413
	Pinetown, RSA	Pinetown2D	EF216414
	Pinetown, RSA	Pinetown3D	EF216415
	Pinetown, RSA	Pinetown4D	EF216416
	Pinetown, RSA	Pinetown5D	EF216417
	Scottburgh, RSA	Scottburgh6D	EF216418
	Scottburgh, RSA	Scottburgh7D	EF216419
	Park Rynie, RSA	Durban Park Rynie324	EF216426
	Park Rynie, RSA	Durban Park Rynie325	EF216427
	Morningside, RSA	Durban Morningside321	EF216424
	Amanzimtoti, RSA	Durban Amanzimtoti322	EF216425
	Kibira NP, Burundi	Burundi415	EF216423
	Sengwa, Zimbabwe	Zimbabwem	EF216421
	Muheza district, Tanzania	Tanzaniam	EF216422
[2] *Otomops* Ivory Coast	Comoé NP, Ivory Coast	Ivory_Coastm	EF216420
[3] *Otomops* north/east Africa	Bale Province, Ethiopia	EthiopiaA1	EF216429
		EthiopiaA2	EF216430

Table 3.1 continued.

Group	Locality	Samples	GenBank #
[3] *Otomops* north/east Africa	Bale Province, Ethiopia	EthiopiaB1	EF216431
		EthiopiaB2	EF216432
		EthiopiaC1	EF216433
		EthiopiaC2	EF216434
		EthiopiaD1	EF216435
		EthiopiaD2	EF216436
	Makuena district, Kenya	Kenya1	EF216428
		Kenya2	EF216438
		Kenya3	EF216439
		Kenya4f	EF216440
		Kenya5m	EF216441
		Kenya7m	EF216442
	Al Mawhit, Yemen	Yemen	EF216437
[4] *Otomops* Madagascar	Mahajanga	Mahajanga2M	EF216373
		Mahajanga3	EF216374
		Mahajanga4M	EF216375
	Fianarantsoa	Fianarantsoa1M	EF216372
	Ambanila, Toliara	Toliara6M	EF216377
		Toliara7M	EF216378
	Bishihiko, Toliara	Toliara8M	EF216379
		Toliara9M	EF216380
	Ankarana, Antsiranana	Ankarana15M	EF216381
		Ankarana18M	EF216382
	Analamerana, Antsiranana	Ankarana5M	EF216376
[5]*Otomops wroughtoni*	Preah Vihear, Cambodia	OwroughtoniM143	
[6]*Otomops formosus*	Luzon Is, Philippines	OformosusM145	
[7]*Mops midas*		Mops midasM701	
[8]*Tadarida fulminans*		Tfulminans	
[9]*Pipistrellus abramus*		Pipistrellusabramus NC005436	

Sequence data sites were examined and results presented in Table 3.2, Table 3.3 and Table 3.4. A full listing of individual nucleotide composition frequencies is given in Appendix 3.

Table 3.2. Number of conserved, variable, parsimony informative and singleton sites out of 1004 nucleotides found in cytochrome *b* sequence data, with and without outgroups included in analysis. Outgroups are *O. wroughtoni*, *O. formosus*, *M. midas*, *T. fulminans* and *P. abramus*.

Variables (out of 1004 nucleotides)	With outgroups	Without outgroups
Conserved sites	631	896
Variable sites	373	108
Parsimony informative sites	204	77
Singleton sites	169	31

Table 3.3. Nucleotide composition for groups using cytochrome *b* data.

Group	% T (U)	% C	% A	% G	Total
[1]*Otomops* south/west Africa	27.1	29.8	29.8	13.3	1003.8
[2] *Otomops* Ivory Coast	27.5	29.1	30.0	13.3	1002
[3]*Otomops* north/east Africa	27.2	29.6	29.8	13.4	1003.9
[4]*Otomops* Madagascar	27.6	29.0	29.6	13.8	1004
Average	27.3	29.5	29.8	13.4	1003.8

Table 3.4. Nucleotide pair frequencies for groups using cytochrome *b* data.

Group	Identical pairs	Transitional pairs (si)	Transversional Pairs (sv)	Ratio (si/sv)	Total
1	995	7	2	3.5	1003.6
3	1000	3	1	3.0	1003.7
4	997	5	2	2.5	1004.0

Without outgroups, analyses reveal that approximately 90% of the sequence data is made up of conserved sites, as opposed to 63% with outgroups included. The number of variable sites drops with the exclusion of outgroups from 373 to 108, as does the number of parsimony informative sites (204 to 77) and singleton sites (169 to 31).

Sequences did not differ by more than 0.8% in nucleotide composition frequency. The *Otomops* mitochondrial cytochrome *b* coding strand is characterized by a low percentage of guanine relative to other nucleotides, as is expected for vertebrate mtDNA (Table 3.3).

The analysis of nucleotide pair frequencies reveals that of the number of nucleotides analysed, 99% were identical pairs for all samples in each group. *Otomops* south/west Africa had the highest number of transitional pairs (7) and both *Otomops* south/west Africa and *Otomops* Madagascar had the highest number of transversional pairs (2). *Otomops* Madagascar had the lowest ratio of transitional to transversional pairs (2.5) whereas *Otomops* south/west Africa had the highest (3.5).

3.3.3 Phenetic analyses

All analyses were performed using the assumptions of the General Time Reversible + Invariant site + Gamma (GTR+I+G) model (section 1.5.1) as specified by MrModeltest v.2 using the Akaike Information Criterion (AIC) (Nylander, 2004).

All genetic distances were calculated using the GTR+I+G model in PAUP 4.0b10 (Swofford, 1993). Individual, between-group and within-group distances were calculated for both data sets.

3.3.3.1 Genetic distances

Individual pairwise genetic distances were calculated and are given in Appendix 4. Within-group means are presented in Table 3.5. Net between-group distances are presented in Table 3.6 and Fig. 3.9.

Table 3.5. Within-group mean GTR+I+G genetic distance for cytochrome *b* (1004 nucleotides).

Groups	Genetic distance	Standard deviation
[1]*Otomops* south/west Africa	0.008	0.001
[3]*Otomops* north/east Africa	0.004	0.001
[4]*Otomops* Madagascar	0.007	0.002

Table 3.6. Net between-group GTR+I+G genetic distances for *Otomops* samples and outgroups for cytochrome *b* (1004 nucleotides) (standard error above diagonal).

Taxon	1	2	3	4	5	6	7	8	9
[1]*Otomops* south/west Africa		0.004	0.004	0.005	0.011	0.011	0.014	0.014	0.018
[2]*Otomops* Ivory Coast	0.021		0.006	0.007	0.011	0.011	0.014	0.014	0.018
[3]*Otomops* north/east Africa	0.021	0.037		0.006	0.011	0.010	0.014	0.014	0.018
[4]*Otomops* Madagascar	0.031	0.049	0.035		0.011	0.011	0.014	0.014	0.019
[5]*Otomops wroughtoni*	0.110	0.116	0.111	0.113		0.008	0.014	0.016	0.018
[6]*Otomops formosus*	0.105	0.108	0.106	0.105	0.066		0.014	0.015	0.019
[7]*Mops midas*	0.158	0.162	0.169	0.171	0.178	0.168		0.014	0.020
[8]*Tadarida fulminans*	0.161	0.166	0.164	0.170	0.197	0.190	0.163		0.019
[9]*Pipistrellus abramus*	0.235	0.240	0.241	0.240	0.250	0.250	0.285	0.274	

Otomops south/west Africa shows the greatest within-group diversity (0.8%), whereas *Otomops* north/east Africa had the least (0.4%). From Table 3.6, it can be seen that the largest amount of divergence exists between *P. abramus* and the other outgroups (28.5% against *M. midas*) as well as the ingroups (24.1% against *Otomops* north/east Africa). There is less divergence between the ingroups, with minimum distances of 2.1% between *Otomops* south/west Africa and both *Otomops* Ivory Coast and *Otomops* north/east Africa, and a maximum distance of 4.9% between *Otomops* Ivory Coast and *Otomops* Madagascar (Fig. 3.9). *Otomops* Madagascar separated from *Otomops* south/west Africa and *Otomops* north/east Africa with distances of 3.1% and 3.5% respectively, but showed greater separation from *Otomops* Ivory Coast, with a distance of 4.9%. As expected, *Otomops* outgroups (*O. wroughtoni* and *O. formosus*) are separated from *Otomops* ingroups by greater distances of between 10.5% and 11.6%. Other outgroups also separated from ingroups with distances greater than 15.8% but less than 18.0%.

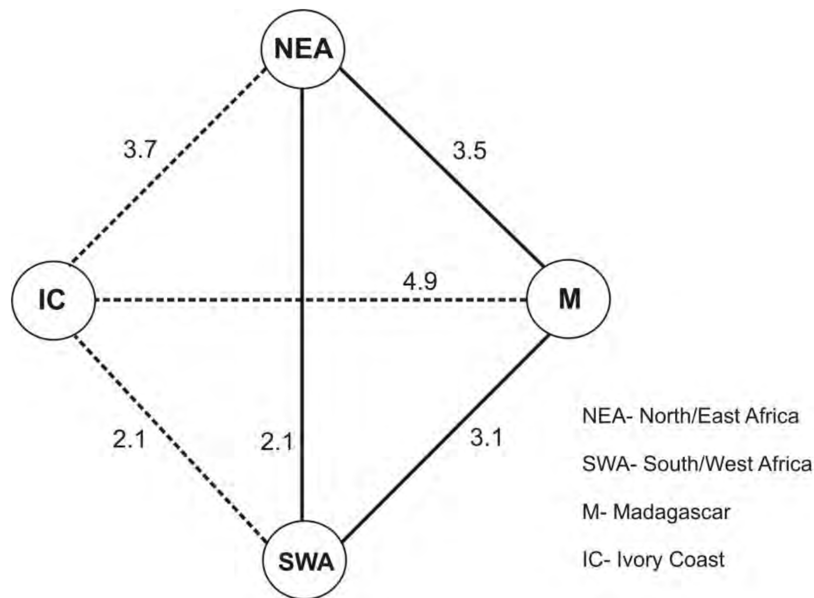

Figure 3.9. Representation of net between-group GTR+I+G genetic distances (percent) for *Otomops* groupings for cytochrome *b* (1004 nucleotides).

3.3.3.2 Neighbour-joining analysis

Cytochrome *b* genetic distances within and between *Otomops* samples and outgroups are represented in Fig. 3.10 as a neighbour-joining tree, with bootstrap support (500 replicates).

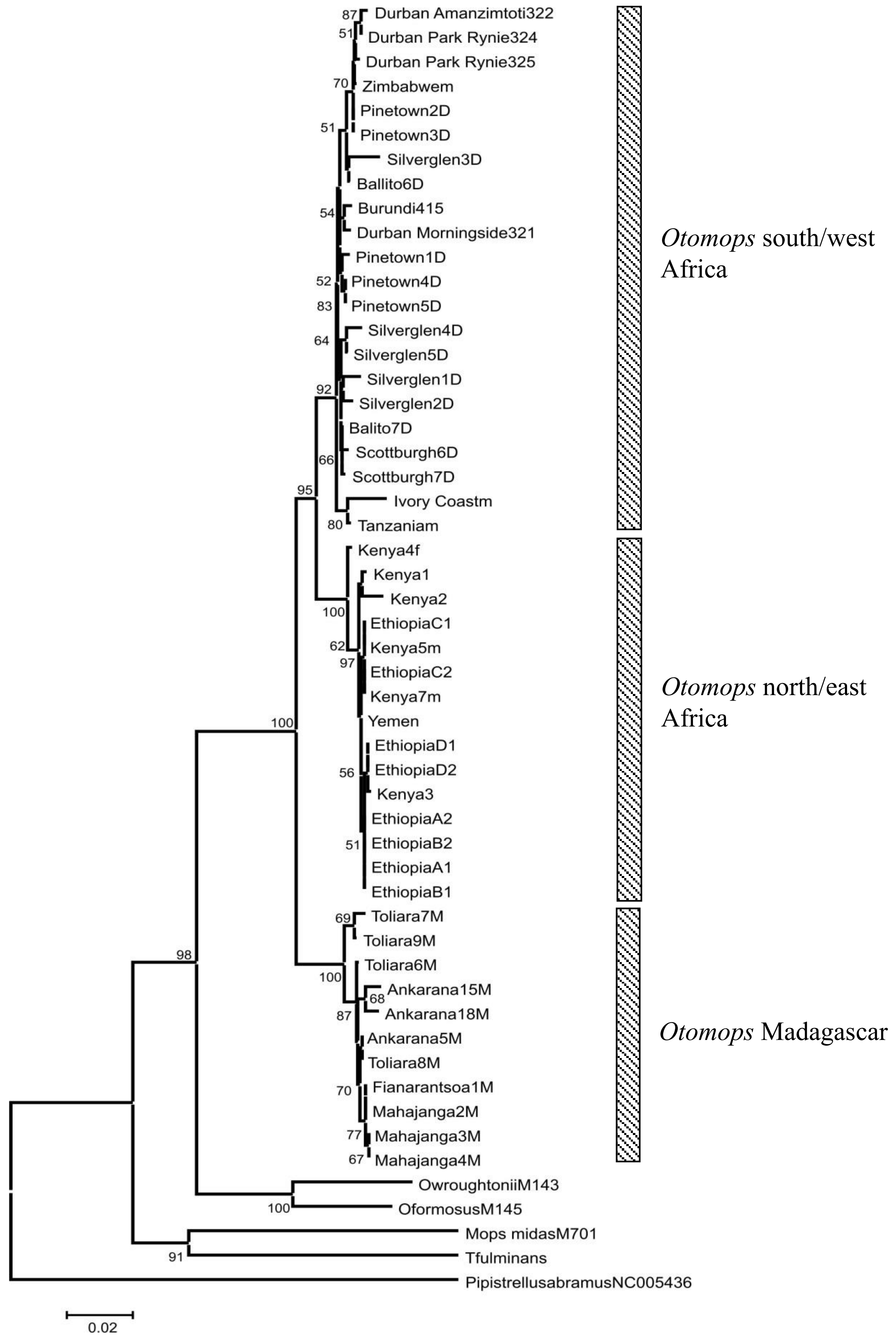

Figure 3.10. Cytochrome *b* neighbour-joining tree using a GTR+I+G distance model (1004 nt) with bootstrap support (500 replicates) showing relationships between 48 samples of *Otomops* with reference to the outgroups *O. wroughtoni*, *O. formosus*, *P. abramus*, *M. midas* and *T. fulminans*. Only bootstrap values above 50% are shown.

All *Otomops* samples formed an exclusive cluster with 98% bootstrap support. There is strong support for subdivision of the *Otomops* grouping into two clusters; these are the sample group from Africa, Madagascar and Yemen (100% bootstrap support) and the Asian species (*O. wroughtoni* and *O. formosus*) (100% bootstrap support). The Africa/Madagascar *Otomops* cluster subdivides into two exclusive sub-clusters (95% and 100% bootstrap support) separating samples according to location (Africa or Madagascar). The African lineage forms two well-defined and well-supported reciprocally-monophyletic sister lineages comprising samples from south/west Africa (Tanzania, Burundi, Ivory Coast, Zimbabwe and South Africa) and north/east Africa (Kenya, Ethiopia and Yemen) with bootstrap support of 92% and 100%, respectively.

3.3.3.3 Analysis of Molecular Variance (AMOVA)

There was significant variance among the three geographically-defined groups (79.37%; *P* (random value >= observed value) = 0.00059) (Table 3.7). Variance among populations within groups accounted for 0.25% of the variation within the data, and was significant (*P* (random value >= observed value) = 0.00010). Differences within populations accounted for 20.38% of variation, and was also significant (*P* (random value >= observed value) = 0.00000).

Table 3.7. Results of Analysis of Molecular Variance (AMOVA) for cytochrome *b*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variance
Among groups	3	432.096	13.32545 Va	79.37
Among populations within groups	5	17.689	0.04240 Vb	0.25
Within populations	39	133.444	3.42165 Vc	20.38
Total	47	583.229	16.78950	

Fixation Indices

FSC	0.01224
FST	0.79620
FCT	0.79368

Significance tests (10100 permutations)

Vc and FST	P (random value < observed value) = 0.00000
	P (random value = observed value) = 0.00000
	P (random value <= observed value) = 0.00000+ -0.00000
Vb and FSC	P (random value > observed value) = 0.00010
	P (random value = observed value) = 0.00000
	P (random value >= observed value) = 0.00010+ -0.00010
Va and FCT	P (random value > observed value) = 0.00000
	P (random value = observed value) = 0.00059
	P (random value >= observed value) = 0.00059+ -0.00028

3.3.4 Phylogenetic analyses

3.3.4.1 Bayesian analysis

Phylogenetic trees were constructed with cytochrome *b* data using Bayesian analysis to create a 50% majority rule tree. The Bayesian tree is represented in Fig. 3.11 with support indicated at the nodes as posterior probabilities (pp).

The topology of the Bayesian tree is similar to that of the neighbour-joining tree (Fig. 3.10). All *Otomops* samples form a monophyletic lineage (0.75 pp). *Otomops wroughtoni* and *O. formosus* may be ancestral to an African and Malagasy *Otomops* clade (1.00 pp), which is subdivided into two reciprocally-monophyletic lineages, i.e. African (0.96 pp) and Malagasy (1.00 pp). The African lineage splits into two well-supported reciprocally-monophyletic sister lineages with samples from south/west Africa forming one clade (1.00 pp) and samples from north/east Africa forming another (1.00 pp).

Figure 3.11. Bayesian phylogram based on 1004 nt of the cytochrome *b* gene showing relationships between 48 samples of *Otomops* with reference to the outgroups *O. wroughtoni*, *O. formosus*, *P. abramus*, *M. midas* and *T. fulminans*. Support is indicated at the nodes as posterior probabilities.

3.3.5 Phylogeographic analyses

3.3.5.1 Haplotype number

DnaSP version 4.0.9 (Rozas *et al*., 2003) was used for haplotype analysis. The number of haplotypes within the cytochrome *b* data set was assessed including and excluding outgroups. There were 36 cytochrome *b* haplotypes when outgroups were excluded, with 8 haplotypes including more than one sample. Over 60% of the samples included in the present analysis are inferred to represent unique haplotypes. Details of haplotypes are given in Table 3.8.

Haplotype analysis of 48 *Otomops* samples revealed that there are 106 variable sites (out of 1004 nucleotides), yielding 36 haplotypes. Results show that, overall, the cytochrome *b* data have a high haplotype diversity (*h*) value (0.9840; standard deviation, 0.009) and a relatively lower nucleotide diversity (π) value (0.02423; standard deviation, 0.0014) (Table 3.17). The average number of nucleotide differences (k) was 24.2544. Values obtained for the three geographically and genetically defined groups are SWA: *h*, 0.9900; π, 0.0082; NEA: *h*, 0.8760; π: 0.0036); and Madagascar: *h*: 0.9450; π: 0.0072.

Table 3.8. Haplotypes within the cytochrome *b* data set, excluding outgroups.

Haplotype number	Number of samples	Sample names	Haplotype network codes
1	1	Silverglen1D	Sg1
2	1	Silverglen2D	Sg2
3	1	Silverglen3D	Sg3
4	1	Silverglen4D	Sg4
5	1	Silverglen5D	Sg5
6	1	Ballito6D	Bt6
7	1	Ballito7D	Bt7
8	1	Pinetown1D	P1
9	2	Pinetown2D; Pinetown3D	P2; P3
10	2	Pinetown4D; Pinetown5D	P4; P5
11	1	Scottburgh6D	Sb6
12	1	Scottburgh7D	Sb7
13	1	Ivory Coastm	IC
14	1	Zimbabwem	Z

Table 3.8 continued.

Haplotype number	Number of samples	Sample names	Haplotype network codes
15	1	Tanzaniam	Tz
16	1	Burundi415	B
17	1	Durban Morningside321	Ms
18	1	Durban Amanzimtoti322	At
19	1	Durban Park Rynie324	Pr1
20	1	Durban Park Rynie325	Pr2
21	2	Fianarantsoa1M; Mahajanga2M	F1; M2
22	2	Mahajanga3M; Mahajanga4M	M3; M4
23	2	Ankarana5M; Toliara8M	A5; T8
24	1	Toliara6M	T6
25	1	Toliara7M	T7
26	1	Toliara9M	T9
27	1	Ankarana15M	A15
28	1	Ankarana18M	A18
29	1	Kenya1	K1
30	4	EthiopiaA1; EthiopiaA2; EthiopiaB1; EthiopiaB2	Ea1; Ea2; Eb1; Eb2
31	4	EthiopiaC1; EthiopiaC2; Kenya5M; Kenya7M	Ec1; Ec2; K5; K7
32	2	EthiopiaD1; EthiopiaD2	Ed1; Ed2
33	1	Yemen	Y
34	1	Kenya2	K2
35	1	Kenya3	K3
36	1	Kenya4f	K4

3.3.5.2 Haplotype network

Statistical parsimony analysis of the cytochrome *b* data using TCS version 1.2.1 generated three haplotype networks when set at the 95% connection limit. These networks were made up of samples from south/west Africa, north/east Africa and Madagascar. The connection limit was set at 100 mutational steps in order to create a single network including all *Otomops* samples (Fig. 3.12).

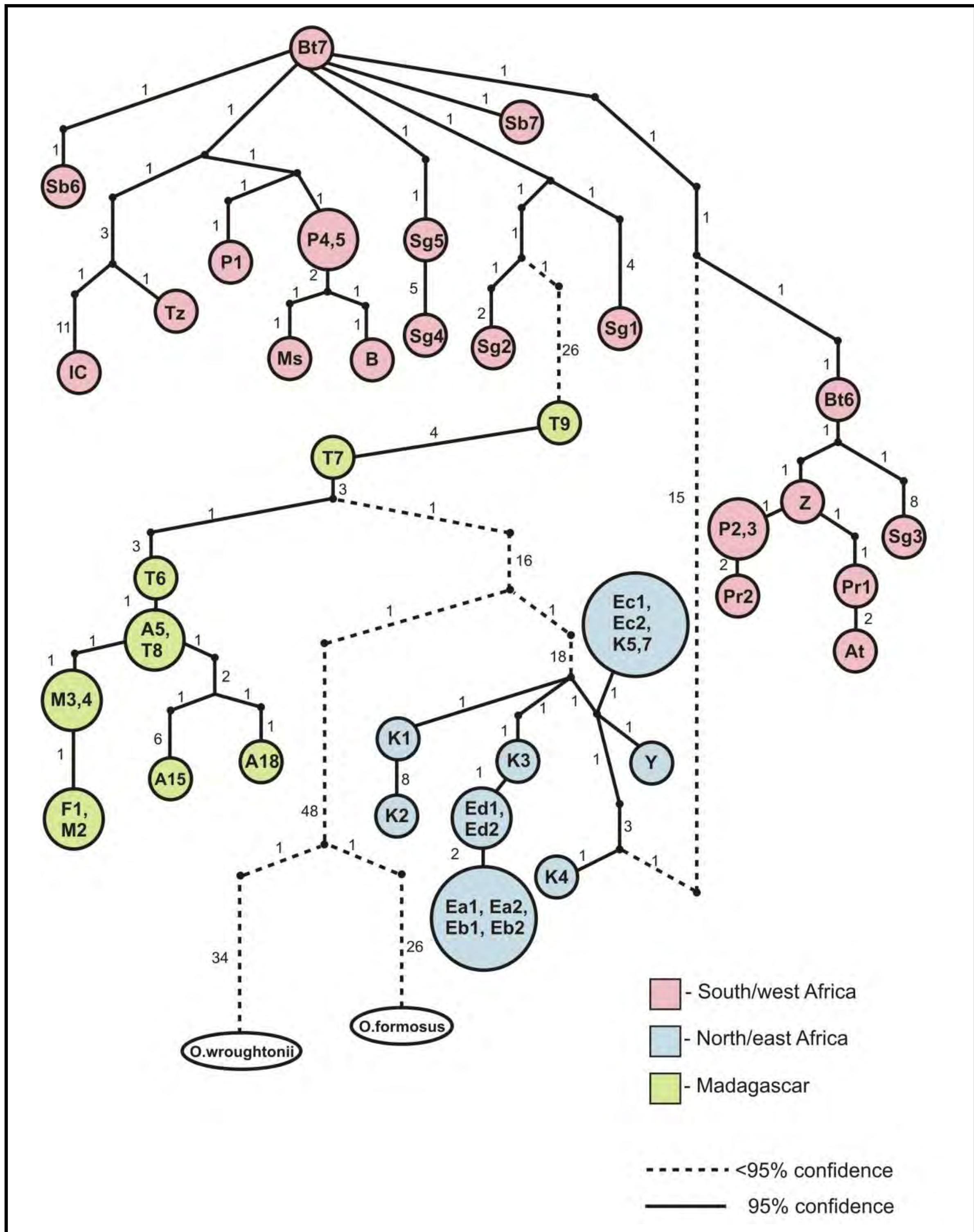

Figure 3.12. Cytochrome *b* haplotype network showing mutational relationships between 36 *Otomops* haplotypes with reference to the outgroups *O. wroughtoni* and *O. formosus*. Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.8.

The haplotype network shows the grouping of haplotypes according to location, as seen in the neighbour-joining (Fig. 3.10) and Bayesian (Fig. 3.11) trees, suggesting genetically and geographically defined species-groups. The south/west Africa group is separated from the north/east Africa and Malagasy groups by a minimum of 19 and 30 steps, respectively. Haplotype Silverglen2D (Sg2) of south/west Africa is closest to haplotype Toliara9M (T9) of Madagascar, whilst haplotype Ballito6D (B6) is closest to haplotype Kenya4f (K4). Samples from Madagascar and north/east Africa are separated by a minimum of 40 steps between haplotypes Toliara7M (T7) and Kenya1 (K1). The Ivory Coast haplotype (IC) is separated from the rest of the south/west Africa group by 13 steps (closest haplotype, Tanzania (Tz)), and by 17 steps from the nearest South African haplotype, Ballito7D (B7). Within the groups, haplotypes are separated by a maximum of 10 steps in south/west Africa, 9 steps in Madagascar and 8 steps in north/east Africa. Outgroups are separated by 70 steps from both Madagascar and north/east Africa and 92 steps from south/west Africa.

3.3.5.3 Haplotype correlations to environmental factors

Statistical parsimony analysis of the cytochrome *b* data using TCS version 1.2.1 generated a single haplotype network (100 mutational steps) including all *Otomops* samples. Environmental factors were superimposed onto haplotype networks in order to determine whether *Otomops* in Africa and Madagascar show genetic structure based on mean annual temperature, mean annual precipitation, altitude or sample locality (Fig. 3.13, Fig. 3.14, Fig. 3.15, Fig. 3.16).

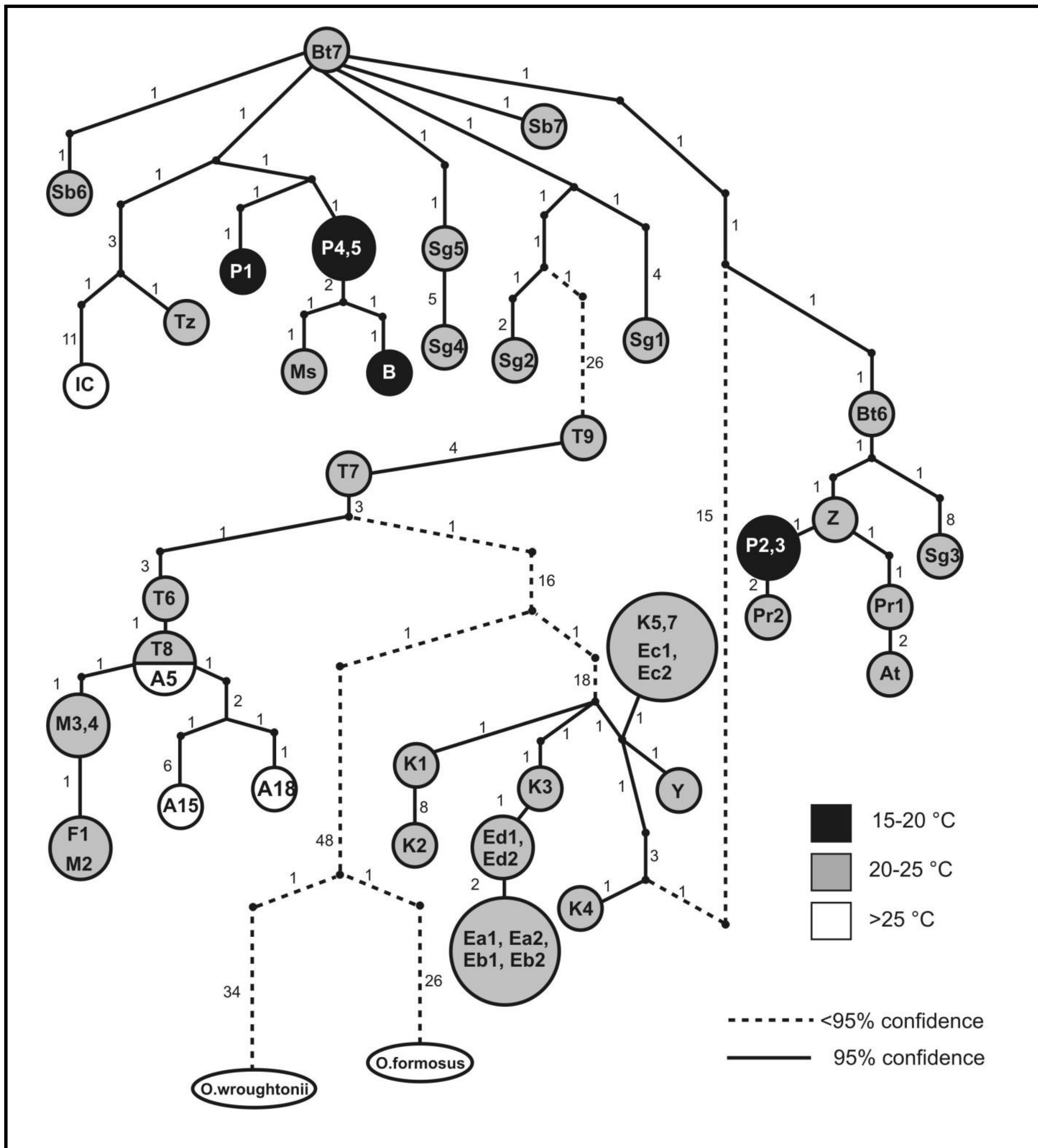

Figure 3.13. Cytochrome *b* haplotype network with superimposition of mean annual temperature (°C). Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.8.

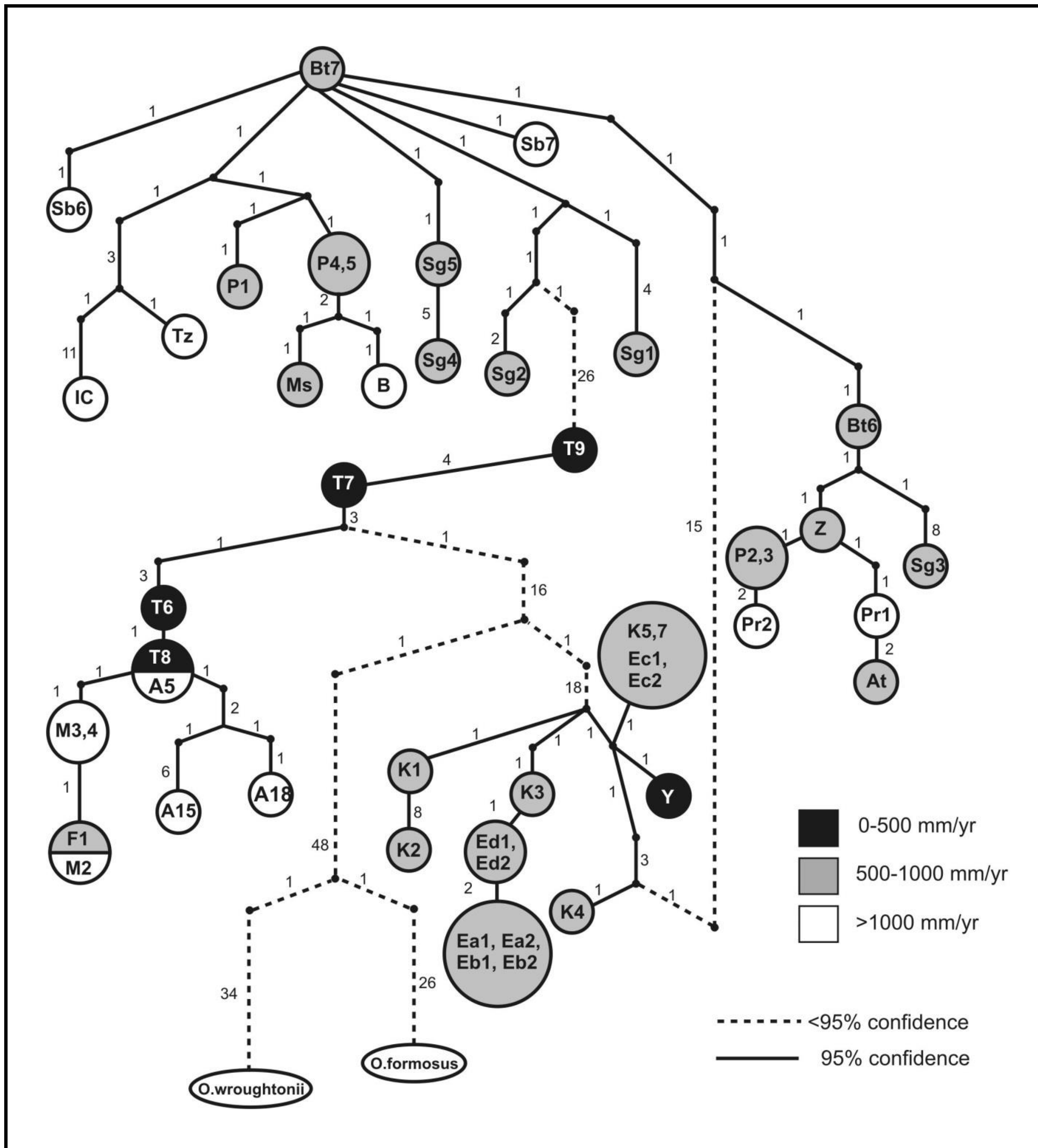

Figure 3.14. Cytochrome *b* haplotype network with superimposition of mean annual precipitation (mm rainfall per year). Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.8.

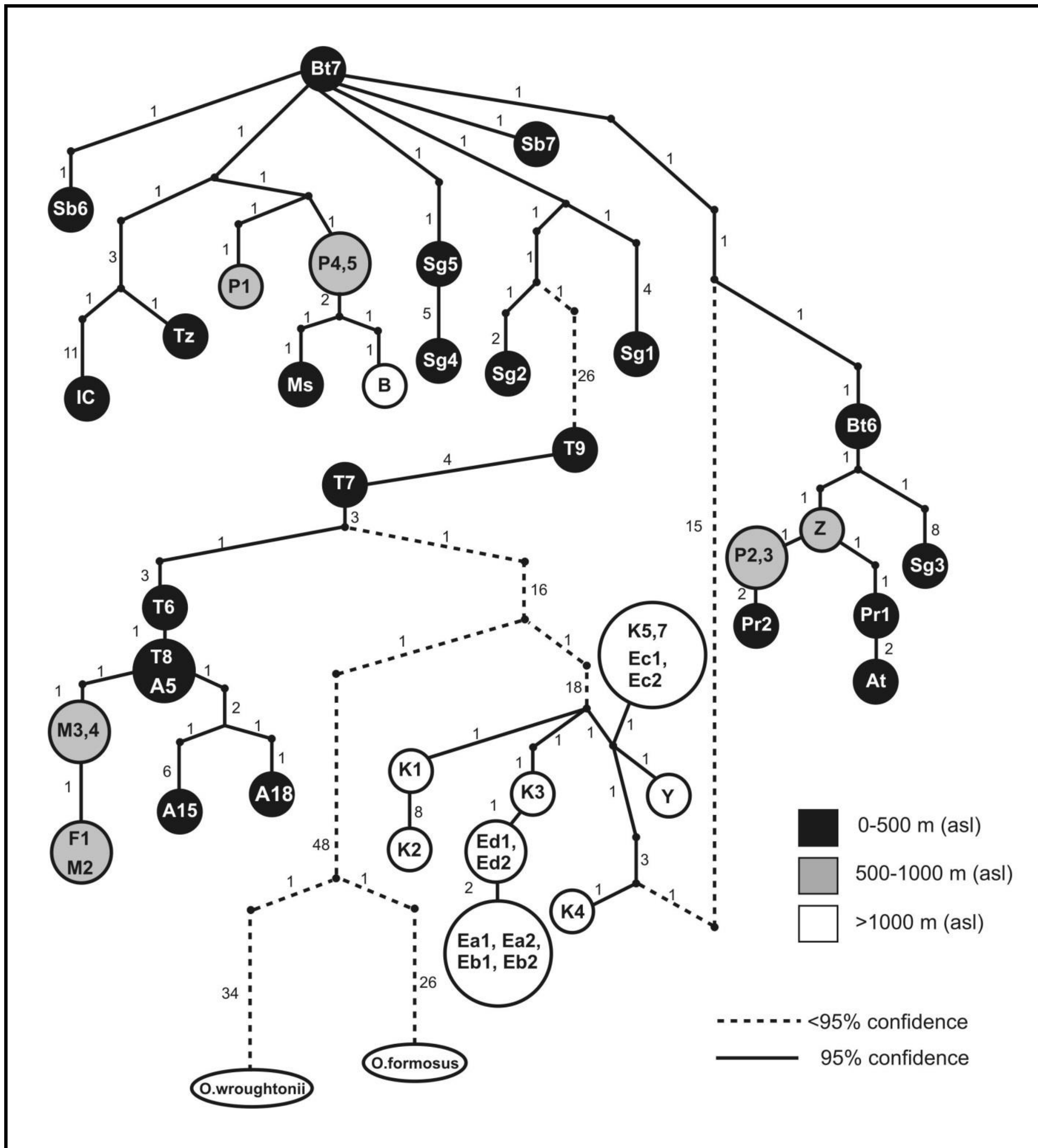

Figure 3.15. Cytochrome *b* haplotype network with superimposition of altitude (m above sea level). Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.8.

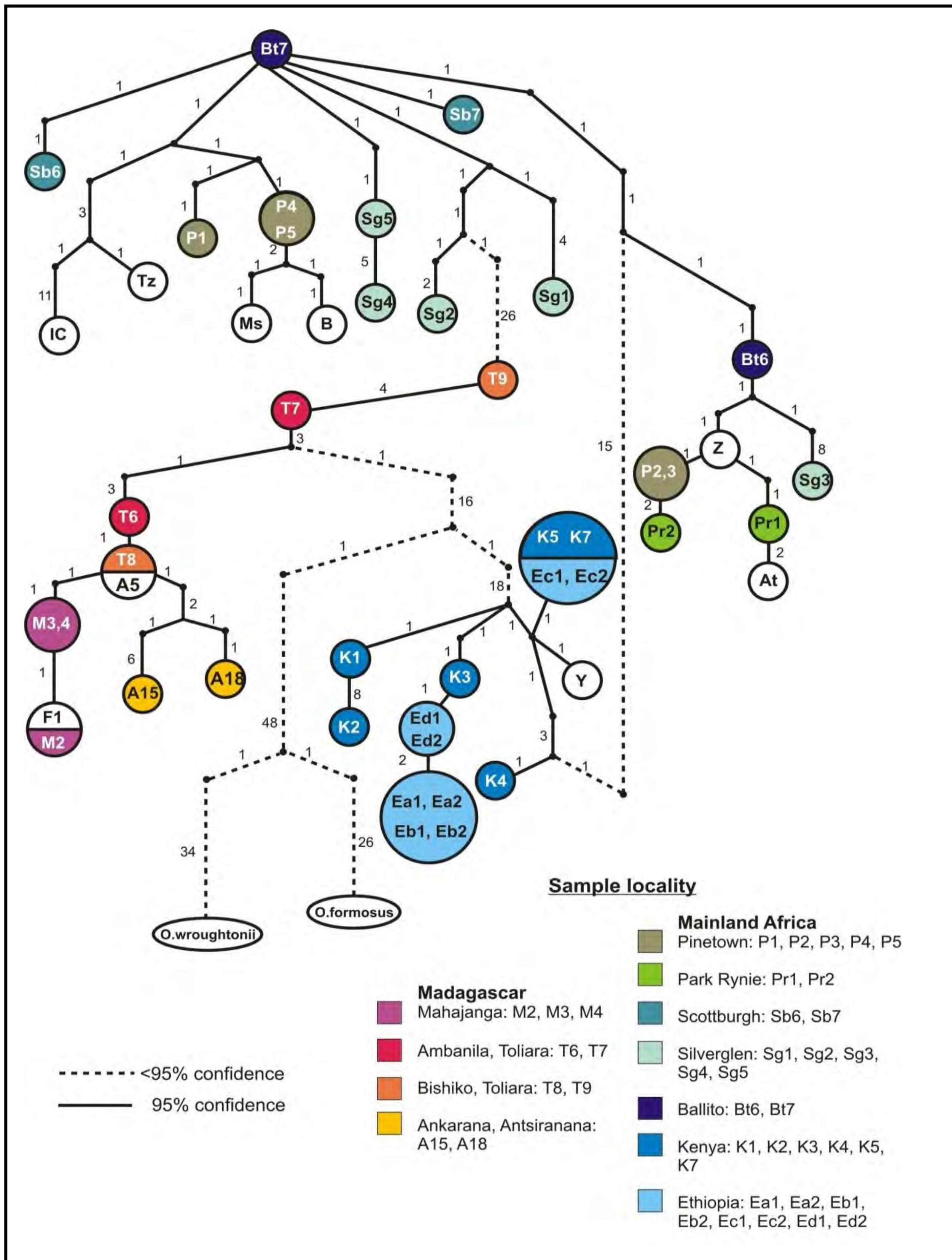

Figure 3.16. Cytochrome *b* haplotype network with superimposition of sample localities. Single sample localities are left uncoloured. Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.8.

Afro-Malagasy *Otomops* tend to live in areas where temperatures are mostly within the 20 – 25 °C range (Fig. 3.13). Samples from the north/east Africa group are found exclusively within this temperature range, whilst there is more variation within the south/west Africa group. The Ivory Coast sample is derived from an area with higher mean annual temperatures (>25 °C). The Malagasy samples tend to be found at temperatures between 20 – 25 °C.

There does not appear to be a strong association of haplotype groups with mean annual precipitation. Members of the north/east Africa group, with the exception of Yemen, which is drier, live in areas which receive 500 – 1000 mm/year (Fig. 3.14). The south/west Africa group tends to inhabit moderate to wet areas (500 – >1000 mm/yr). The Malagasy group is found in either dry (0 – 500 mm/yr) areas such as Toliara (south west) or wet areas (>1000 mm/yr) in the more northerly regions.

Altitude appears to be associated with haplotype groups, in that all samples from the north/east Africa group are found in areas over 1000 m above sea level (Fig. 3.15). Most south/west African and Malagasy samples are found at lower altitudes (0 – 500 m) and are less common above 500 m. There appears to be little relationship between the localities of samples and haplotype network structure, with the exception of the Ethiopian *Otomops* individuals, which shows mother-foetus associations (Fig. 3.16).

3.4 Analysis of D-loop sequence data

3.4.1 Data saturation

The proportion of transitions and transversions in the D-loop data set were plotted against divergence to assess whether or not the data was saturated (Fig. 3.17).

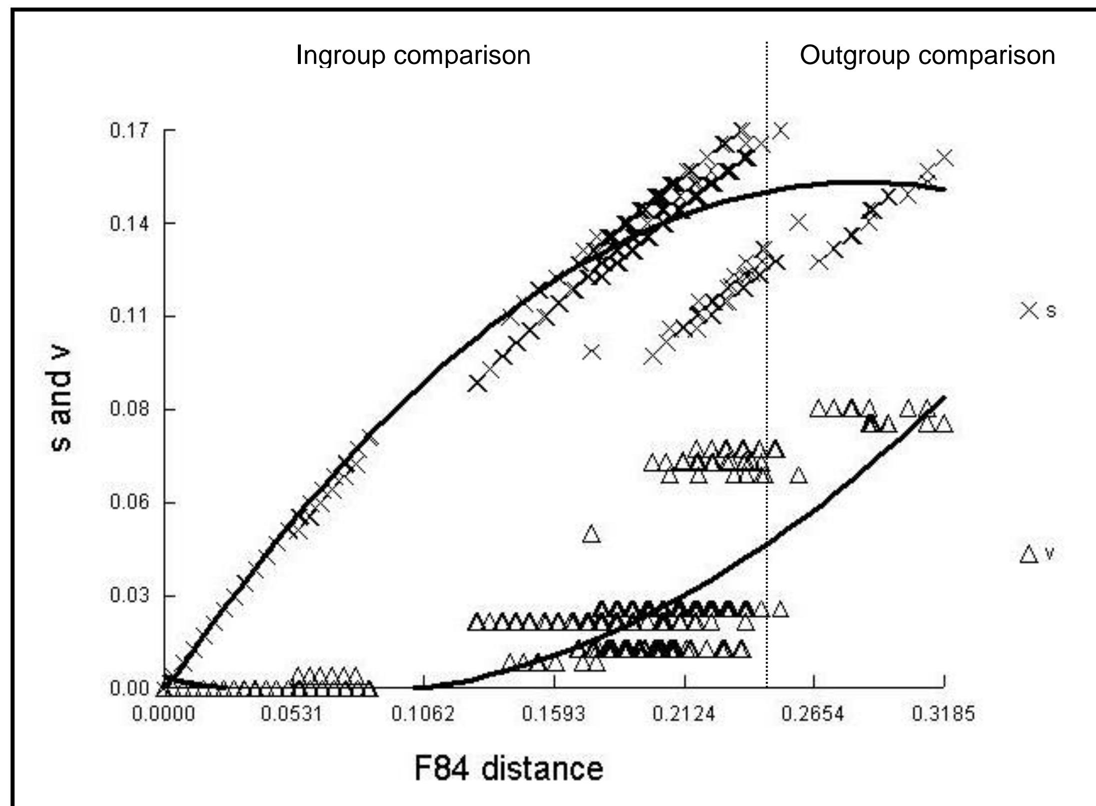

Figure 3.17. Transitions and transversions versus genetic divergence for the D-loop sequences; s = transitions and v = transversions. Solid lines represent the least squares best-fit line. *O. wroughtoni* and *O. formosus* were outgroups in analysis.

There appears to be no saturation in transversions. Transitions begin to plateau above a genetic distance of 0.2124, where comparisons with outgroups are represented. There appears to be little saturation at lower genetic distances, which represent ingroup comparisons.

The Xia *et al*. (2003) test revealed that the index of substitution saturation (Iss = 0.284) is significantly lower than the critical value when assuming a symmetrical topology (Iss.cSym = 0.675) but is not significant when assuming an assymetrical topology (Iss.cAsym = 0.336), with probabilities of 0.000 and 0.4388 respectively. This indicates that there is some

saturation in the data but the data is still viable for use in phylogenetic and phylogeographic analysis.

3.4.2 Statistical analyses of sequence data

Groupings comprised *Otomops* from south/west Africa (excluding Ivory Coast), Ivory Coast, north/east Africa, Madagascar and the outgroups, *O. wroughtoni*, *O. formosus* and *M. jugularis* (Table 3.9).

Table 3.9. Sample groupings used in D-loop analyses.

Group	Locality	Samples	GenBank #
[1] *Otomops* south/west Africa	Silverglen, RSA	Durban Silverglen2	EF216451
	Silverglen, RSA	Durban Silverglen4	EF216452
	Silverglen, RSA	Durban Silverglen5	EF216453
	Pinetown, RSA	Durban Pinetown1	EF216447
	Pinetown, RSA	Durban Pinetown2	EF216448
	Pinetown, RSA	Durban Pinetown3	EF216449
	Pinetown, RSA	Durban Pinetown4	EF216450
	Park Rynie, RSA	Durban Park RynieM324	EF216446
	Morningside, RSA	Durban MorningsideM321	EF216444
	Amanzimtoti, RSA	Durban AmanzimtotiM322	EF216445
	Kibira NP, Burundi	Burundi M415	EF216443
[2] *Otomops* Ivory Coast	Comoé NP, Ivory Coast	Ivory Coast	EF216454
[3] *Otomops* north/east Africa	Bale Province, Ethiopia	EthiopiaA1	EF216461
		EthiopiaA2	EF216462
		EthiopiaB1	EF216463
		EthiopiaB2	EF216464
		EthiopiaC1	EF216465
		EthiopiaC2	EF216466
		EthiopiaD1	EF216467
		EthiopiaD2	EF216468
	Makuena district, Kenya	Kenya1	EF216455
		Kenya3	EF216456

Table 3.9 continued.

Group	Locality	Samples	GenBank #
[3] *Otomops* north/east Africa	Makuena district, Kenya	Kenya4	EF216457
		Kenya5	EF216458
		Kenya6	EF216459
		Kenya7	EF216460
	Al Mawhit, Yemen	Yemen	EF216469
[4] *Otomops* Madagascar	Mahajanga	Mahajanga2	EF216384
		Mahajanga3	EF216385
		Mahajanga4	EF216386
	Fianarantsoa	Fianarantsoa1	EF216383
		Fianarantsoa19	EF216401
	Ambanila, Toliara	Toliara6	EF216388
		Toliara7	EF216389
		Toliara12	EF216394
		Toliara13	EF216395
	Bishihiko, Toliara	Toliara8	EF216390
		Toliara9	EF216391
		Toliara10	EF216392
		Toliara11	EF216393
		Toliara14	EF216396
	Ankarana, Antsiranana	Antsiranana15	EF216397
		Antsiranana16	EF216398
		Antsiranana17	EF216399
		Antsiranana18	EF216400
	Analamerana, Antsiranana	Antsiranana5	EF216387
		Antsiranana20	EF216402
		Antsiranana21	EF216403
		Antsiranana22	EF216404
		Antsiranana25	EF216405
[5]*Otomops wroughtoni*	Preah Vihear, Cambodia	O.wroughtoni	
[6]*Otomops cf. formosus*	Luzon Is, Philippines	O.formosus	
[7] *Mormopterus jugularis*		Mormopterus_jugularis I1P	

Sequence data sites were examined and results presented in Table 3.10, Table 3.11 and Table 3.12. A full listing of individual nucleotide composition frequencies is given in Appendix 3. Informative sites of each haplotype are represented in Appendix 5.

Table 3.10. Number of conserved, variable, parsimony informative and singleton sites out of 290 nucleotides found in D-loop sequence data, with and without outgroups included in analysis. Outgroups are *O. wroughtoni, O. formosus* and *M. jugularis*.

Variables (out of 290 nucleotides)	With outgroups	Without outgroups
Conserved sites	135	191
Variable sites	151	88
Parsimony informative sites	95	82
Singleton sites	54	6

Table 3.11. Nucleotide composition for groups using D-loop data.

Group	% T (U)	% C	% A	% G	Total
[1]*Otomops* south/west Africa	28.7	17.6	42.5	11.3	277
[2] *Otomops* Ivory Coast	28.2	18.1	44.4	9.4	277
[3]*Otomops* north/east Africa	27.1	18.6	43.8	10.5	278
[4]*Otomops* Madagascar	28.8	16.1	44.2	10.8	278
Average	28.2	17.2	43.7	10.8	277.8

Table 3.12. Nucleotide pair frequencies for groups using D-loop data.

Group	Identical pairs	Transitional pairs (si)	Transversional pairs (sv)	Ratio (si/sv)	Total
1	268	9	0	-	277.0
3	270	8	0	-	278.0
4	273	5	0	-	278.0

Approximately 65% of the ingroup sequence data comprises conserved sites, compared with 47% when outgroups are included in the analysis. The number of variable sites decreases upon exclusion of outgroups from 151 to 88 sites, as does the number of parsimony-informative sites (95 to 82).

D-loop sequences did show some difference in nucleotide composition frequencies across the groups. Group averages differed by a maximum of 2.5 in % C between *Otomops* north/east Africa and *Otomops* Madagascar, as well as 1.9 in both % A and % G between *Otomops* Ivory Coast and *Otomops* south/west Africa. Sequence data has high A and low G frequencies with averages of 43.7 and 10.8, respectively.

The analysis of nucleotide pair frequencies reveals that, of the number of nucleotides analysed, 96 – 98% were identical pairs for all samples in each group. *Otomops* south/west Africa had the highest number of transitional pairs (9), closely followed by *Otomops* north/east Africa (8). *Otomops* from Madagascar, north/east Africa and south/west Africa had no transversional pairs.

3.4.3 Phenetic analyses

All analyses were performed using the assumptions of the General Time Reversible + Invariant site + Gamma (GTR+I+G) model (section 1.5.1) as specified by MrModeltest v.2 using the Akaike Information Criterion (AIC) (Nylander, 2004).

All genetic distances were calculated using the GTR+I+G model in PAUP 4.0b10 (Swofford, 1993). Individual, between-group and within-group distances were calculated for both data sets.

3.4.3.1 Genetic distances

Individual pairwise genetic distances were calculated and are given in Appendix 4. Within-group distances are presented in Table 3.13. Net between-group distances are presented in Table 3.14 and Fig. 3.18.

Table 3.13. Within-group mean GTR+I+G genetic distance for D-loop (290 nt).

Groups	Genetic distance	Standard deviation
[1]*Otomops* south/west Africa	0.036	0.007
[3]*Otomops* north/east Africa	0.031	0.006
[4]*Otomops* Madagascar	0.020	0.004

Table 3.14. Net between-group GTR+I+G distances for *Otomops* samples and outgroups for D-loop (290 nt) (standard error above diagonal).

Taxon	1	2	3	4	5	6	7
[1]*Otomops* south/west Africa		0.014	0.025	0.028	0.031	0.033	0.059
[2]*Otomops* Ivory Coast	0.053		0.024	0.030	0.035	0.035	0.059
[3]*Otomops* north/east Africa	0.143	0.128		0.029	0.037	0.039	0.067
[4]*Otomops* Madagascar	0.155	0.170	0.166		0.033	0.032	0.058
[5]*Otomops wroughtoni*	0.212	0.221	0.260	0.199		0.029	0.057
[6]*Otomops formosus*	0.191	0.227	0.249	0.214	0.161		0.066
[7]*Mormopterus jugularis*	0.490	0.480	0.544	0.471	0.552	0.474	

Mean within-group genetic distances show *Otomops* south/west Africa to be the most diverse population (3.6%), and *Otomops* Madagascar to be the least diverse population (2.0%). Table 3.14 shows that the greatest divergence lies in the comparison of *M. jugularis* with the other outgroups, with distances of 55.2% and 47.4% from *O. wroughtoni* and *O. formosus*, respectively. The lowest divergence is seen between *Otomops* south/west Africa and *Otomops* Ivory Coast (5.3%), whereas divergence between *Otomops* north/east Africa and *Otomops* Ivory Coast is 14.3%. *Otomops* Madagascar separates from *Otomops* found in Africa with an average distance of 16.4% but shows greatest separation from *Otomops* Ivory Coast with a distance of 17%. *Otomops* outgroups are separated from *Otomops* ingroups by a minimum distance 19.1% between *O. formosus* and *Otomops* south/west Africa and a maximum of 26.0% between *O. wroughtoni* and *Otomops* north/east Africa.

Figure 3.18. Representation of net between-group GTR+I+G genetic distances (percent) for *Otomops* groupings for D-loop (290 nucleotides).

3.4.3.2 Neighbour-joining analysis

Genetic distances within and between *Otomops* samples and outgroups are represented in Fig. 3.19 as a neighbour-joining tree. The topology of the D-loop neighbour-joining tree was found to be similar to that of the corresponding cytochrome *b* tree. *Otomops* samples formed an exclusive cluster with 100% bootstrap support. Within the *Otomops* cluster, the outgroups, *O. wroughtoni* and *O. formosus*, separated from the *Otomops* Africa/Madagascar cluster with moderate bootstrap support (72 – 78%). The Africa/Madagascar *Otomops* cluster subdivides into two exclusive lineages separating samples according to location, i.e. Africa (85% bootstrap support) or Madagascar (93% bootstrap support). The African lineage splits into two well-defined and well-supported exclusive sister lineages comprising samples from south/west Africa (Tanzania, Burundi, Ivory Coast, Zimbabwe and South Africa) (96% bootstrap support) and north/east Africa (Kenya, Ethiopia and Yemen) (100% bootstrap support). Internal structure within these groups had varied support values ranging from 9% to 100%.

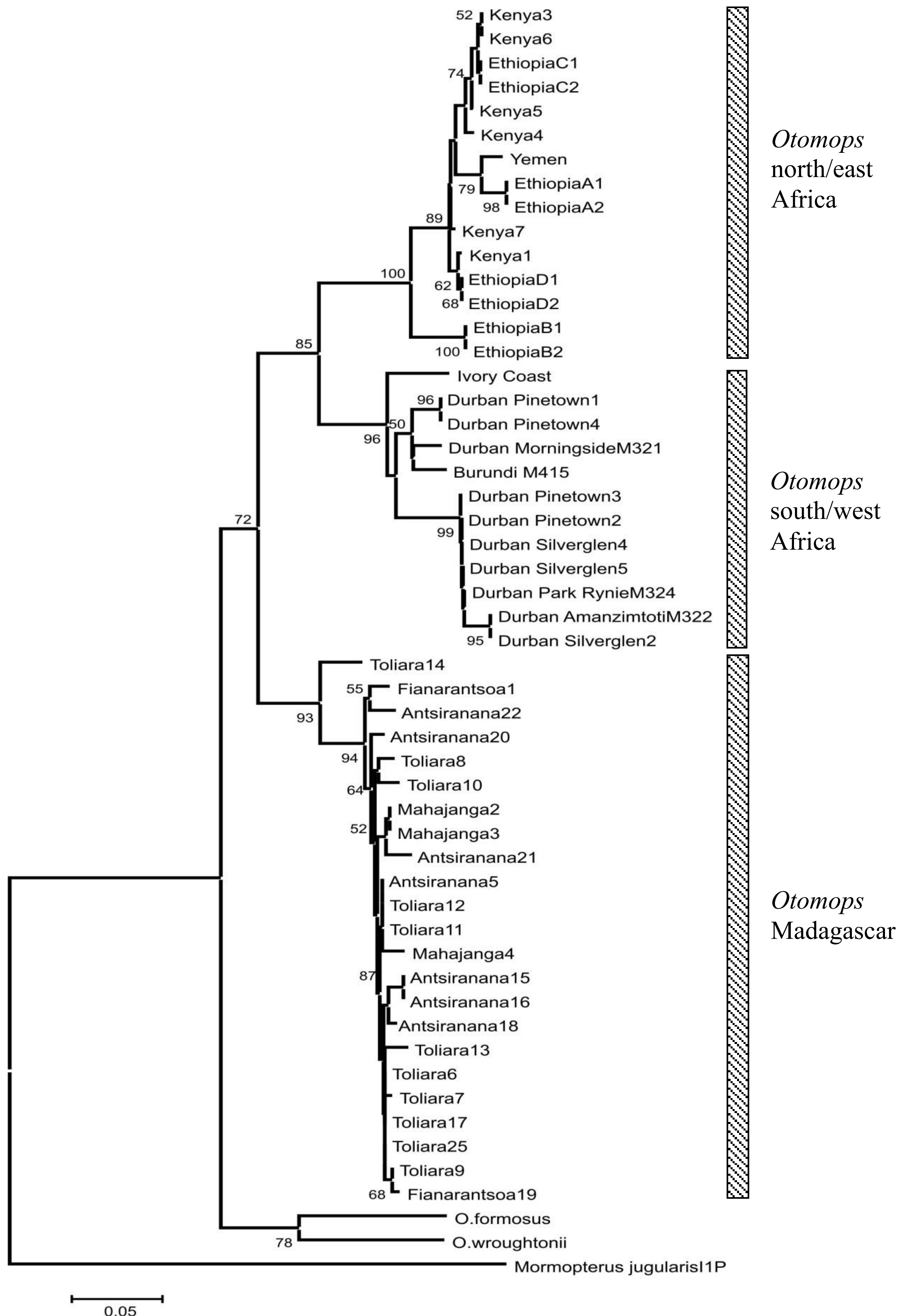

Figure 3.19. D-loop neighbour-joining tree using a GTR+I+G distance model (290 nt) with bootstrap support (500 replicates) showing relationships between 50 samples of *Otomops* with reference to the outgroups *O. wroughtoni*, *O. formosus* and *M. jugularis*. Only bootstrap values above 50% are shown.

3.4.3.3 Analysis of Molecular Variance (AMOVA)

Seventy nine percent of the variance was distributed among groups; this was significant (*P* (random value >= observed value) = 0.00426) (Table 3.15). Variance among populations within groups (5.16% of total) was significant (*P* (random value >= observed value) = 0.04921). Variance within populations (15.54% of total) was highly significant (*P* (random value >= observed value) = 0.00000).

Table 3.15. Results of Analysis of Molecular Variance (AMOVA) for D-loop.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variance
Among groups	3	584.238	16.83002 Va	79.30
Among populations within groups	3	20.948	1.09615 Vb	5.16
Within populations	43	141.813	3.29798 Vc	15.54
Total	49	747.000	21.22415	

Fixation Indices

FSC	0.24946
FST	0.84461
FCT	0.79297

Significance tests (10100 permutations)

Vc and FST	P (random value < observed value) = 0.00000
	P (random value = observed value) = 0.00000
	P (random value <= observed value) = 0.00000+ -0.00000
Vb and FSC	P (random value > observed value) = 0.04871
	P (random value = observed value) = 0.00050
	P (random value >= observed value) = 0.04921+ -0.00196
Va and FCT	P (random value > observed value) = 0.00000
	P (random value = observed value) = 0.00426
	P (random value >= observed value) = 0.00426+ -0.00069

3.4.4 Phylogenetic analysis

3.4.4.1 Bayesian analysis

Bayesian analysis was used to construct a 50% majority rule tree illustrating phylogenetic relationships between D-loop sequences (Fig. 3.20).

The topology of the Bayesian tree (Fig. 3.20) paralleled that of the neighbour-joining tree (Fig. 3.19). The outgroups, *O. wroughtoni* and *O. formosus*, may be ancestral to the monophyletic African and Malagasy *Otomops* ingroup clade (1.00 pp) however it should be noted that additional species and larger sample number would be needed to resolve this with confidence. Within this, the Malagasy and African clades formed reciprocally-monophyletic sister clades (1.00 pp and 1.00 pp, respectively). Within the Africa clade, the south/west and north/east clades also formed reciprocally-monophyletic sister clades (1.00 pp and 1.00 pp, respectively).

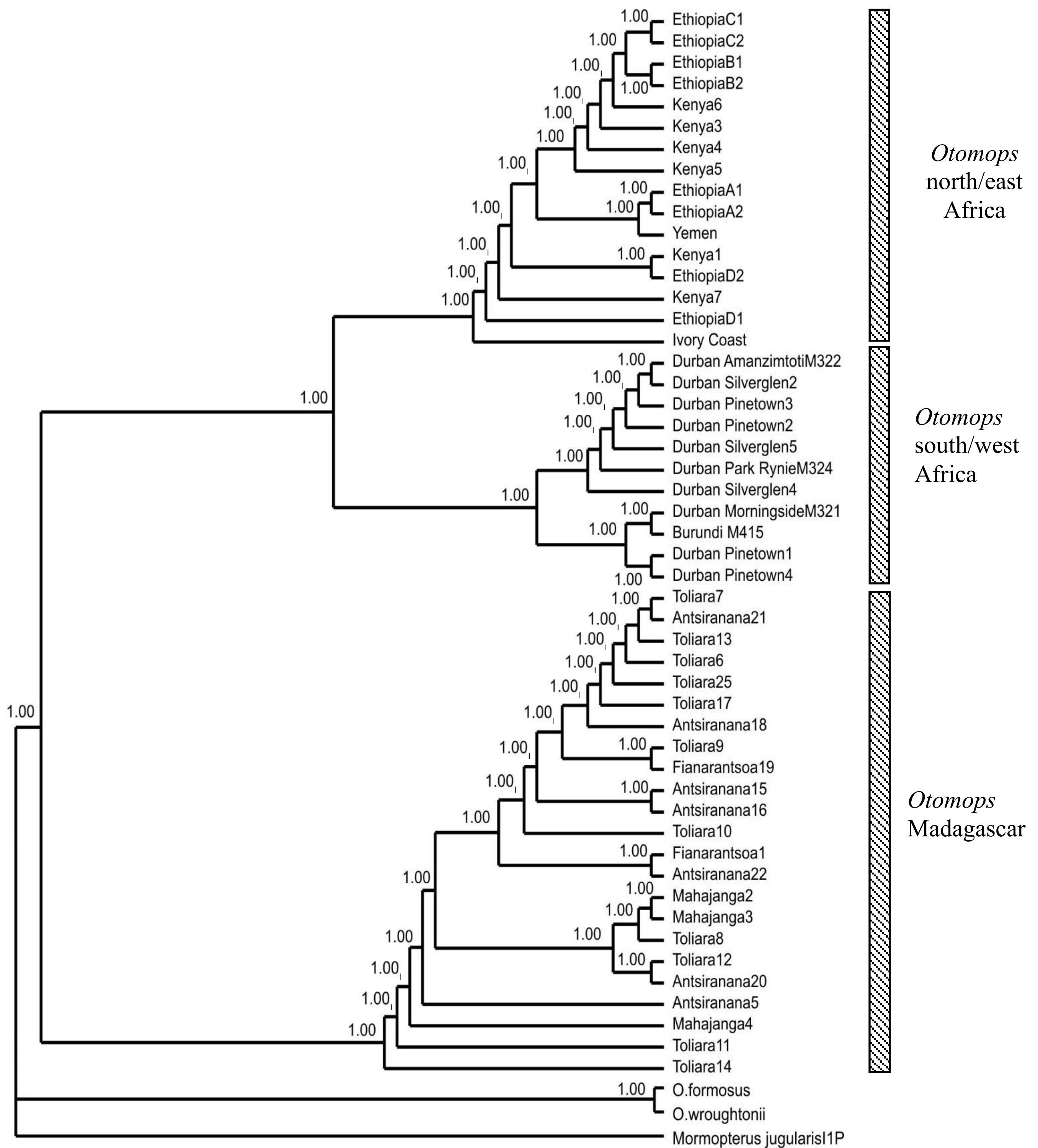

Figure 3.20. Bayesian phylogram based on 290 nt of the D-loop region showing relationships between 50 samples of *Otomops* with reference to the outgroups *O. wroughtoni*, *O. formosus* and *M. jugularis*. Support is indicated at the nodes as posterior probabilities.

3.4.5 Phylogeographic analysis

3.4.5.1 Haplotype number

The D-loop data set comprised 35 haplotypes when outgroups were excluded. Individual haplotypes formed 45% of the complete data set. Details of haplotypes are given in Table 3.16.

Table 3.16. Haplotypes within the D-loop data set, excluding outgroups.

Haplotype number	Number of samples	Sample names	Haplotype network codes
1	2	Durban Pinetown1; Durban Pinetown4	P1; P4
2	1	Durban MorningsideM321	Ms
3	1	Burundi M415	B
4	2	Durban Pinetown3; Durban Silverglen5	P3; Sg5
5	2	Durban Silverglen4; Durban Park RynieM324	Sg4; Pr1
6	1	Durban Pinetown2	P2
7	2	Durban AmanzimtotiM322; Durban Silverglen2	At; Sg2
8	1	Ivory Coast	IC
9	1	Kenya1	K1
10	2	Kenya3; Kenya6	K3; K6
11	1	Kenya4	K4
12	1	Kenya5	K5
13	1	Kenya7	K7
14	2	EthiopiaA1; EthiopiaA2	Ea1; Ea2
15	2	EthiopiaB1; EthiopiaB2	Eb1; Eb2
16	2	EthiopiaC1; EthiopiaC2	Ec1; Ec2
17	2	EthiopiaD1; EthiopiaD2	Ed1; Ed2
18	1	Yemen	Y
19	1	Fianarantsoa1	F1
20	2	Mahajanga2; Mahajanga3	M2; M3
21	1	Mahajanga4	M4
22	3	Antsiranana5; Toliara11; Toliara12	A5; T11; T12
23	3	Toliara6; Antsiranana17; Antsiranana25	T6; A17; A25

Table 3.16 continued.

Haplotype number	Number of samples	Sample names	Haplotype network codes
24	1	Toliara7	T7
25	1	Toliara8	T8
26	1	Toliara9	T9
27	1	Toliara10	T10
28	1	Toliara13	T13
29	1	Toliara14	T14
30	2	Antsiranana15; Antsiranana16	A15; A16
31	1	Antsiranana18	A18
32	1	Fianarantsoa19	F19
33	1	Antsiranana20	A20
34	1	Antsiranana21	A21
35	1	Antsiranana22	A22

3.4.5.2 Population genetic analyses

The demographic history of *Otomops* was analysed using a variety of diversity tests, neutrality tests and mismatch distribution analyses using D-loop data (Russell *et al.*, 2005). Data was analysed both as a complete D-loop set and according to genetically-defined species-groups, i.e. south/west Africa, north/east Africa and Madagascar. Results of these analyses are represented in Table 3.17.

Table 3.17. Diversity tests, neutrality tests and mismatch distribution analysis for the D-loop.

	Overall	South/West Africa	North/East Africa	Madagascar	Expectation #
Nucleotide diversity (π)	0.1064	0.0337	0.0302	0.0196	Low
Haplotype diversity (*h*)	0.9860	0.9270	0.9520	0.9680	High
Expansion co-efficient (*S/d*)	2.9513	2.7821	3.3409	5.8752	High
Fu & Li's (1993) F*	1.8112	0.4482	0.7216	-1.5592	Not significant
Fu & Li's (1993) D*	1.4998	0.4477	0.9102	-1.2941	Not significant
Fu's (1997) F_S	-2.7035	1.2093	-0.5091	-7.5225**	Significant
Raggedness (*rg*)	0.0049	0.0853	0.0434	0.0144**	
Mismatch distribution	Multimodal	Multimodal	Multimodal	Unimodal	Unimodal
τ (in mutational units)	13.440	4.767	4.382	2.621	
Time since expansion (yr BP)				27 388 – 52 242	

Significant results are indicated by asterisks: ** $p < 0.05$

Expected trends for a model of demographic population expansion (Peck and Congdon, 2004)

Haplotype analysis of 50 *Otomops* samples revealed that there are 87 variable sites (out of 290 nucleotides), yielding 35 haplotypes. Results show that the D-loop data have a high overall *h* value (0.9860; standard deviation, 0.006) and a low π value for each population (SWA, 0.0337; NEA, 0.0302; MAD, 0.0196) (Table 3.17), comparable to those found in Russell *et al*. (2005). The average number of nucleotide differences (k) was 29.4784. The Malagasy group has the highest haplotype diversity (0.9680), low nucleotide diversity (0.0198) in relation to other groups and the largest expansion co-efficient (5.8752).

Neutrality tests suggest population growth for Malagasy *Otomops* populations, where Fu & Li's (1993) *F** and *D** are not significant ($F^* = -1.5592$, $P > 0.10$; $D^* = -1.2941$, $P > 0.10$) while Fu's (1997) F_S was significant ($F_S = -7.5225$, $P < 0.05$). Both north/east and south/west Africa groups, however, have neither a significant Fu & Li's (1993) *F** and *D** nor a significant Fu's (1997) F_S.

The overall mismatch distribution for the D-loop data was multimodal (Fig. 3.21). The Malagasy population, however, has a unimodal distribution with significantly low raggedness (*rg* = 0.0144, $P < 0.05$), which is expected in cases of population expansion. South/west and north/east African populations show a pattern closer to a multimodal mismatch distribution according to a model describing a constant population size through time, and raggedness statistics for both groups are not significant (south/west Africa: *rg* = 0.0853, $P > 0.05$; north/east Africa: *rg* = 0.0434, $P > 0.05$).

The Malagasy group meets the expectations set by Hull and Girman (2005) and may thus be considered to be an expanding population. Estimates of the time since population expansion were taken from the mismatch distribution analysis and calculated, as outlined in Rogers and Harpending (1992), for the Malagasy group. The population(s) in Madagascar have been expanding for 27 388 – 52 242 years BP.

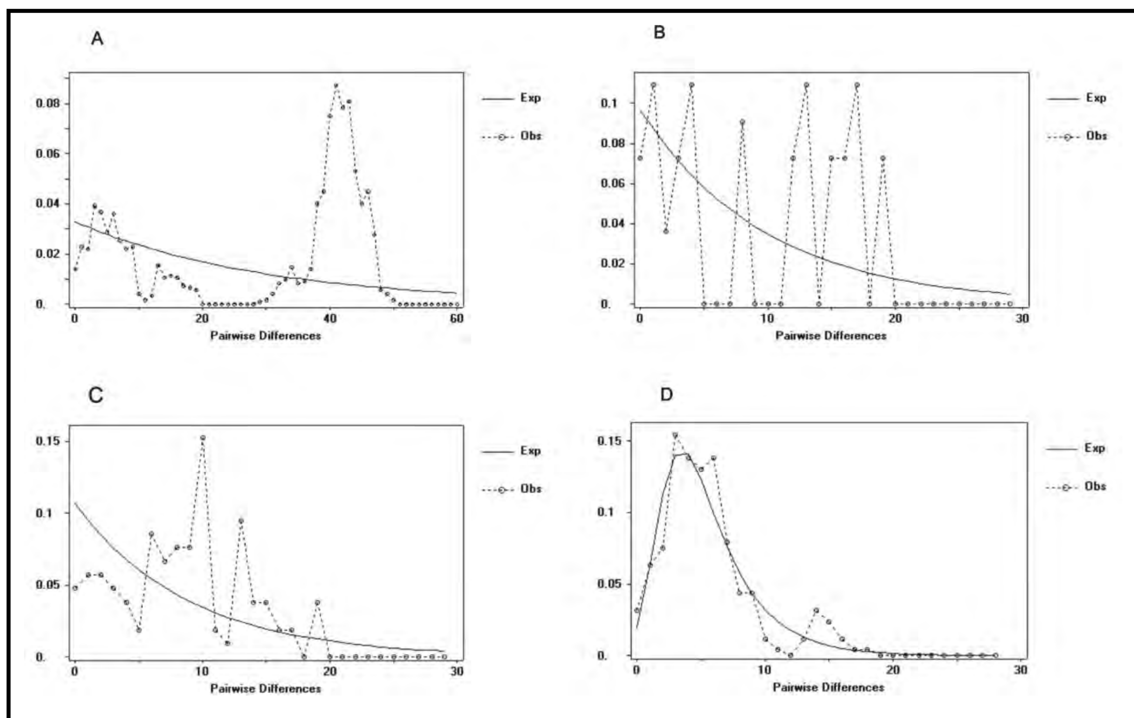

Figure 3.21. Mismatch distributions for the D-loop data set with expected distribution based on a model of constant population size though time (A, B and C) and a model of exponential population growth (D). A: overall; B: south/west Africa; C: north/east Africa; D: Madagascar.

3.4.5.3 Haplotype network

Statistical parsimony analysis of the D-loop data using TCS version 1.2.1 generated three haplotype networks when set at the 95% connection limit. These networks comprised

samples from south/west Africa, north/east Africa and Madagascar. The connection limit was set at 80 mutational steps in order to create a single network including all *Otomops* samples (Fig. 3.22).

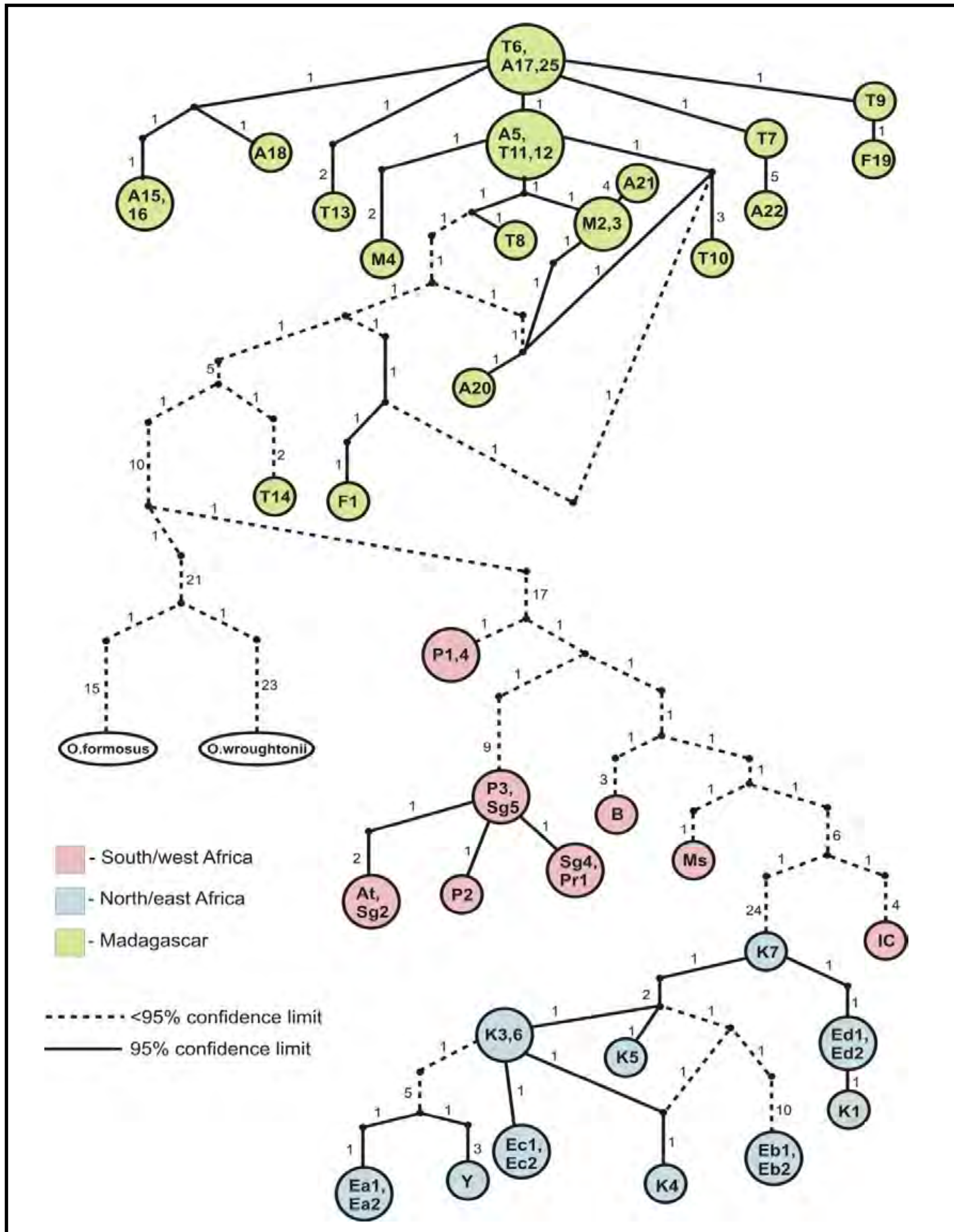

Figure 3.22. D-loop haplotype network showing mutational relationships between 35 *Otomops* haplotypes with reference to the outgroups *O. wroughtoni* and *O. formosus*. Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.16.

Haplotypes are separated according to location which, as in the cytochrome *b* data, indicates the presence of geographically-defined genetic groups. Samples from south/west Africa are joined to those from north/east Africa through haplotypes Durban MorningsideM321 (Ms) and Kenya7 (K7) by 34 mutational steps and to those from Madagascar though the Pinetown1 and Pinetown4 (P1, 4) haplotype and Toliara14 (T14) haplotype by 33 steps. Samples from Madagascar and north/east Africa are separated by a minimum of 69 steps between haplotypes Toliara14 (T14) and Kenya7 (K7). Within the groups, haplotypes are separated by a maximum of 16 steps in south/west Africa, 13 steps in Madagascar and 15 steps in north/east Africa. A similar trend is found in the cytochrome *b* haplotype network. Ingroups are separated from both outgroups by 37, 42 and 78 steps to Madagascar, south/west Africa and north/east Africa, respectively.

3.4.5.4 Haplotype correlations to environmental factors

Statistical parsimony analysis of the D-loop data using TCS version 1.2.1 generated a single haplotype network (100 mutational steps) including all *Otomops* samples. Environmental factors were superimposed onto haplotype networks in order to determine whether *Otomops* in Africa and Madagascar show genetic structure based on mean annual temperature, mean annual precipitation, altitude or sample locality (Fig. 3.23, Fig. 3.24, Fig. 3.25, Fig. 3.26).

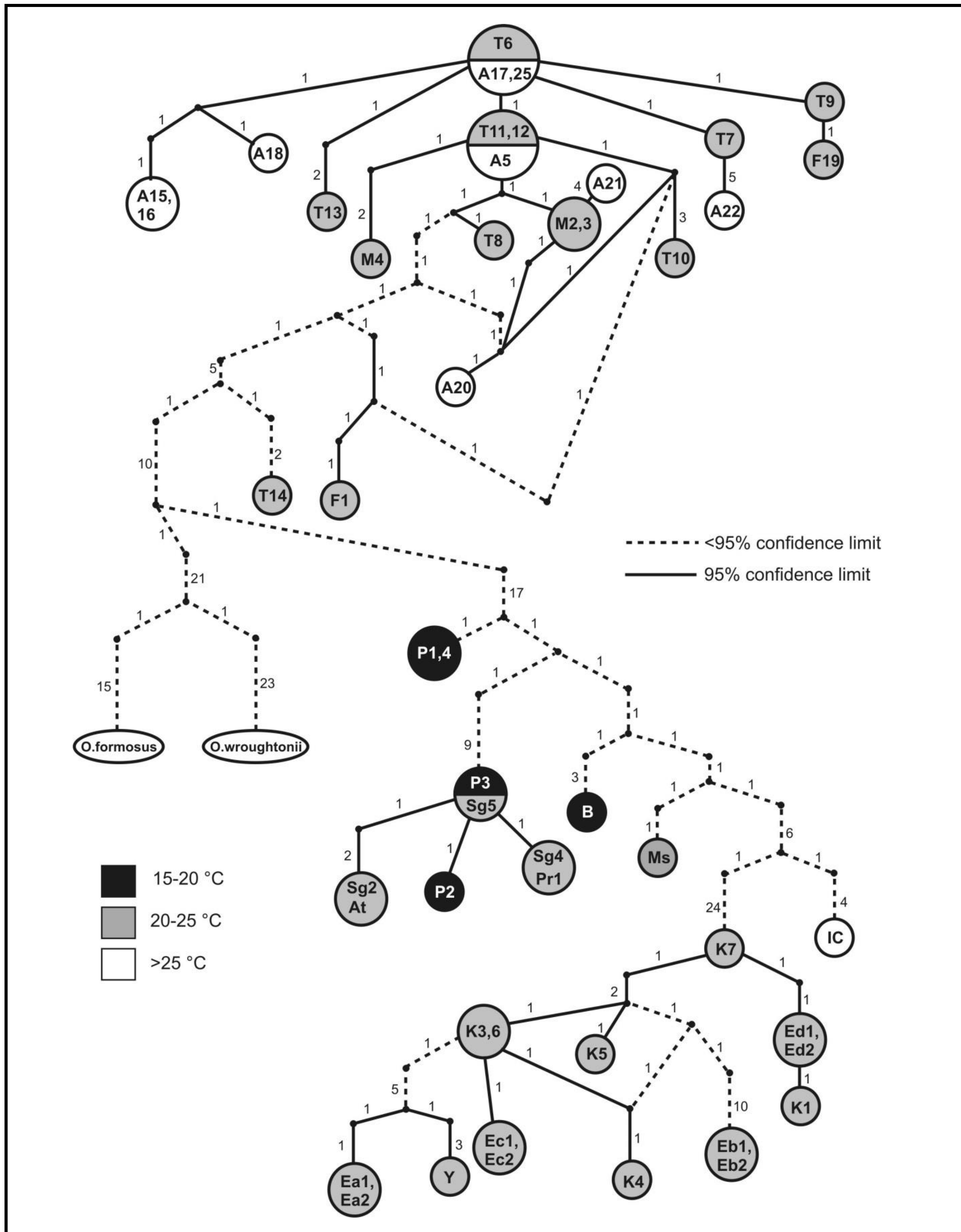

Figure 3.23. D-loop haplotype network with superimposition of mean annual temperature (°C). Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.16.

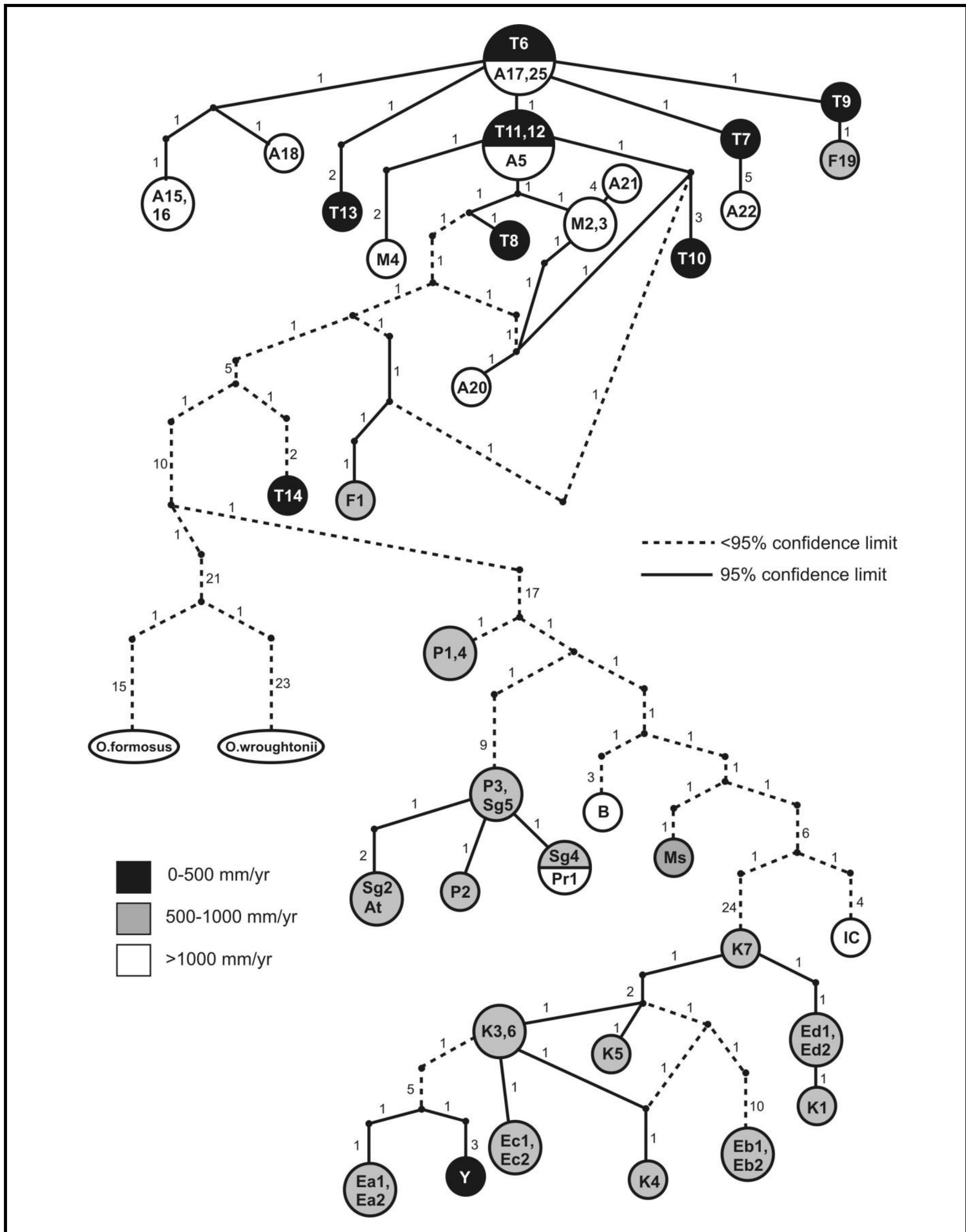

Figure 3.24. D-loop haplotype network with superimposition of mean annual precipitation (mm rainfall per year). Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.16.

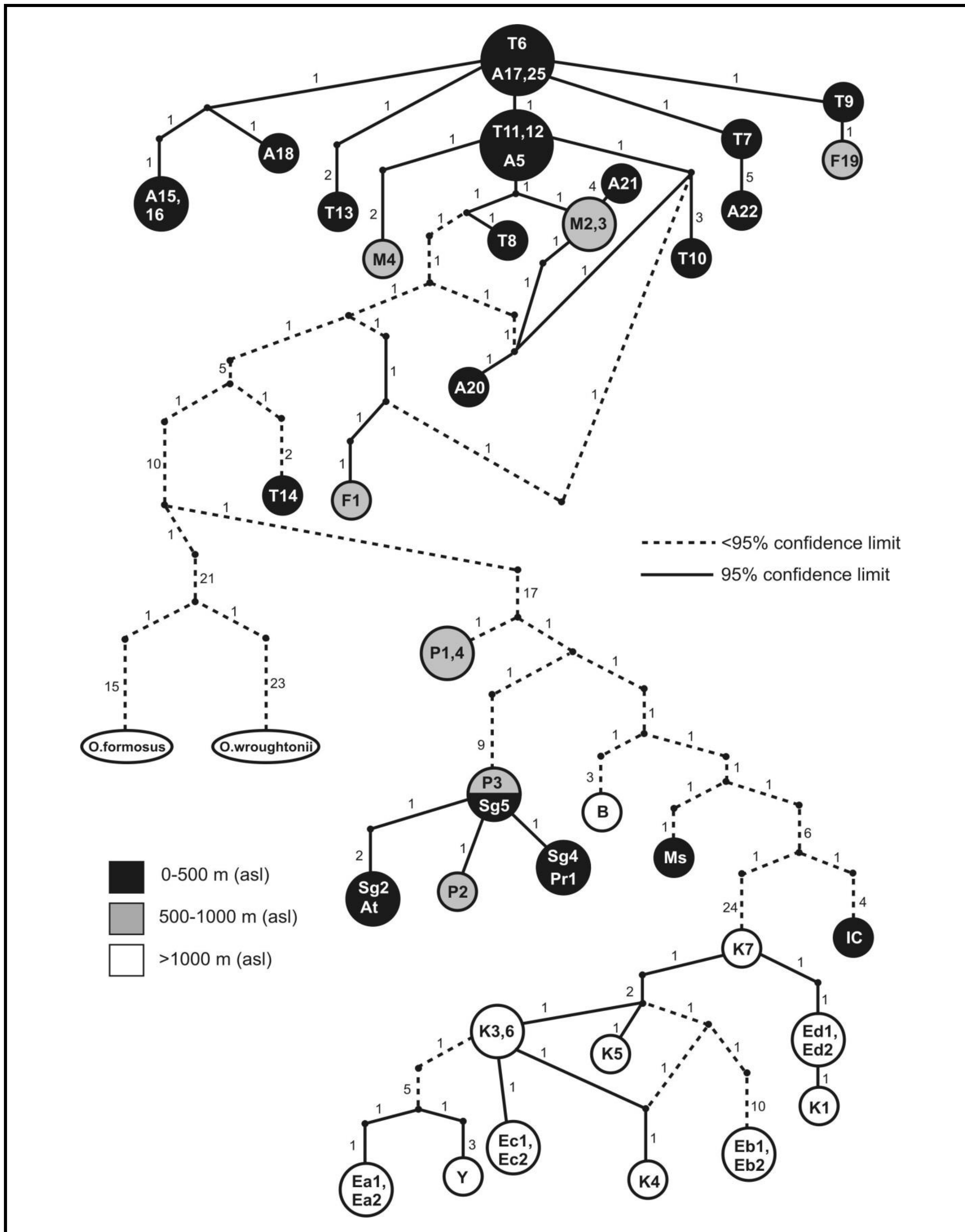

Figure 3.25. D-loop haplotype network with superimposition of altitude (m above sea level). Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.16.

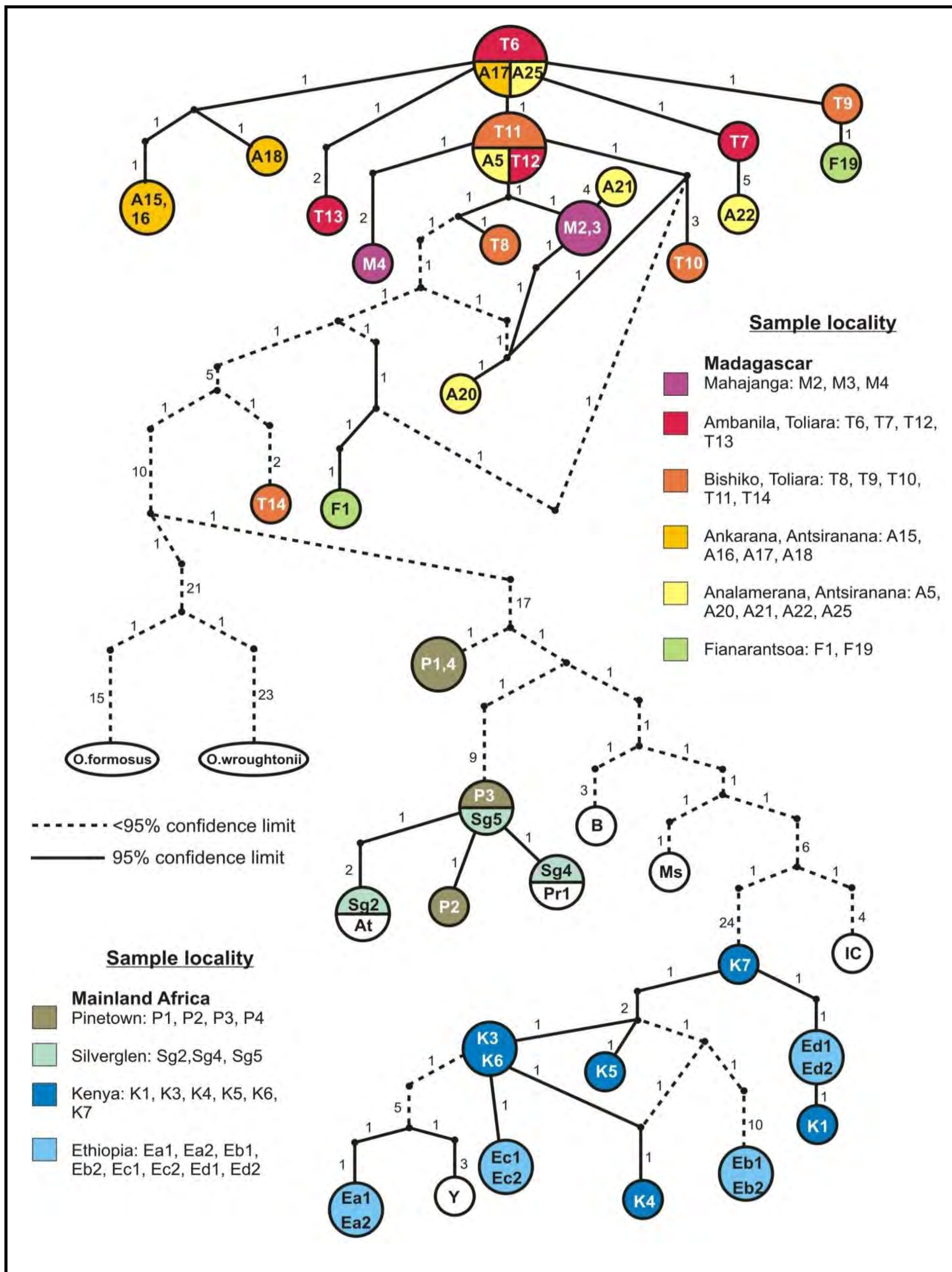

Figure 3.26. D-loop haplotype network with superimposition of sample localities. Single sample localities are left uncoloured. Numbers along branches indicate mutational steps. Circle size is representative of the number of samples per haplotype. Haplotypes are defined in Table 3.16.

As would be expected in the case of geographically defined genetic groups, patterns of their association with temperature, precipitation, altitude and sample localities are the same, based on the D-loop, as they were for the cytochrome *b* region (section 3.3.5.3).

4. DISCUSSION

4.1 Taxonomy of Afro-Malagasy Otomops

Phenetic, phylogenetic and phylogeographical analyses of cytochrome *b* and D-loop data suggest the existence of two reciprocally-monophyletic *Otomops* clades (Madagascar and Africa), with the African group further divided into two reciprocally-monophyletic clades, north/east Africa (NEA) and south/west Africa (SWA). There are thus three distinct, reciprocally-monophyletic *Otomops* clades, as seen in neighbour-joining and Bayesian trees of both cytochrome *b* and D-loop. All trees show congruence in their topology (Fig. 3.10, Fig. 3.11, Fig. 3.19, Fig. 3.20). The presence of three *Otomops* clades is also supported by haplotype networks which show the division of samples into three geographically and genetically-defined groups, and AMOVA which showed 80% of the variance to be significant among groups for both cytochrome *b* and D-loop markers (Table 3.7, Table 3.15).

The clade from Madagascar is recognized as *Otomops madagascariensis*, a conclusion that is also supported by the fact that these individuals are geographically isolated and exhibit morphological differences from the mainland variety (Peterson *et al*., 1995). Within the African group, the SWA clade includes individuals that were found close to the type localities of both *O. martiensseni* (Magrotto Plantation, west of Tanga, at the foot of the East Usambara Mountains, Tanzania) and *O. icarus* (Durban, South Africa), hence *icarus* can be regarded as a junior synonym of *martiensseni* (Matschie, 1897; Chubb, 1917). If the NEA and SWA clades were to be regarded as separate species, the SWA clade would then be regarded as *O. martiensseni* and the NEA clade would constitute an as yet undescribed taxon. Provisionally, the NEA and SWA lineages might be better classified as major clades or subspecies since levels of divergence for species are comparatively low (section 1.3.3).

A study by Baker and Bradley (2006) of the cytochrome *b* sequences of twelve bat genera, revealed the ranges of genetic distance (under the Genetic Species Concept) separating animals at different taxonomic levels: genus level differences ranged from 8.4% to 15.7%; species level differences from 3.3% to 14.7%; and within-species differences from 0.6% to 2.3%. *Otomops madagascariensis* is essentially equidistant genetically from NEA *Otomops* (cytochrome *b* divergence = 3.5%; D-loop divergence = 16.6%) and SWA *Otomops* (cytochrome *b* divergence = 3.1%; D-loop divergence = 15.5%), whilst the continental NEA and SWA groupings separate with a genetic distance of 2.1 % (Fig. 3.9, Fig. 3.18). This result lends support to the finding of Lamb *et al*. (2006) that *Otomops* populations from East Africa

and South Africa are reciprocally-monophyletic in cytochrome *b* and D-loop sequences, albeit at a low percentage sequence divergence for cytochrome *b* (2.5%).

Lower levels of sequence divergence, like those between *O. madagascarensis* and NEA and SWA *Otomops*, could separate valid species which have recently diverged and which might manifest incomplete lineage sorting (artefact of retained ancestral polymorphism) and/or a low level of ongoing gene flow (Ditchfield, 2000; Bradley and Baker, 2001; Mayer and von Helversen, 2001; Hoffman and Baker, 2003; Juste *et al*., 2003; Ramon *et al*., 2003; Russell *et al*., 2005; Baker and Bradley, 2006; Jacobs *et al*., 2006). For example, using the mitochondrial ND1 gene, Mayer and von Helversen (2001) found that *Eptesicus serotinus* and *E. nilssonii* differed by 0.7 – 1.4% and similarly, *Myotis blythii* and *M. myotis* differed by 0.25 – 2.6%; however, both are classified as separate species. The mitochondrial ND1 gene and cytochrome *b* gene are both protein-coding regions and are known to evolve at a similar rate (Mayer *et al*., 2007). Piaggio *et al*. (2002) also found distances between certain recognized *Myotis* species to be relatively low (*M. velifer – M. yumanensis* cytochrome *b* divergence = 3.0%).

The work of Benda *et al*. (2004) on *Pipistrellus* has parallels with this study. As with this study, they too found that African populations represent two genetically, geographically and morphologically separate units within the *P. pipistrellus* complex. *P. pygmaeus* inhabits the Mediterranean part of Libyan Cyrenaica and showed 6 – 7% divergence from their European counterparts, whilst *P. pipistrellus* inhabits Mediterranean parts of the north-west African countries, namely Morocco, Algeria and Tunisia (the Maghreb) and showed 3 – 5% divergence from the European populations, divergence values which are similar to those found in this study. Jacobs *et al*. (2006) identified two sympatric phonic types of *Scotophilus dinganii* which were reciprocally-monophyletic for cytochrome *b* sequences (3.3% divergence), suggesting that they are sibling species.

Although the West African sample from Ivory Coast shows a phylogenetic affinity with the South African group, the cytochrome *b* divergence between the Ivory Coast and the SWA species-groups is equivalent to the distance observed between the NEA and SWA groups (divergence = 2.10%). This is of interest in that NEA and SWA groups have been provisionally defined as major clades or possible separate subspecies based on findings from this study. Individual cytochrome *b* pairwise distances show that the greatest distances are between South African and Ivory Coast samples (Appendix 4). These distances may be indicative of a recent or incomplete speciation event occurring within the Ivory Coast population since the taxonomic status of this population is poorly resolved (it is

geographically distant, yet closely-related to the southern African populations) (Burgess *et al*., 2004; Russell *et al*., 2007). Haplotype network structure lends support to this since it shows that the Ivory Coast sample is most closely linked to the Tanzanian sample, although the number of steps between them is greater than the number of steps between any other two samples within the group (Fig. 3.12). The Ivory Coast sample is thus more distinctly separated from the rest of the south/west Africa group, which suggests the possibility of a speciating population in this region. Ivory Coast *Otomops* could thus be regarded as a potential MU or even as ESU (section 1.3.3, section 1.3.4). There is clearly a need for more data from this locality before any specific distinction can be credibly established and supported.

It has been suggested that within Africa, the Usambara Mountains and the Rift Valley in the region of the African Great Lakes form a geographical border/barrier between the two African species groups, thereby allowing for the possible ongoing speciation of *Otomops* into the NEA and SWA clades. A similar situation is seen within populations of Starred Robins (*Pogonocichla stellata*) around the montane circle of Africa (Bowie *et al*., 2006). Genetic structure and diversity of these birds has been largely influenced by at least two major vicariance events (one separating the Albertine Rift from all but the Kenyan Highlands (1.3 – 1.2 MYA), and another separating the Kenyan Highlands from the northern Eastern Arc, and the northern Eastern Arc from the south-central Eastern Arc (0.9 – 0.8 MYA)) giving rise to four major ancestral populations, i.e. Kenyan Highlands (subspecies *keniensis*), Albertine Rift (*ruwenzori*), northern Eastern Arc (*helleri*) and south-central Eastern Arc, Ufipa and the Malawi Rift (*orientalis*) (Bowie *et al*., 2006). In Europe, the Alps prevented the northward expansion of Italian and Balkan haplotypes of *M. myotis*, and in South America, the uplifting of the Andes presented a strong barrier to gene flow and dispersal for *Carollia* and *Uroderma* species, thereby shaping present-day distributions and biodiversity (Hoffman and Baker, 2003; Ruedi and Castella, 2003).

Since *Otomops* is not limited by flight capability, populations of *Otomops* in mainland Africa must have also been separated by some other means. The distinct genetic separation of the NEA and SWA lineages could be influenced by insect migration patterns whereby *Otomops* in the southern hemisphere follows an austral cycle of migration and *Otomops* in the northern hemisphere follows a boreal cycle (section 4.4.2). In this way, *Otomops* from the north (Ethiopia, Kenya and Yemen) and south (South Africa, Burundi, Zimbabwe, Tanzania) might be kept somewhat separate despite the fact that their ranges apparently adjoin. This suggestion, however, is speculative at this stage since data does not fully corroborate the theory. Ivory Coast samples should group with the NEA clade since they would follow the

boreal cycle of insect migration and insects would have had to have been migrating in these patterns since before the arrival of *Otomops* on the African continent (2.4 – 2.6 MYA).

4.2 Phylogeny and phylogeography of Afro-Malagasy Otomops

According to the guidelines outlined for Avise's phylogeographic categories, *Otomops* would be defined as Category I, having deep mitochondrial DNA divergence and an allopatric geographic distribution of variants (Ruedi and McCracken, 2009) (section 1.5.5). This particular category is typical of conspecific isolates (Avise, 2000). A similar situation is seen in the case of *Miniopterus schreibersii natalensis*, where D-loop data revealed the separation of haplotypes into three geographically separate groups (maximum 6% divergence between groups) (Miller-Butterworth *et al*., 2003). Category I is unexpected in species capable of large migratory movements and therefore suggests that females are strongly faithful to their breeding colonies and migrate in relatively closed societies (Ruedi and McCracken, 2009).

The Afro-Malagasy *Otomops* clade consists of three species-groups or lineages, with Asian *Otomops* occupying a position basal to this clade. Asian *Otomops* species appear to be older than their Afro-Malagasy counterparts, since they are separated from them by larger genetic distances (cytochrome *b*, ~10 – 11%; D-loop, ~19 – 26%) than those seen between the species-groups within the Afro-Malagasy clade (cytochrome *b*, ~2 – 5%; D-loop, ~14 – 17%) (Table 3.6; Table 3.14). Greater species diversity of Asian *Otomops* and older divergences for the two species sampled suggest that this was the region of origin and hence may be ancestral to the Afro-Malagasy species, as seen in the cytochrome *b* Bayesian tree (Fig. 3.11). Similar cytochrome *b* divergences have also been reported between lineages within African *Chaerephon* species (*C. ansorgei* – *C. pumilus* divergence = 11.18%, range = 10.31 – 11.18%) (Jacobs *et al*., 2004) and *Scotophilus* species (*S. heathi* – *S. dinganii* divergence = 11.4%, range 10.9 – 13.5%) (Jacobs *et al*., 2006). Jacobs *et al*. (2006) also report D-loop divergences of 14.9 – 18% (average 16.4%) between *S. viridis* and *S. dinganii*.

Fossil records for most bat species, i.e. Molossidae (including *Otomops*), Phyllostomidae, Pteropodidae, Vespertilionidae and Rhinolophidae, are lacking, and this makes diagnosing dispersal patterns and dates of origin difficult (Jones *et al*., 2005). Characterization of population structure at the microgeographic level, using statistical parsimony haplotype networks, may help infer patterns of historical dispersal and colonisation (Burland *et al*., 1999; Burland and Worthington Wilmer, 2001; Abbott and Double, 2003; Newton *et al*., 2003). Since the Malagasy lineage is largely equidistant genetically between the SWA and NEA mainland lineages, it is difficult to establish the pattern of dispersal and speciation

within the Afro-Malagasy clade. In addition, haplotype networks for cytochrome *b* and D-loop show slightly different results and thus different patterns of possible historical dispersal.

The cytochrome *b* phylogenetic trees suggest that modern-day Afro-Malagasy species dispersed simultaneously from Asia to Madagascar and north/east Africa. The NEA form later dispersed southward to South Africa and western Africa (Fig. 3.12). According to dating analysis conducted by Lamb *et al*. (2008), this Asia-Africa divergence may have occurred ~7.7 MYA during the late Miocene (Appendix 6). Many species from the Afrotropical region appear most closely related to Asian fauna and have very little in common with fauna from other southern continents, e.g. tooth-combed (strepsirrhine) primates which are found in the Old World tropics and subtropics, and the Udzungwa partridge from Tanzania which is a relict animal species that has its strongest associations with species groups in Asia (Sclater, 1894; Bigalke, 1968; Burgess *et al*., 2004; Masters *et al*., 2007). Bat genera found in both Africa and Asia include *Chaerephon* (*C. ansorgei*, *C. bemmeleni* and *C. bivittatus* from Africa and *C. jobensis*, *C. johorensis* and *C. plicatus* from Asia), *Myotis* (*M. welwitschii*, *M. tricolor* and *M. morrisi* from the Afrotropical region and *M. formosus* and *M. emarginatus* from Asia), *Hipposideros* and *Nycteris* (Griffiths, 1997; Taylor, 1999b; Goodman and Cardiff, 2004; Stadelmann *et al*., 2004b). Using tracheal characters, Griffiths (1997) suggests that two *Nycteris* species from southeastern Asia (*N. javanica* and *N. tragata*) are possibly derived from species in western Africa (*N. arge*, *N. nana*, *N. major* and *N. intermedia*), and subsequently speciated due to the loss of suitable intermediate habitat in northeastern Africa and southwestern Asia.

The theory of a downward dispersal from north/east Africa is supported by the fact that eastern Africa, from the Albertine Rift montane forests and the Victoria Basin forest-savannah mosaic down toward the central Zambezian Miombo woodlands, has the highest species richness and biodiversity in mammals (Pomeroy, 1993; Burgess *et al*., 2004). Hence, Eastern Africa is considered part of the Afrotropical realm from which numerous species have evolved, radiated and subsequently speciated (Bigalke, 1968; Villee *et al*., 1989; Burgess *et al*., 2004). This theory is also supported by airflow patterns across Africa, including those during the Pliocene-Pleistocene era (DeMenocal, 2004). A north-easterly wind moves south from December to March and these airflows may assist dispersal in animals with long-distance flight capabilities (Crowe, 1949, 1950; Hills, 1979). Divergence between the NEA and SWA groups may have taken place during the Pliocene-Pleistocene ~2.4 MYA (Lamb *et al*., 2008).

Most island populations arise as a result of colonisation; Malagasy fauna exhibit close ties to both Afrotropical and Asian fauna (Berry, 1996). Theories relating to the origin of Malagasy bats involve biogeographical scenarios of dispersal because isolation of the island from the African mainland (165 MYA) and India (88 MYA) predates the evolution of Chiroptera (71 – 58 MYA) (Eizirik *et al*., 2001; Hingston *et al*., 2005; Russell *et al*., 2007). Malagasy and African lineages appear to have diverged during the Pleistocene, ~3.1 MYA. Analyses of the biogeographic affinities of the Malagasy bat fauna indicate that the majority of taxa are of Afrotropical origin, e.g. molecular analysis places *M. goudoti* from Madagascar close to *M. scotti* from Ethiopia and in a derived position (Eger and Mitchell, 2003; Stadelmann *et al*., 2004 b; Andriafidison *et al*., 2006). Findley (1972) ascertained that *M. goudoti* was found in a clade consisting of *M. tricolor* from Africa and *M. emarginatus* from Asia. A new species of *Pipistrellus* from Madagascar, recently described by Bates *et al*. (2006) on the basis of several morphological characters, was found to have a close affinity to its Asian counterpart, whilst other members of the Malagasy *Pipistrellus* group are of African origin (Koopman, 1966). There are other bat genera found in Madagascar which have African and/or Asian links: *Emballonura* has a range extending from islands in the Pacific Ocean, through southeastern Asia to their western limit on Madagascar; *Chaerephon* has a broad distribution across most of the Old World, including much of Africa, the Middle East, Madagascar, New Guinea, Australia and Asia; *Tadarida*, *Neoromicia*, *Hypsugo*, *Pteropus*, *Eidolon* and *Rousettus* may also fit the scenario regarding the dispersal of NEA *Otomops*, SWA *Otomops* and *O. madagascariensis* and their affiliation to the Indo-Malayan *Otomops* species (Taylor, 1999b, Koopman, 1966; Juste *et al*., 1997; Eger and Mitchell, 2003; Goodman and Cardiff, 2004; Andriafidison *et al*., 2006; Bates *et al*., 2006, Goodman *et al*., 2006).

The D-loop phylogenetic trees suggest an alternative scenario where dispersal from Asia occurred simultaneously to Madagascar and South Africa, followed by northwards dispersal in Africa (Fig. 3.22). As reviewed above, it has been established that Asia-Africa faunal connections exist; hence, dispersal of *Otomops* to Madagascar and South Africa is plausible. Dispersal from South Africa to northeastern Africa may be supported by potential resource availability in northeastern Africa, i.e. *Otomops* may have dispersed northward in search of both food and suitable roost habitats in the cave systems of northeastern Africa. The Albertine Rift area is a source of high biodiversity, as countries in this area have high species richness for butterflies (and possibly moths) (Stuart and Adams, 1991 *loc. cit*. Pomeroy, 1993). This theory is also supported by wind patterns across Africa, which show a south-westerly wind moving north from May to August, thereby enabling dispersal in this direction (Crowe, 1949, 1950; Hills, 1979). It should be noted that further analysis would be required to properly address scenarios of dispersal.

Common to both haplotype networks is the connection between the Malagasy species and the Asian outgroups (Fig. 3.12, Fig. 3.22). This observation lends itself to the idea that Madagascar was occupied first from Asia and that from there, subsequent dispersal to and colonisations in Africa took place, thereby giving rise to the two putative mainland subspecies/species. Madagascar has been hailed as a source of biodiversity with ancient connections to mainland Africa, i.e. many endemic, mainland African species are derived from Madagascar and/or vice versa (Bigalke, 1968; Yoder *et al*., 2005; Yoder and Nowak, 2006). Phylogenetic analysis of certain mammal groups in Madagascar showed each group to be monophyletic with a sister group found in Africa (Yoder and Nowak, 2006). *Chaerephon*, as a genus, shows a great range regarding species dispersal but also reveal the existence of remote island endemics with an insular range (Goodman and Cardiff, 2004). According to Goodman and Cardiff (2004), these insular species indicate "members of this genus (*Chaerephon*) are capable of dispersal over water and subsequent colonisation and speciation on distant oceanic islands". Other species shown to surpass water barriers include Barbastelle bats which are capable of crossing the Gibraltar Strait, and members of the *Artibeus* species (*Artibeus jamaicensis* and *Artibeus lituratus*) which are able to disperse among the Antillean (Caribbean) islands (Burland and Worthington Wilmer, 2001; Juste *et al*., 2003). It is possible that the same applies to *Otomops* as it is a member of the Molossidae family and therefore possesses the necessary wing morphology and traits required for long distance flights to alternate habitats (Vaughan, 1966; Goodman and Cardiff, 2004). Many bird species have also been shown to arrive on Madagascar via transoceanic dispersal (Yoder and Nowak, 2006).

Prevailing winds also support the theory of colonisation of Africa by *Otomops* from Madagascar. Year-long easterly trade winds and strong, seasonal (austral winter) south-easterly monsoon winds are present between mainland Africa and Madagascar (Crowe, 1949, 1950; Kirk, 1962; Hills, 1979; Cooke *et al*., 2003; Jury, 2003; Wells, 2003). These winds could have helped facilitate the dispersal of fast and high-flying *Otomops* from Madagascar to Africa (Jury, 2003). The easterly trade winds that affect southern Madagascar and southern Africa could explain the origin of the South Africa lineage from Madagascar. And the strong south-easterly monsoon winds that affect northern Madagascar in winter could account for the origin of the north/eastern lineage, taking into consideration that these winds blow towards the coast of Ethiopia (Hill, 1979; Jury, 2003).

Triaenops is an example of a bat genus that has shown a definite inter-relation between the mainland African species variety and both its Malagasy and Asian counterparts, where *T. auritus*, *T. furculus* and *T. rufus* are endemic to Madagascar and *T. persicus* is distributed in eastern Africa and southwest Asia (Eger and Mitchell, 2003; Russell *et al*., 2007). Results

from cytochrome *b* analysis revealed each species to be a separate lineage. However, *T. rufus* and *T. periscus*, and *T. auritus* and *T. furculus* were sister to each other, suggesting that the Malagasy species did not have a single origin, but rather stemmed from at least two independent dispersal events. Phylogeny alone could not help ascertain whether Africa or Madagascar was the geographical centre of origin for species radiation, but population genetic patterns suggested two dispersal events of *Triaenops* species from Africa to Madagascar (Russell *et al*., 2007). Colonisation of Madagascar from mainland Africa supports the theory that certain bat species are able to traverse bodies of water separating the landmasses, and this, in turn, supports the theory that, in the case of *Otomops*, colonisation from Madagascar (or Africa) is plausible (Dobson and Wright, 2000).

Alternative theories for the general colonisation of Madagascar from mainland Africa have been put forward, e.g. island-hopping across the Mozambique Channel during mid-Eocene (45 MYA) to lower-Miocene (26 MYA) period during which there was regional uplift and hence, exposure of dry areas in the Davie Fracture Zone in the channel (Förster, 1975; Bassias, 1992; McCall, 1997; Houle, 1998; Wells, 2003). A similar scenario is suggested for the transatlantic dispersal of animals from Africa to South America where it is suggested that, after the Cretaceous period, island hopping across wide water barriers (at times of sea-level lowering) or rafting would have been the only modes of dispersal (Eick *et al*., 2005). According to dating, *Otomops* would have colonized Africa during the Pliocene-Pleistocene epoch (2.5 – 3.1 MYA) when ice ages brought about sea level lowering (range ~65 – 200 m), thereby exposing previously-submerged areas of land and creating land connections for dispersal (Gascoyne *et al*., 1979; Villee *et al*., 1986; Campbell *et al*., 2004; Lamb *et al*., 2008) (Appendix 6). These exposed dry land areas could have been used by *Otomops* for island-hopping purposes and, even though it is thought that only small dots of land would have been exposed during this time, the ability of bats to fly would enable overseas crossing to occur (Krause, 2003). The second suggested theory involves rafting across the channel on mats of flotsam or floating islands via wind-powered assistance (McCall, 1997; Houle, 1998; Goodman *et al*., 2003; Krause, 2003; Yoder and Nowak, 2006; Masters *et al*., 2007). According to Eick *et al*. (2005), dispersal via rafting has also been suggested for Platyrrhine monkeys and caviomorph rodents traveling from Africa to South America sometime during the late Eocene or early Oligocene.

Without a wider sampling range and increased sample number, it cannot be definitely assumed that any of these modes of dispersal and colonisation for *Otomops* are accurate. Therefore other theories of dispersal cannot be excluded, e.g. possible dispersal to north-east Africa only, followed by subsequent dispersal back to Madagascar, or dispersal to southern

and western Africa and then to Madagascar. There is also the possibility that there are dispersal routes that we have not yet examined due to the paucity of the data sets. A similar situation is seen with African and Malagasy tenrecs. Although it has never been disputed that Malagasy tenrecs are of African origin, no fewer than five separate theories have been put forward to explain the sequence and number of colonisations that would explain the present distribution of tenrecs on Madagascar (Olson and Goodman, 2003).

4.3 Population genetics

Analysis of D-loop data suggests that Madagascar contains an expanding *Otomops* population. The Malagasy population has the highest haplotype and lowest nucleotide diversity, as well as the highest expansion coefficient and a unimodal mismatch distribution, indicative of population expansion (Hull and Girman, 2005) (Table 3.17). Neutrality tests of the D-loop region also show strong support for population expansion of *Otomops* in Madagascar where a significant Fu's (1997) F_S and insignificant Fu & Li's (1993) D^* and F^* indicate an over-abundance of uncommon haplotypes as a result of possible population expansion (Hingston *et al*., 2005; Russell *et al*., 2005).

Although little data is available on the life history traits of *Otomops*, i.e. dispersal and seasonal movements, some observations can be made from the data. The absence of uniform genetic structure within a group, as seen in the haplotype networks, neighbour joining and Bayesian trees, may be indicative of populations undergoing rapid demographic expansion since much of the original genetic diversity is conserved and gene flow remains high, even though the population range has expanded (Campbell *et al*., 2004; Hingston *et al*., 2005; Russell *et al*., 2005). In certain cases, range expansion is usually associated with an increased dispersal potential, much like the potential observed in *Otomops* for mobility which gives them access to a range of habitats, making them less dependent on one particular setting (Fenton, 1997; Campbell *et al*., 2004).

Population expansion of Malagasy *Otomops* appears to have occurred recently, 27 388 – 52 242 years ago. This assertion is supported by the fact that *Otomops* were not found among the variety of bat fossils recovered from breccia deposits within karst system of Anjohibe (northern area of the Mahajanga Province) dating back ~80 – 10 thousand years (Sammonds, 2007 *loc*. *cit*. Lamb *et al*., 2008). *O. madagascariensis* must have recently colonized the Anjohibe cave system because, although *Otomops* was not present ~80 – 10 thousand years ago, *O. madagascariensis* currently inhabit this cave system today (Goodman *et al*., 2005).

4.4 Genetic structure

4.4.1 Local scale genetic structure and distribution patterns

On a local scale, it has been suggested that *Otomops* colonies comprise harem structures, most particularly within the Durban area (Richardson and Taylor, 1995; Fenton *et al*., 2002, 2004). This harem structure could promote female philopatry, even in the presence of dispersal ability, which would reveal definite female genetic structuring at the local level since mtDNA is a maternally-inherited marker (Burland *et al*., 1999; Castella *et al*., 2001; Kerth *et al*., 2002b). Female philopatry has been documented in a number of bat species including *Glossophaga longirostris*, *Macroderma gigas*, *Miniopterus australis*, *Miniopterus schreibersii*, *Myotis bechsteini*, *Myotis myotis*, *Nyctalus noctula*, *Nycticeus humeralis*, *Plecotus auritus*, *Pipistrellus pipistrellus* and *Desmodus rotundus*, and is thought to be a consequence of either the presence of dispersal barriers between colonies, where males and females have equal dispersal potential, or behavioural mechanisms such as colony faithfulness, both of which result in high genetic structure among populations (Worthington Wilmer *et al*., 1994, 1999; Fenton, 1997; Petit and Mayer, 1999, 2000; Kerth *et al*., 2000, 2002b; Castella *et al*., 2001; Newton *et al*., 2003).

In contrast to the norms expected for harem structure, i.e. the promotion of female philopatry, mtDNA data for *Otomops* presents a scenario of little definite genetic structuring inferred within genetically-defined species-groups. Individuals from particular colonies or areas do not group together with definite structure/association in the neighbour-joining or Bayesian trees and do not show close linking in haplotype networks, e.g. Ballito7 is connected to Ballito6 and Scottburgh7 by five and one mutational steps, respectively. These findings are also supported by haplotype networks depicting haplotype association with sample locality where little genetic structuring is seen (Fig. 3.16, Fig. 3.26). Most individuals represent unique haplotypes with the exception of a few Pinetown, Ethiopian, Kenyan and Malagasy (Mahajanga) samples. However, Ethiopian samples showed a definite relationship, as expected, for mother and foetus pairs, and female Pinetown samples showed some genetic associations in the D-loop haplotype network (Fig. 3.26). From this study, it appears that *Otomops* does not exhibit strict colony faithfulness in any of the species-groups (SWA, NEA and/or Madagascar) and therefore does not group to form genetically-stable harems (Richardson and Taylor, 1995; Fenton *et al*., 2002, 2004). Rather, although there is a harem structure and possible female philopatry within each colony, factors may exist that influence and alter the genetic uniformity of the colony, e.g. mating with individuals from other colonies (Burland and Worthington Wilmer, 2001; Miller-Butterworth *et al*., 2003).

In certain bat species, extra-harem mating has been suggested as the reason for genetic heterogeneity within a colony, i.e. dominant males rarely father all, if not the majority, of offspring within the colony and females may mate with extra-harem males, as seen in *Saccopteryx bilineata* and *Plecotus auritus* (Burland *et al*., 1999; Burland and Worthington Wilmer, 2001; Storz *et al*., 2001; Heckel and von Helversen, 2003; Miller-Butterworth *et al*., 2003). In the case of *Phyllostomus hastatus* and *Desmodus rotundus*, 10 – 40% and 55% of the colonies respectively, were fathered by outside males (Burland and Worthington Wilmer, 2001). In order to investigate colony structure of *Otomops* more thoroughly, more extensive sampling would need to be done and a technique used to elucidate fine-scale relationships among individuals within each colony, e.g. microsatellites (Worthington Wilmer *et al*., 1994; Petri *et al*., 1997; Burland *et al*., 1999; Petit and Mayer, 1999; Abbott and Double, 2003; Heckel and von Helversen, 2003; Miller-Butterworth *et al*., 2003). This approach would need to be employed before any definite assumptions about female or male philopatry could be made.

This lack of genetic relatedness within colonies could be attributed to the dispersal ability of *Otomops*, which may maintain gene flow within the species groups. Dispersal in animal species has often been attributed to inbreeding avoidance and usually occurs in small populations, much like the small aggregations of *Otomops* observed in the metropolitan areas of Durban, South Africa (Lewis, 1995; Richardson and Taylor, 1995; Burland *et al*., 1999; Storz *et al*., 2001; Fenton *et al*., 2002; Kerth *et al*., 2002a). Dispersal ability also allows for individuals from different colonies to move to other suitable roosts/locations, e.g. when young individuals move out of the primary roost in order to establish new colonies of their own or when males have been evicted from the colony after reaching maturity (Richardson and Taylor, 1995; Long, 1995; Burland *et al*., 1999; Storz *et al*., 2001). If new colonies were then sampled, it would be expected that this newly established roost would be made up of a variety of haplotypes/genotypes since recruitment into the new colony would have been quite recent (Kerth *et al*., 2002a). In addition, sampling of non-natal or temporary roosts may also reveal increased haplotype diversity, as was seen in the case of *Nyctalus noctula* which has been observed to change its roost daily (Lewis, 1995; Petit and Mayer, 2000; Kerth *et al*., 2002b). *Otomops* may also take advantage of more than one roost, e.g. day roosts versus night roosts. Therefore if a day/night roost is not the primary roost, then individuals within these roosts might consist of different genotypes and show a lack of homogeneity within the roost (Worthington Wilmer *et al*., 1999; Fenton, 1997). In addition, bats have also been known to evacuate their roosting sites due to disturbance (human or otherwise) and in order to avoid predators (Lewis, 1995). Other factors that may influence roost-switching include the

location of the foraging range, microclimate, roost structure and parasitism. Bats may then either re-establish the old colony in a new suitable roost or may disperse and establish a new colony in a new roost via recruitment of other individuals, and this encourages diversity of the genotypes within the colony (Lewis, 1995; Fenton, 1997; Burland *et al*., 1999).

4.4.2 Broad scale genetic structure and distribution patterns

Glacial refugia are believed to have influenced speciation and distribution and hence phylogeographic structuring of taxa through range contraction and expansion of suitable habitats. Contraction causes fragmentation of the original population into smaller or subpopulations and expansion allows individuals to extend their range and combine to form modern populations (DeMenocal, 1995; Nichol, 1999; Lloyd, 2003; Ruedi and Castella, 2003; Weyandt and Van Den Bussche, 2007). *Otomops* populations of north/east Africa (NEA) and south/west Africa (SWA) could have become genetically-distinct during the Last Glacial Maximum (~18 000 – 20 000 BP) where the disappearance of suitable forest/vegetation cover may have created separate refugia in southern Africa and eastern Africa (Ethiopian highlands/Rift Valley) for these populations to inhabit (Ambrose, 1998; Reynolds, 2007). Although habitat range expansion would have occurred thereafter, this broad population structure, i.e. division of mainland African *Otomops* into two species groups, might have persisted through social behaviours such as philopatry, or ecological factors, e.g. following the migratory patterns of potential food sources (see below). A similar scenario has been proposed for the diversification of the bats *Antrozous pallidus* and *Mystacina tuberculata* (Lloyd, 2003; Weyandt and Van den Bussche, 2007)

Within the SWA *Otomops* group, it is of interest to note the close genetic association between the South African (Durban) population and the Ivory Coast (5000 km), Burundi (3,000 km), Tanzania (2,800 km), and Zimbabwe (1,300 km) populations, even though they are widely separated both geographically and ecologically. This suggests that there may be ongoing gene flow (as documented in the case of *T. brasiliensis*) which could be attributed to both high dispersal ability of *Otomops* (as documented in other bat species, e.g. *M. myotis*, *T. brasiliensis*, *Pteropus* species, *Leptonycteris curasoae* and other molossid species) and a lack of effective dispersal barriers (Vaughan, 1966; Castella *et al*., 2000, 2001; Russell *et al*., 2005, 2007). Additionally, the bat species (*Otomops*) would have to find a suitable habitat in which to establish itself after dispersal (Castella *et al*., 2000).
Due to the paucity of records, *Otomops* appears to have a somewhat sparse distribution throughout Africa. However, those countries that have documented the presence of *Otomops* all consist of different habitats: montane grasslands and shrublands (Ethiopia); tropical and

subtropical grasslands, savannahs, shrublands and woodlands (Burundi, Zimbabwe, Kenya, Ivory Coast); deserts and xeric shrublands (Madagascar); tropical and subtropical dry broadleaf forests (Madagascar); tropical and subtropical moist broadleaf forests (Durban and Tanzania) (Dufils, 2003; Du Puy and Moat, 2003; Wells, 2003; Burgess *et al*., 2004).

Correlating the haplotype network structure to environmental factors such as temperature reveal that the majority of *Otomops* individuals exhibit a preference for temperatures ranging from 20 – 25 °C (Fig. 3.13, Fig. 3.23). Some correlation with temperature is seen in Pinetown samples and the individual Burundi sample where they are the only individuals sharing a preference for temperatures ranging from 15 – 20 °C and are linked by 4 mutational steps in the cytochrome *b* network. In addition, Zimbabwe and Tanzania samples also share the same temperature range as other Durban samples. Ivory Coast is the only sample that is in an area with temperatures higher than 25 °C, and this could relate to this particular sample displaying a relatively large genetic distance from the rest of the SWA group. Correlation with precipitation also shows *Otomops* from Ivory Coast being differentiated from the rest of the SWA group; it is in an area that receives more than 1000 mm of precipitation per year, whereas the majority of Durban samples receive 500 – 1000 mm/year (Fig. 3.14, Fig. 3.24). Malagasy *Otomops* live in areas with a precipitation range of either 0 – 500 mm/year (in the west) or above 1000 mm/year (in the east). All NEA samples, with the exception of those from Yemen, live in areas receiving 500 – 1000 mm precipitation per year. Altitude correlation reveals a general association of most haplotypes with areas 0 – 500 m above sea level (Fig. 3.15, Fig. 3.25). In Madagascar, individuals from different regions are found in areas of similar altitude range (0 – 500 m above sea level). This could then explain haplotype associations between individuals living in different parts of the island, e.g. Toliara in the south shares a haplotype with Antsiranana in the north. All individuals of the NEA group showed a preference for altitudes above 1000 m, which could be the cause for their association and/or separation from other individuals in mainland Africa.

Occupancy patterns, predicted by models, help elucidate species dispersal patterns and/or distribution which may be affected by the level of habitat fragmentation, temperature and water availability (Goodman *et al*., 1996; Fenton, 1997; Worthington Wilmer *et al*., 1999; Burland and Worthington Wilmer, 2001; Menéndez and Thomas, 2006; McCain, 2007). Thus, using current topography, climate (temperature and precipitation) and land cover data, the MaxEnt model utilizes the current distribution of a species to predict its potential distribution throughout a given area. The MaxEnt model for *O. martiensseni* predicts a generally continuous distribution throughout most of Sub-Saharan Africa due to the largely uninterrupted habitat present in these areas, i.e. woodland and grassland areas are extensive in

this region of Africa (Fig. 1) (Keast, 1968). Countries in which *Otomops* are predicted to be present include those used in this study as well as much of western Africa, i.e. Ghana, Togo, Benin, Nigeria, Equatorial Guinea, Gabon, Republic of Congo, Democratic Republic of Congo, Angola and other parts of southern and eastern Africa, i.e. Rwanda, Uganda, Zambia, Malawi, Mozambique, Swaziland and Lesotho. Some locations have a higher probability of hosting *Otomops* populations than others, e.g. places within Angola, Democratic Republic of Congo, Uganda and Rwanda have a 50.9 – 100% probability, whereas places within, e.g. Mozambique, Togo and Benin would only have a 20.8 – 50.9% chance. *Otomops* is thought to be found and, in some cases, has been documented in Republic of Congo, Zimbabwe, Angola, Zambia, Sierra Leone, Gambia, Ghana and Malawi (Hill and Carter, 1941; Verschuren, 1957; Harrison, 1965; Ansell, 1978; Ansell and Dowset, 1988; Crawford-Cabral, 1989; Decher *et al*., 1997; Grubb *et al*., 1998). More extensive sampling would need to be carried out in order to confirm the presence of *Otomops* in these regions.

Otomops can travel relatively easily to these areas and establish new colonies/roosts or perhaps use these regions as rest stops or “stepping stones”, on their way to other areas since these regions contain a suitable habitat (Burland and Worthington Wilmer, 2001). Results support the idea of a “stepping stone" strategy of dispersal and distribution since, according to cytochrome *b* pairwise distances, South African *Otomops* are most closely related to those from Burundi and Zimbabwe which are, in turn, most closely related to those from Tanzania and then Ivory Coast. Thus, there is a progression in genetic distance that appears to be correlated with geographical distance and continued gene flow due to a possibly continuous distribution and a lack of isolation by distance (Burland and Worthington Wilmer, 2001).

It has been established that *Otomops* may be capable of large-scale migrations based on seasonal and periodic cave evacuations observed in Kenyan colonies. This is supported by previous observations and conclusions regarding wing morphology and its ability for fast high flight (Kock *et al*. 2005). One reason for *Otomops* migrations may be that they go in search of and follow the migration patterns of airborne prey such as moths (Burland and Worthington Wilmer, 2001). The African armyworm moth (*Spodoptera exempta*) (Lepidoptera: Noctuidae) is a pest of grasslands and cereal crops in much of tropical Africa and southwest Arabia, with infestations occurring throughout eastern Africa (Aidley and Lubega, 1979; Odiyo, 1979, 1990; Rose, 1979; Wilson and Gatehouse, 1993). Larval outbreaks tend to be associated with the rains, with a southward movement from Malawi, Mozambique, Zambia or Zimbabwe across southern Africa (including South Africa) in December/January and a northward movement from Tanzania to the whole of East Africa, i.e. Uganda, Kenya, Ethiopia, Somalia and as far north as Yemen in March/June (Aidley and Lubega, 1979;

Odiyo, 1979, 1990; Rose, 1979; Wilson and Gatehouse, 1993; Jury, 2003; Wells, 2003). Many of these countries have been predicted to contain *Otomops* as part of their fauna. Seasonal, long-distance migration of the African armyworm usually occurs in low-level wind-convergence zones such as the intertropical convergence zone (ITCZ) or the African Rift convergence zone. Observation of mass outbreaks and migrations reveals the movement of these insects to be associated with the movement of the ITCZ (Odiyo, 1979, 1990; Rose, 1979). Migration of bats according to insect migration has also been documented in *T. brasiliensis* where bats exploit the migration of insects from Mexico to the southern United States (Lee and McCracken, 2005).

If *Otomops* follows these African armyworm migrations, this may explain the close association among *Otomops* found within the geographically distant countries of the SWA group, i.e. following insect migrations in these areas would allow for possible gene flow to occur and lead to the gradual subsequent development of relatedness and broad genetic structure (Burland and Worthington Wilmer, 2001). *Otomops* from the north (Ethiopia, Kenya and Yemen) and south (South Africa, Burundi, Zimbabwe, Tanzania) could therefore be kept separate despite their shared ranges. Individuals of *Leptonycteris curasoae* have been found to share identical mtDNA haplotypes even when separated by distances of up to 1800 km, much like the scenario presented for south/west African *Otomops* individuals (Wilkinson and Fleming, 1996). The theory that *Otomops* does in fact migrate, is therefore supported by the findings of this study. Seasonal changes in East African colony numbers have been documented; studies have shown that during November, there are greater numbers of *Otomops* in the Mount Suswa breeding caves in Kenya relative to June where it is thought that caves are vacated *en masse* (Kock *et al*., 2005).

Alternatively, the separation of the NEA and SWA groups could be attributed to the fact that dispersal and migration do not necessarily always equate with gene flow, i.e. although individuals may have the ability to migrate, this does not necessarily mean that they mate during their migration, and thus genetic population structure may not be as weak as expected (Burland and Worthington Wilmer, 2001).

Mainland African and Malagasy *Otomops* could have become genetically-distinct due to the presence of a physical barrier, such as the Mozambique Channel. Similar scenarios are seen in *Cynopterus nusatenggara* and *Rhinolophus affinis* in Indonesia where population subdivision and inter-island genetic distances are significantly correlated with either sea crossing or geographic distances (Burland and Worthington Wilmer, 2001).

4.5 Conservation implications and management

Knowledge of the population genetic structure of a threatened species can significantly contribute toward evaluating the sustainability of populations and in the identification of appropriate conservation strategies (Abbott and Double, 2003). In addition, a broad understanding of the ecology and behaviour of bat species is also essential for effective conservation and management implications, and for an understanding of the underlying evolutionary processes which have affected their adaptive radiation (Burland and Worthington Wilmer, 2001).

According to this study, it appears that there are three genetically-distinct species groups that warrant protection, i.e. *Otomops* of north/east Africa, *Otomops* of south/west Africa and *O. madagascariensis* of Madagascar. A previous study by Fenton *et al.* (2002) addressed the issue of conservation of *Otomops* in Africa by focusing on "three fundamental questions" or issues, i.e. population, roles of roost and food resources and the use of vocalizations, to study the biology and distribution of this species. It is of interest to note, however, that this study was conducted under the assumption that *Otomops* of the Afrotropical region were all part of the same species, *O. martiensseni* (ranging from East Africa to Madagascar).

In recent years, it been suggested that population sizes of *Otomops* from mainland Africa are higher than previously thought (Fenton *et al*., 2002; Kock *et al*., 2005). Although this may be the case, it is the threats presented predominantly by human activity to this species that warrant their current classification as "Vulnerable" according to the IUCN (section 1.2.4). Population sizes of *Otomops* have not been confirmed, although it has been observed that there is an abundance of *Otomops* (approximately 600 individuals per m^2) within the caves of East Africa (Kock *et al*., 2005). These are thought to migrate at certain times of the year. *Otomops* individuals in more urban areas, such as in South Africa, are found in smaller aggregations which may be due to the availability of suitable roosts. In order to obtain a more accurate estimate of population numbers throughout Africa and Madagascar, more behavioural and ecological studies of *Otomops* would have to be conducted and for future research and monitoring, all known roosts would have to be reassessed to establish the numbers and status of colonies (Hutson *et al*., 2001).

Roost availability does not appear to be a constraint for *Otomops*. MaxEnt models predict a continuous distribution of *Otomops* throughout Africa with the exception of desert climate regions, e.g. Sahara and Namib deserts; it would appear that *Otomops* is able to find a suitable

habitat in most areas, whether it be in large aggregations in the lava caves of Mt Suswa in East Africa or the karst formations of Madagascar, or in fewer numbers in houses and buildings. Within urban areas, such as Durban (South Africa), roost availability is anthrophilic and the establishment and/or destruction of buildings influence individual dispersal and roost location. For this reason, Fenton *et al*. (2002) proposed that there was no shortage of suitable roost sites within this region; this was also the reason why they observed dispersing populations in this area. In terms of available food resources, *Otomops* appears capable of locating suitable food sources and feeds predominantly on Lepidoptera such as moths. Within the Malagasy regions, a great diversity and quantity of Lepidoptera can be found, e.g. Lees and Minet (2003) give an estimate of 4219 moth and 311 butterfly species. Thus moths can usually be found in abundance throughout the Afrotropical region, as outlined in section 4.4.2.

It is apparent that further study is required to fully understand the biology and distribution of the (three) species groups. Therefore additional biological, behavioural and ecological studies should be conducted, such as the vocalization studies suggested by Fenton *et al*. (2002). In addition, identification of key roost sites, distribution and foraging behaviour could be resolved by monitoring echolocation calls. Further research and conservation action will be based on the outcomes of this research (Hutson *et al*., 2001; Russ *et al*., 2001). It is hoped that conservation efforts will be made more focused and that it will be easier to monitor any progress through the identification of genetically-distinct populations that must be protected.

It is recommended that the Ivory Coast population be classified as being at an MU level. ESU and/or MU classifications guide the prioritisation of units for conservation below classical taxonomic levels and aid management plans aimed at preserving current levels of genetic diversity (Abbott and Double, 2003). Ivory Coast *Otomops* may represent a unique conservation priority within the putative SWA *Otomops* grouping and warrants further study to determine its taxonomic rank (section 1.3.3, section 1.3.4). Under the AEC definition, ESUs should consist of individuals of a lineage demonstrating highly restricted gene flow from other lineages. Thus, in this case, it may be suggested that Ivory Coast *Otomops* be defined as an MU. An MU is defined by shallow population genetic subdivisions, which are the most sensible units for population monitoring (Abbott and Double, 2003). A similar scenario to that of Ivory Coast *Otomops* was observed in studies of the shy and white-capped albatrosses where it was found that, although there was sufficient divergence to reject the hypothesis that they should be considered genetically equivalent, it was not possible to define them as ESUs under the AEC definition. This, indeed, may the case of Ivory Coast *Otomops*

which appears to share a lineage with the rest of the SWA group, rather than being an independent lineage.

Conservation of *Otomops* species relies heavily upon the drawing up and enforcement of legislation within different countries. As previously mentioned, the species *O. martiensseni* has fallen under a number of protective legislations due to its Vulnerable status within Africa. *Otomops martiensseni* (1) was selected as one of eight potential candidates for the CMS Appendices list, (2) has been classified globally as having a Vulnerable status according to the 2006 IUCN (The World Conservation Union) Red List of Threatened Species, (3) is listed as a Protected Wild Animal under Schedule 2 of the old Cape Province Ordinance No. 19 of 1974, (4) has provincial government protection and is listed under Endangered Mammals in KwaZulu-Natal (Schedule 6 of the Natal Provincial Ordinance No. 15 of 1974), and (5) is acknowledged as either Specially Protected or Protected under the KwaZulu-Natal Ordinance of 1999 (section 1.2.4) (Taylor, 1999a, 2000a, 2000b; Hutson, 2002; Mickleburgh *et al*., 2004). An Action Plan has been outlined by the International Union for Conservation of Nature's Species Survival Commission (IUCN/SSC) Chiroptera Specialist Group and conservation recommendations have been made in order to meet Action Plan objectives: to assess threats; identify and implement research and monitoring programmes; protect known key roosts; and develop a more specific Recovery Plan (Hutson *et al*., 2001). It was also recommended that key roost sites should be protected and/or managed so as to reduce disturbances and that all known roosts should be considered threatened for site/species safeguarding and management (Hutson *et al*., 2001; Mickleburgh *et al*., 2002). However, *Otomops madagascariensis* has found protection through the establishment of local reserves and/or national parks throughout Madagascar, where it is found in at least five protected areas (Goodman *et al*., 2003, 2005).

Another integral part of conservation is that of publicity and public awareness. Conservation Acts, Treaties and/or Agreements spark public awareness and with it, the formation of bat groups that offer assistance in enforcing these Agreements (Taylor, 1999a). These groups promote public awareness and knowledge of bats and bat conservation, e.g. the establishment of rehabilitation centres to care for sick, injured or young bats, and increased media coverage which draws attention to the rarity of the species (Taylor, 1999a, 2000a, 2000b; Hutson *et al*., 2001). Public awareness is key since humans prove to be the biggest threat, both direct and indirect, not only to *Otomops* but also other bat and mammal species.

Another fundamental issue that must be addressed in order to effectively conserve the species, is to ascertain the conservation status of each of the putative new ESUs or species, i.e. *O.*

martiensseni, *O. icarus* and *O. madagascariensis*. With these new taxonomic classifications arise many questions that must be addressed, e.g. should all the species be classified as Vulnerable or are some species perhaps more threatened than others? It may also be possible that certain species belong on a Blue List, i.e. a list of those species that were once threatened and/or endangered that now show populations to be stabilizing or increasing in abundance (Gigon *et al*., 2000; Fenton *et al*., 2002). Factors affecting *Otomops* populations may also differ in each situation, e.g. the large roosts of north/east Africa versus the smaller, urban-based roosts of south/west Africa, the extent of human threat, urban development and level of public awareness in each region. It is for these reasons that additional research, encompassing many areas of expertise, needs to be done.

4.6 Future research

In order to properly manage and conserve MUs, ESUs and/or species, genetically-distinct populations need to be identified. The identification of such populations will also lead to a greater understanding of historical patterns of colonisation and of current dispersal and/or migratory patterns (Burland and Worthington Wilmer, 2001). Such is the case for *Otomops* in this study, where genetically-distinct species-groups have been defined and suggestions as to their dispersal, both historical and current, made. Although there has been much progress in this regard, further studies are required to gain a more complete understanding of interactions within the various populations, from an ecological and behavioural standpoint.

Molecular techniques, such as microsatellite genotyping, have been used to investigate population structure, dispersal rates, relatedness within and among colonies, and mating systems in bats (Kerth *et al*., 2002b). With this in mind, proposed future research would include the addition of such techniques to our current study. Mitochondrial DNA, used in this study, is maternally-inherited and therefore reflects the roost fidelity and dispersal patterns only of females. Nuclear gene(s), which are bi-parentally inherited, should be employed to reveal a possibly different pattern or confirm currently-suggested patterns of dispersal behaviour (Ruedi and McCracken, 2009).

Future research goals for this project are:

(1) Increased sample numbers of current population groups used in the study as well as new, additional populations, i.e. Ethiopia, Kenya, Yemen, South Africa, Ivory Coast, Burundi, Zimbabwe, Tanzania and Madagascar.

(2) Increased sampling range across mainland Africa and parts of Madagascar. The inclusion of *Otomops* samples from additional countries will help gain a fuller and more complete understanding of dispersal behaviour, consequent dispersal patterns, and the inter-relation between the different populations and will therefore help ascertain whether distribution is continuous, as suggested in this study.

(3) Mitochondrial cytochrome *b* and D-loop sequencing of new samples added to the study. This will be added to the current data set in order to provide a more complete mitochondrial DNA data set for analysis.

(4) Sequencing of nuclear DNA regions. Porter *et al*. (2003), Hoofer *et al*. (2003) and Van den Bussche and Weyandt (2003) recommended this as it can help in elucidating male-mediated patterns of dispersal.

(5) Microsatellite fingerprint analysis. This technique gives better within-population data which can be used to establish population structure, dispersal rates, within- and among-colony relatedness, as well as mating systems in bats (Castella *et al*., 2000, 2001; Kerth *et al*., 2002b).

An increased sample size and sampling range, and the addition of other molecular techniques will all allow for the inclusion of more stringent and revealing analyses such as nested clade analysis and population-specific AMOVAs, and thus, contribute to a more holistic interpretation of results. With future research, the colony structure and philopatry of *Otomops* can be investigated and, from a broader standpoint, the taxonomic status of contentious populations such as Ivory Coast *Otomops*, can be resolved. This will also contribute to the long-term goal of management and conservation of these genetically-distinct population groups (MUs, ESUs and/or species).

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6. APPENDICES

Appendix 1. Agarose gel electrophoresis stock solutions

10x TBE (Tris-borate/EDTA)

0.89 M Tris-HCl (53.89 g)
0.89 M boric acid powder (24.96 g)
0.01 M disodium EDTA (1.86 g)

Make up to 500 ml with distilled water and adjust pH to 8.3 with NaOH or HCl. Autoclave before use.

0.5x TBE (Tris-borate/EDTA)

10x TBE (Tris-borate/EDTA)
Distilled water

TBE and water are mixed in a 1:19 dilution

Ethidium bromide stock (10 mg/ml EtBr)

10 mg EtBr
1 ml distilled water

0.05 mg/ml EtBr (1:200 dilution)

0.1 ml EtBr (10mg/ml)
19.9 ml distilled water

Loading dye solution

0.1% (w/v) bromophenol blue
0.02% (w/v) xylene cyanol FF
15% (w/v) Ficoll (Type 400, Pharamacia) in water

Appendix 2. Fluorometry stock solutions

Hoechst 33258 stock dye solution (1mg.ml^{-1})

10mg Hoechst 33258
10ml sterile water

Can be stored at 4 ˚C in an amber bottle for up to 6 months.

10x TNE (Tris/NaCl/EDTA)

100mM Tris (12.11 g)
10mM EDTA $Na_2.2H_2O$ (3.72 g)
2M NaCl (116.89 g)

Dissolve in approximately 800 ml distilled water. Adjust pH to 7.4 with concentrated hydrochloric acid. Made up to 1000 ml with distilled water. Autoclave and store in a dark bottle at 4 °C for up to 3 months.

Low Range Assay Solution

1 µl Hoechst 33258 stock solution
1 ml 10 X TNE
9 ml filtered distilled water

The assay solution is prepared fresh before use at room temperature.

Calf Thymus DNA (100 ng/µl)

12.5 µl calf thymus DNA standard (8 ng/ml)
12.5 µl 10 X TNE
75 µl distilled water

Appendix 3. Individual nucleotide frequencies

Table 6.1. Individual nucleotide composition for cytochrome *b* data.

Sample	T(U)	C	A	G	Total
Silverglen1D	26.8	29.8	30.0	13.4	1004
Silverglen2D	26.9	30.1	29.9	13.1	1004
Silverglen3D	27.6	29.3	30.1	13.0	1004
Silverglen4D	27.1	29.9	30.0	13.0	1004
Silverglen5D	27.3	29.8	29.9	13.0	1004
Ballito6D	27.1	29.9	29.9	13.1	1004
Balito7D	27.2	29.8	29.9	13.1	1004
Pinetown1D	27.3	29.8	29.6	13.3	1004
Pinetown2D	27.1	29.9	29.7	13.3	1004
Pinetown3D	27.1	29.9	29.7	13.3	1004
Pinetown4D	27.2	29.8	29.6	13.4	1004
Pinetown5D	27.2	29.8	29.6	13.4	1004
Scottburgh6D	27.0	29.8	30.1	13.1	1004
Scottburgh7D	27.1	29.8	30.0	13.1	1004
Ivory Coastm	27.5	29.1	30.0	13.3	1002
Zimbabwem	27.2	29.7	29.6	13.4	1002
Tanzaniam	27.1	29.8	29.8	13.2	1002
Burundi415	27.0	29.9	29.8	13.3	1004
Durban MorningsideM321	27.1	29.9	29.7	13.3	1004
Durban AmanzimtotiM322	27.0	30.0	29.7	13.3	1004
Durban Park Rynie324	27.0	29.9	29.7	13.4	1004
Durban Park Rynie325	27.0	30.0	29.6	13.4	1004
Fianarantsoa1M	27.8	28.8	29.6	13.8	1004
Mahajanga2M	27.8	28.8	29.6	13.8	1004
Mahajanga3M	27.7	28.9	29.6	13.8	1004
Mahajanga4M	27.7	28.9	29.6	13.8	1004
Ankarana5M	27.5	29.1	29.6	13.8	1004
Toliara6M	27.5	29.1	29.7	13.7	1004
Toliara7M	27.7	29.0	29.7	13.6	1004
Toliara8M	27.5	29.1	29.6	13.8	1004
Toliara9M	27.7	29.0	29.9	13.4	1004

Table 6.1 continued.

Sample	T(U)	C	A	G	Total
Ankarana15M	27.7	29.2	29.2	13.9	1004
Ankarana18M	27.4	29.3	29.4	13.9	1004
Kenya1	27.2	29.5	29.9	13.4	1004
EthiopiaA1	27.1	29.6	29.8	13.5	1003
EthiopiaA2	27.2	29.6	29.8	13.4	1004
EthiopiaB1	27.2	29.6	29.8	13.4	1004
EthiopiaB2	27.1	29.6	29.8	13.5	1003
EthiopiaC1	27.2	29.6	29.8	13.4	1004
EthiopiaC2	27.2	29.6	29.8	13.4	1004
EthiopiaD1	27.2	29.6	29.9	13.3	1004
EthiopiaD2	27.2	29.6	29.9	13.3	1004
Yemen	27.3	29.6	29.7	13.4	1004
Kenya2	26.9	29.5	30.1	13.5	1004
Kenya3	27.1	29.7	29.9	13.3	1004
Kenya4f	27.4	29.4	29.8	13.4	1004
Kenya5m	27.2	29.6	29.8	13.4	1004
Kenya7m	27.2	29.6	29.8	13.4	1004
Pipistrellus abramusNC005436	31.0	26.9	27.9	14.2	1003
Otomops wroughtoniM143	26.4	30.9	28.5	14.2	1004
Otomops formosusM145	26.2	30.8	29.3	13.7	1004
Mops midasM701	29.4	27.0	30.0	13.6	1004
Tadarida fulminans	26.0	30.4	28.7	14.9	1004
Average with outgroups	27.3	29.5	29.7	13.5	1003.8

Table 6.2. Individual nucleotide composition for D-loop data.

Sample	T(U)	C	A	G	Total
Durban Pinetown1	28.2	18.1	43.0	10.8	277
Durban MorningsideM321	27.8	18.4	43.3	10.5	277
Burundi M415	27.1	19.1	42.6	11.2	277
Durban Pinetown4	28.2	18.1	43.0	10.8	277
Durban Pinetown3	28.9	17.3	42.2	11.6	277
Durban Silverglen4	28.5	17.7	42.2	11.6	277
Durban Silverglen5	28.9	17.3	42.2	11.6	277
Durban Park RynieM324	28.5	17.7	42.2	11.6	277
Durban Pinetown2	29.2	17.0	42.2	11.6	277
Durban AmanzimtotiM322	30.0	16.2	42.2	11.6	277
Durban Silverglen2	30.0	16.2	42.2	11.6	277
Ivory Coast	28.2	18.1	44.4	9.4	277
Kenya1	27.0	18.7	44.2	10.1	278
Kenya3	27.3	18.3	43.5	10.8	278
Kenya4	27.3	18.3	43.5	10.8	278
Kenya5	27.0	18.7	43.9	10.4	278
Kenya6	27.3	18.3	43.5	10.8	278
Kenya7	27.7	18.0	44.6	9.7	278
EthiopiaA1	26.6	19.1	43.5	10.8	278
EthiopiaA2	26.6	19.1	43.5	10.8	278
EthiopiaB1	26.6	19.1	43.9	10.4	278
EthiopiaB2	26.6	19.1	43.9	10.4	278
EthiopiaC1	27.0	18.7	43.5	10.8	278
EthiopiaC2	27.0	18.7	43.5	10.8	278
EthiopiaD1	27.3	18.3	44.2	10.1	278
EthiopiaD2	27.3	18.3	44.2	10.1	278
Yemen	27.0	18.7	43.9	10.4	278
Fianarantsoa1	29.5	15.5	43.9	11.2	278
Mahajanga2	29.1	15.8	44.6	10.4	278
Mahajanga3	29.1	15.8	44.6	10.4	278
Mahajanga4	28.8	16.2	44.6	10.4	278
Antsiranana5	28.8	16.2	44.2	10.8	278
Toliara6	28.8	16.2	43.9	11.2	278

Table 6.2 continued.

Sample	T(U)	C	A	G	Total
Toliara7	28.4	16.5	43.9	11.2	278
Toliara8	28.8	16.2	44.6	10.4	278
Toliara9	28.4	16.5	43.9	11.2	278
Toliara10	28.8	16.2	44.2	10.8	278
Toliara11	28.8	16.2	44.2	10.8	278
Toliara12	28.8	16.2	44.2	10.8	278
Toliara13	28.8	16.2	44.2	10.8	278
Toliara14	29.5	15.8	44.2	10.4	278
Antsiranana15	28.4	16.5	44.6	10.4	278
Antsiranana16	28.4	16.5	44.6	10.4	278
Antsiranana17	28.8	16.2	43.9	11.2	278
Antsiranana18	29.1	15.8	44.2	10.8	278
Fianarantsoa19	28.4	16.5	43.5	11.5	278
Antsiranana20	29.5	15.5	44.6	10.4	278
Antsiranana21	28.8	16.2	44.2	10.8	278
Antsiranana22	28.8	16.2	43.9	11.2	278
Antsiranana25	28.8	16.2	43.9	11.2	278
O.formosus	31.7	15.1	44.8	8.5	259
O.wroughtoni	28.9	17.3	45.8	8.1	284
Mormopterus jugularis I1P	31.1	14.6	41.8	12.5	280
Average with outgroups	28.4	17.2	43.7	10.7	277.6

Appendix 4. Individual pairwise genetic distances

Table 6.3. Pairwise genetic distances of cytochrome *b* samples against samples 1 to 14.

	Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Silverglen1D														
2	Silverglen2D	0.010													
3	Silverglen3D	0.021	0.019												
4	Silverglen4D	0.013	0.011	0.018											
5	Silverglen5D	0.008	0.008	0.013	0.005										
6	Ballito6D	0.011	0.009	0.01	0.012	0.007									
7	Balito7D	0.006	0.006	0.015	0.007	0.002	0.005								
8	Pinetown1D	0.010	0.010	0.017	0.011	0.006	0.009	0.004							
9	Pinetown2D	0.013	0.011	0.012	0.014	0.009	0.004	0.007	0.007						
10	Pinetown3D	0.013	0.011	0.012	0.014	0.009	0.004	0.007	0.007	0.000					
11	Pinetown4D	0.009	0.009	0.016	0.010	0.005	0.006	0.003	0.003	0.006	0.006				
12	Pinetown5D	0.009	0.009	0.016	0.010	0.005	0.006	0.003	0.003	0.006	0.006	0.000			
13	Scottburgh6D	0.008	0.008	0.017	0.009	0.004	0.007	0.002	0.006	0.009	0.009	0.005	0.005		
14	Scottburgh7D	0.007	0.007	0.016	0.008	0.003	0.006	0.001	0.005	0.008	0.008	0.004	0.004	0.003	
15	Ivory_Coastm	0.023	0.021	0.03	0.022	0.019	0.019	0.017	0.019	0.019	0.019	0.018	0.018	0.019	0.018
16	Zimbabwem	0.013	0.011	0.011	0.014	0.009	0.003	0.007	0.007	0.001	0.001	0.006	0.006	0.009	0.008
17	Tanzaniam	0.012	0.010	0.018	0.013	0.008	0.008	0.006	0.008	0.008	0.008	0.007	0.007	0.008	0.007

Table 6.3 continued.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
18	Burundi415	0.011	0.011	0.018	0.012	0.007	0.008	0.005	0.007	0.008	0.008	0.004	0.004	0.007	0.006
19	Durban MorningsideM321	0.011	0.011	0.018	0.012	0.007	0.008	0.005	0.007	0.008	0.008	0.004	0.004	0.007	0.006
20	Durban AmanzimtotiM322	0.018	0.016	0.015	0.019	0.014	0.007	0.012	0.012	0.005	0.005	0.011	0.011	0.014	0.013
21	Durban Park Rynie324	0.016	0.014	0.013	0.017	0.012	0.005	0.010	0.010	0.003	0.003	0.009	0.009	0.012	0.011
22	Durban Park Rynie325	0.015	0.013	0.014	0.016	0.011	0.006	0.009	0.009	0.002	0.002	0.008	0.008	0.011	0.010
23	Fianarantsoa1M	0.040	0.036	0.044	0.039	0.038	0.037	0.036	0.040	0.039	0.039	0.037	0.037	0.038	0.037
24	Mahajanga2M	0.040	0.036	0.044	0.039	0.038	0.037	0.036	0.040	0.039	0.039	0.037	0.037	0.038	0.037
25	Mahajanga3M	0.041	0.037	0.045	0.040	0.039	0.038	0.037	0.041	0.040	0.040	0.038	0.038	0.039	0.038
26	Mahajanga4M	0.041	0.037	0.045	0.040	0.039	0.038	0.037	0.041	0.040	0.040	0.038	0.038	0.039	0.038
27	Ankarana5M	0.039	0.035	0.042	0.038	0.037	0.036	0.035	0.039	0.038	0.038	0.036	0.036	0.037	0.036
28	Toliara6M	0.038	0.034	0.041	0.037	0.036	0.035	0.034	0.038	0.037	0.037	0.035	0.035	0.036	0.035
29	Toliara7M	0.039	0.035	0.045	0.038	0.037	0.038	0.035	0.039	0.040	0.040	0.038	0.038	0.037	0.036
30	Toliara8M	0.039	0.035	0.042	0.038	0.037	0.036	0.035	0.039	0.038	0.038	0.036	0.036	0.037	0.036
31	Toliara9M	0.035	0.031	0.040	0.036	0.033	0.034	0.031	0.035	0.036	0.036	0.034	0.034	0.033	0.032
32	Ankarana15M	0.044	0.040	0.05	0.043	0.042	0.043	0.040	0.044	0.046	0.046	0.043	0.043	0.042	0.041
33	Ankarana18M	0.044	0.040	0.048	0.043	0.042	0.041	0.040	0.045	0.043	0.043	0.041	0.041	0.042	0.041
34	Kenya1	0.028	0.025	0.031	0.024	0.028	0.025	0.026	0.030	0.027	0.027	0.027	0.027	0.028	0.027
35	EthiopiaA1	0.027	0.024	0.030	0.026	0.027	0.024	0.025	0.029	0.026	0.026	0.026	0.026	0.027	0.026
36	EthiopiaA2	0.027	0.024	0.030	0.025	0.027	0.024	0.025	0.029	0.026	0.026	0.026	0.026	0.027	0.026

Table 6.3 continued.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
37	EthiopiaB1	0.027	0.024	0.030	0.025	0.027	0.024	0.025	0.029	0.026	0.026	0.026	0.026	0.027	0.026
38	EthiopiaB2	0.027	0.024	0.030	0.026	0.027	0.024	0.025	0.029	0.026	0.026	0.026	0.026	0.027	0.026
39	EthiopiaC1	0.027	0.024	0.030	0.023	0.027	0.024	0.025	0.029	0.026	0.026	0.026	0.026	0.027	0.026
40	EthiopiaC2	0.027	0.024	0.030	0.023	0.027	0.024	0.025	0.029	0.026	0.026	0.026	0.026	0.027	0.026
41	EthiopiaD1	0.028	0.025	0.031	0.027	0.028	0.025	0.026	0.030	0.027	0.027	0.027	0.027	0.028	0.027
42	EthiopiaD2	0.028	0.025	0.031	0.027	0.028	0.025	0.026	0.030	0.027	0.027	0.027	0.027	0.028	0.027
43	Yemen	0.026	0.022	0.030	0.025	0.027	0.023	0.024	0.029	0.026	0.026	0.026	0.026	0.027	0.026
44	Kenya2	0.033	0.031	0.036	0.028	0.033	0.030	0.031	0.035	0.032	0.032	0.032	0.032	0.033	0.032
45	Kenya3	0.029	0.027	0.032	0.028	0.029	0.026	0.027	0.031	0.028	0.028	0.028	0.028	0.029	0.028
46	Kenya4f	0.022	0.020	0.025	0.025	0.022	0.019	0.020	0.024	0.021	0.021	0.021	0.021	0.022	0.021
47	Kenya5m	0.027	0.024	0.030	0.023	0.027	0.024	0.025	0.029	0.026	0.026	0.026	0.026	0.027	0.026
48	Kenya7m	0.027	0.024	0.030	0.023	0.027	0.024	0.025	0.029	0.026	0.026	0.026	0.026	0.027	0.026
49	PabramusNC005436	0.245	0.246	0.245	0.249	0.246	0.241	0.243	0.249	0.244	0.244	0.247	0.247	0.244	0.244
50	OwroughtoniM143	0.118	0.117	0.121	0.121	0.120	0.114	0.118	0.122	0.116	0.116	0.119	0.119	0.120	0.119
51	OformosusM145	0.112	0.110	0.118	0.114	0.112	0.111	0.110	0.115	0.113	0.113	0.114	0.114	0.112	0.111
52	Mops_midasM701	0.17	0.172	0.179	0.171	0.169	0.168	0.167	0.166	0.166	0.166	0.168	0.168	0.169	0.168
53	Tfulminans	0.171	0.170	0.177	0.166	0.167	0.166	0.165	0.167	0.170	0.170	0.168	0.168	0.166	0.166

Table 6.4. Pairwise genetic distances of cytochrome *b* samples against samples 15 to 28.

		15	16	17	18	19	20	21	22	23	24	25	26	27	28
15	Ivory_Coastm														
16	Zimbabwem	0.020													
17	Tanzaniam	0.013	0.009												
18	Burundi415	0.020	0.008	0.009											
19	Durban MorningsideM321	0.016	0.008	0.007	0.004										
20	Durban AmanzimtotiM322	0.022	0.004	0.011	0.011	0.011									
21	Durban Park Rynie324	0.020	0.002	0.009	0.009	0.009	0.002								
22	Durban Park Rynie325	0.021	0.003	0.010	0.008	0.010	0.005	0.003							
23	Fianarantsoa1M	0.054	0.040	0.042	0.039	0.039	0.045	0.043	0.041						
24	Mahajanga2M	0.054	0.040	0.042	0.039	0.039	0.045	0.043	0.041	0.000					
25	Mahajanga3M	0.055	0.042	0.043	0.040	0.040	0.046	0.044	0.042	0.001	0.001				
26	Mahajanga4M	0.055	0.042	0.043	0.040	0.040	0.046	0.044	0.042	0.001	0.001	0.000			
27	Ankarana5M	0.053	0.039	0.040	0.038	0.038	0.044	0.041	0.040	0.003	0.003	0.002	0.002		
28	Toliara6M	0.051	0.038	0.039	0.037	0.037	0.043	0.040	0.039	0.004	0.004	0.003	0.003	0.001	
29	Toliara7M	0.048	0.041	0.038	0.040	0.038	0.044	0.041	0.042	0.009	0.009	0.010	0.010	0.008	0.007
30	Toliara8M	0.053	0.039	0.040	0.038	0.038	0.044	0.041	0.040	0.003	0.003	0.002	0.002	0.000	0.001
31	Toliara9M	0.044	0.037	0.034	0.036	0.034	0.039	0.037	0.038	0.013	0.013	0.014	0.014	0.012	0.011
32	Ankarana15M	0.058	0.047	0.046	0.046	0.046	0.051	0.049	0.048	0.013	0.013	0.012	0.012	0.010	0.011
33	Ankarana18M	0.058	0.045	0.046	0.043	0.044	0.049	0.047	0.046	0.008	0.008	0.007	0.007	0.005	0.006

Table 6.4 continued.

		15	16	17	18	19	20	21	22	23	24	25	26	27	28
34	Kenya1	0.037	0.028	0.029	0.029	0.029	0.032	0.030	0.029	0.040	0.040	0.041	0.041	0.039	0.038
35	EthiopiaA1	0.036	0.027	0.028	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.041	0.041	0.038	0.037
36	EthiopiaA2	0.036	0.027	0.028	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.040	0.040	0.038	0.037
37	EthiopiaB1	0.036	0.027	0.028	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.040	0.040	0.038	0.037
38	EthiopiaB2	0.036	0.027	0.028	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.041	0.041	0.038	0.037
39	EthiopiaC1	0.036	0.027	0.028	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.040	0.040	0.038	0.037
40	EthiopiaC2	0.036	0.027	0.028	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.040	0.040	0.038	0.037
41	EthiopiaD1	0.037	0.028	0.029	0.029	0.029	0.032	0.030	0.029	0.040	0.040	0.042	0.042	0.039	0.038
42	EthiopiaD2	0.037	0.028	0.029	0.029	0.029	0.032	0.030	0.029	0.040	0.040	0.042	0.042	0.039	0.038
43	Yemen	0.035	0.026	0.027	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.040	0.040	0.038	0.037
44	Kenya2	0.037	0.033	0.031	0.034	0.034	0.037	0.035	0.034	0.048	0.048	0.049	0.049	0.047	0.046
45	Kenya3	0.038	0.029	0.030	0.030	0.030	0.033	0.031	0.030	0.042	0.042	0.043	0.043	0.040	0.039
46	Kenya4f	0.034	0.022	0.023	0.023	0.023	0.027	0.024	0.023	0.039	0.039	0.040	0.040	0.038	0.037
47	Kenya5m	0.036	0.027	0.028	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.040	0.040	0.038	0.037
48	Kenya7m	0.036	0.027	0.028	0.028	0.028	0.031	0.029	0.028	0.039	0.039	0.040	0.040	0.038	0.037
49	PabramusNC005436	0.247	0.246	0.243	0.244	0.247	0.248	0.245	0.242	0.248	0.248	0.250	0.250	0.250	0.248
50	OwroughtoniM143	0.116	0.118	0.116	0.120	0.116	0.118	0.116	0.117	0.118	0.118	0.117	0.117	0.114	0.113
51	OformosusM145	0.108	0.115	0.111	0.112	0.111	0.116	0.113	0.114	0.113	0.113	0.112	0.112	0.109	0.108
52	Mops_midasM701	0.169	0.166	0.164	0.169	0.168	0.172	0.170	0.168	0.181	0.181	0.182	0.182	0.179	0.178
53	Tfulminans	0.169	0.169	0.165	0.164	0.163	0.171	0.168	0.171	0.178	0.178	0.180	0.180	0.177	0.175

Table 6.5. Pairwise genetic distances of cytochrome *b* samples against samples 29 to 42.

		29	30	31	32	33	34	35	36	37	38	39	40	41	42
29	Toliara7M														
30	Toliara8M	0.008													
31	Toliara9M	0.004	0.012												
32	Ankarana15M	0.016	0.010	0.020											
33	Ankarana18M	0.013	0.005	0.017	0.009										
34	Kenya1	0.041	0.039	0.041	0.043	0.045									
35	EthiopiaA1	0.040	0.038	0.040	0.042	0.044	0.003								
36	EthiopiaA2	0.040	0.038	0.040	0.042	0.044	0.003	0.000							
37	EthiopiaB1	0.040	0.038	0.040	0.042	0.044	0.003	0.000	0.000						
38	EthiopiaB2	0.040	0.038	0.040	0.042	0.044	0.003	0.000	0.000	0.000					
39	EthiopiaC1	0.040	0.038	0.040	0.042	0.044	0.003	0.002	0.002	0.002	0.002				
40	EthiopiaC2	0.040	0.038	0.040	0.042	0.044	0.003	0.002	0.002	0.002	0.002	0.000			
41	EthiopiaD1	0.041	0.039	0.042	0.043	0.045	0.004	0.001	0.001	0.001	0.001	0.003	0.003		
42	EthiopiaD2	0.041	0.039	0.042	0.043	0.045	0.004	0.001	0.001	0.001	0.001	0.003	0.003	0.000	
43	Yemen	0.040	0.038	0.040	0.042	0.044	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003
44	Kenya2	0.049	0.047	0.047	0.051	0.052	0.008	0.011	0.011	0.011	0.011	0.009	0.009	0.012	0.012
45	Kenya3	0.043	0.040	0.043	0.045	0.046	0.003	0.002	0.002	0.002	0.002	0.004	0.004	0.001	0.001
46	Kenya4f	0.038	0.038	0.034	0.042	0.044	0.007	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.007
47	Kenya5m	0.040	0.038	0.040	0.042	0.044	0.003	0.002	0.002	0.002	0.002	0.000	0.000	0.003	0.003
48	Kenya7m	0.040	0.038	0.040	0.042	0.044	0.003	0.002	0.002	0.002	0.002	0.000	0.000	0.003	0.003

Table 6.5 continued.

		29	30	31	32	33	34	35	36	37	38	39	40	41	42
49	PabramusNC005436	0.245	0.250	0.244	0.257	0.257	0.247	0.246	0.245	0.245	0.246	0.247	0.247	0.247	0.247
50	OwroughtoniM143	0.114	0.114	0.114	0.125	0.119	0.115	0.117	0.117	0.117	0.117	0.117	0.117	0.118	0.118
51	OformosusM145	0.107	0.109	0.110	0.117	0.115	0.107	0.109	0.109	0.109	0.109	0.109	0.109	0.110	0.110
52	Mops_midasM701	0.175	0.179	0.173	0.188	0.186	0.179	0.178	0.178	0.178	0.178	0.178	0.178	0.179	0.179
53	Tfulminans	0.171	0.177	0.169	0.180	0.182	0.170	0.169	0.169	0.169	0.169	0.169	0.169	0.167	0.167

Table 6.6. Pairwise genetic distances of cytochrome *b* samples against samples 43 to 52.

		43	44	45	46	47	48	49	50	51	52
43	Yemen										
44	Kenya2	0.011									
45	Kenya3	0.004	0.011								
46	Kenya4f	0.006	0.012	0.008							
47	Kenya5m	0.002	0.009	0.004	0.006						
48	Kenya7m	0.002	0.009	0.004	0.006	0.000					
49	PabramusNC005436	0.248	0.249	0.245	0.247	0.247	0.247				
50	OwroughtoniM143	0.117	0.119	0.117	0.117	0.117	0.117	0.254			
51	OformosusM145	0.108	0.109	0.108	0.109	0.109	0.109	0.256	0.066		
52	Mops_midasM701	0.177	0.179	0.18	0.175	0.178	0.178	0.285	0.184	0.173	
53	Tfulminans	0.171	0.171	0.166	0.172	0.169	0.169	0.274	0.201	0.193	0.166

Table 6.7. Pairwise genetic distances of D-loop samples against samples 1 to 15.

	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Durban Pinetown1															
2	Durban MorningsideM321	0.030														
3	Burundi M415	0.030	0.030													
4	Durban Pinetown4	0.000	0.030	0.030												
5	Durban Pinetown3	0.046	0.063	0.063	0.046											
6	Durban Silverglen4	0.050	0.067	0.067	0.050	0.004										
7	Durban Silverglen5	0.046	0.063	0.063	0.046	0.000	0.004									
8	Durban Park RynieM324	0.050	0.067	0.067	0.050	0.004	0.000	0.004								
9	Durban Pinetown2	0.050	0.067	0.067	0.050	0.004	0.007	0.004	0.007							
10	Durban AmanzimtotiM322	0.058	0.075	0.075	0.058	0.011	0.015	0.011	0.015	0.015						
11	Durban Silverglen2	0.058	0.075	0.075	0.058	0.011	0.015	0.011	0.015	0.015	0.000					
12	Ivory Coast	0.063	0.055	0.072	0.063	0.071	0.067	0.071	0.067	0.076	0.076	0.076				
13	Kenya1	0.135	0.141	0.156	0.135	0.176	0.170	0.176	0.170	0.182	0.195	0.195	0.132			
14	Kenya3	0.144	0.150	0.177	0.144	0.186	0.180	0.186	0.180	0.192	0.205	0.205	0.141	0.026		
15	Kenya4	0.148	0.155	0.181	0.148	0.180	0.175	0.180	0.175	0.187	0.200	0.200	0.145	0.034	0.007	
16	Kenya5	0.134	0.140	0.166	0.134	0.175	0.169	0.175	0.169	0.181	0.193	0.193	0.131	0.026	0.007	0.015
17	Kenya6	0.144	0.150	0.177	0.144	0.186	0.180	0.186	0.180	0.192	0.205	0.205	0.141	0.026	0.000	0.007
18	Kenya7	0.125	0.131	0.157	0.125	0.165	0.159	0.165	0.159	0.171	0.184	0.184	0.122	0.011	0.015	0.023
19	EthiopiaA1	0.163	0.170	0.198	0.163	0.208	0.202	0.208	0.202	0.214	0.227	0.227	0.159	0.034	0.030	0.039
20	EthiopiaA2	0.163	0.170	0.198	0.163	0.208	0.202	0.208	0.202	0.214	0.227	0.227	0.159	0.034	0.030	0.039

Table 6.7 continued.

	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
21	EthiopiaB1	0.143	0.148	0.175	0.143	0.185	0.179	0.185	0.179	0.190	0.203	0.203	0.139	0.062	0.050	0.050
22	EthiopiaB2	0.143	0.148	0.175	0.143	0.185	0.179	0.185	0.179	0.190	0.203	0.203	0.139	0.062	0.050	0.050
23	EthiopiaC1	0.149	0.156	0.183	0.149	0.192	0.186	0.192	0.186	0.198	0.212	0.212	0.146	0.030	0.004	0.011
24	EthiopiaC2	0.149	0.156	0.183	0.149	0.192	0.186	0.192	0.186	0.198	0.212	0.212	0.146	0.030	0.004	0.011
25	EthiopiaD1	0.135	0.141	0.156	0.135	0.176	0.170	0.176	0.170	0.182	0.195	0.195	0.132	0.004	0.022	0.030
26	EthiopiaD2	0.135	0.141	0.156	0.135	0.176	0.170	0.176	0.170	0.182	0.195	0.195	0.132	0.004	0.022	0.030
27	Yemen	0.143	0.159	0.175	0.143	0.174	0.168	0.174	0.168	0.180	0.192	0.192	0.140	0.034	0.038	0.047
28	Fianarantsoa1	0.172	0.161	0.171	0.172	0.182	0.176	0.182	0.176	0.177	0.202	0.202	0.175	0.190	0.201	0.190
29	Mahajanga2	0.166	0.167	0.165	0.166	0.177	0.171	0.177	0.171	0.171	0.195	0.195	0.169	0.174	0.196	0.185
30	Mahajanga3	0.166	0.167	0.165	0.166	0.177	0.171	0.177	0.171	0.171	0.195	0.195	0.169	0.174	0.196	0.185
31	Mahajanga4	0.172	0.173	0.171	0.172	0.193	0.187	0.193	0.187	0.187	0.213	0.213	0.175	0.18	0.202	0.202
32	Antsiranana5	0.177	0.178	0.176	0.177	0.187	0.181	0.187	0.181	0.182	0.207	0.207	0.180	0.174	0.195	0.184
33	Toliara6	0.182	0.183	0.181	0.182	0.193	0.187	0.193	0.187	0.187	0.212	0.212	0.185	0.179	0.201	0.189
34	Toliara7	0.188	0.189	0.187	0.188	0.199	0.192	0.199	0.192	0.193	0.218	0.218	0.191	0.184	0.206	0.195
35	Toliara8	0.172	0.173	0.171	0.172	0.182	0.176	0.182	0.176	0.176	0.202	0.202	0.188	0.174	0.195	0.185
36	Toliara9	0.188	0.189	0.187	0.188	0.199	0.192	0.199	0.192	0.193	0.218	0.218	0.191	0.184	0.206	0.195
37	Toliara10	0.177	0.178	0.165	0.177	0.187	0.181	0.187	0.181	0.182	0.207	0.207	0.180	0.174	0.195	0.184
38	Toliara11	0.177	0.178	0.176	0.177	0.187	0.181	0.187	0.181	0.182	0.207	0.207	0.180	0.174	0.195	0.184
39	Toliara12	0.177	0.178	0.176	0.177	0.187	0.181	0.187	0.181	0.182	0.207	0.207	0.180	0.174	0.195	0.184
40	Toliara13	0.165	0.166	0.165	0.165	0.176	0.170	0.176	0.170	0.170	0.194	0.194	0.167	0.163	0.184	0.173

Table 6.7 continued.

	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
41	Toliara14	0.135	0.147	0.168	0.135	0.145	0.140	0.145	0.140	0.140	0.162	0.162	0.149	0.153	0.164	0.153
42	Antsiranana15	0.178	0.179	0.177	0.178	0.199	0.193	0.199	0.193	0.193	0.219	0.219	0.181	0.163	0.184	0.184
43	Antsiranana16	0.178	0.179	0.177	0.178	0.199	0.193	0.199	0.193	0.193	0.219	0.219	0.181	0.163	0.184	0.184
44	Antsiranana17	0.182	0.183	0.181	0.182	0.193	0.187	0.193	0.187	0.187	0.212	0.212	0.185	0.179	0.201	0.189
45	Antsiranana18	0.183	0.184	0.182	0.183	0.194	0.187	0.194	0.187	0.188	0.214	0.214	0.186	0.168	0.190	0.179
46	Fianarantsoa19	0.193	0.194	0.192	0.193	0.193	0.187	0.193	0.187	0.187	0.213	0.213	0.196	0.190	0.212	0.200
47	Antsiranana20	0.161	0.162	0.171	0.161	0.171	0.165	0.171	0.165	0.165	0.189	0.189	0.163	0.180	0.190	0.179
48	Antsiranana21	0.165	0.178	0.165	0.165	0.176	0.170	0.176	0.170	0.170	0.194	0.194	0.180	0.197	0.220	0.208
49	Antsiranana22	0.160	0.173	0.171	0.160	0.170	0.165	0.170	0.165	0.165	0.189	0.189	0.175	0.190	0.201	0.189
50	Antsiranana25	0.182	0.183	0.181	0.182	0.193	0.187	0.193	0.187	0.187	0.212	0.212	0.185	0.179	0.201	0.189
51	O.formosus	0.223	0.223	0.245	0.223	0.217	0.218	0.217	0.218	0.217	0.240	0.240	0.246	0.286	0.288	0.282
52	O.wroughtoni	0.251	0.227	0.252	0.251	0.246	0.252	0.246	0.252	0.245	0.267	0.267	0.247	0.289	0.290	0.284
53	Mormopterus_jugularisI1P	0.585	0.571	0.556	0.585	0.531	0.521	0.531	0.521	0.522	0.520	0.520	0.505	0.592	0.594	0.605

Table 6.8. Pairwise genetic distances of D-loop samples against samples 16 to 30.

	Sample	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
16	Kenya5															
17	Kenya6	0.007														
18	Kenya7	0.015	0.015													
19	EthiopiaA1	0.038	0.030	0.038												
20	EthiopiaA2	0.038	0.030	0.038	0.000											
21	EthiopiaB1	0.050	0.050	0.050	0.076	0.076										
22	EthiopiaB2	0.050	0.050	0.050	0.076	0.076	0.000									
23	EthiopiaC1	0.011	0.004	0.018	0.034	0.034	0.054	0.054								
24	EthiopiaC2	0.011	0.004	0.018	0.034	0.034	0.054	0.054	0.000							
25	EthiopiaD1	0.022	0.022	0.007	0.038	0.038	0.058	0.058	0.026	0.026						
26	EthiopiaD2	0.022	0.022	0.007	0.038	0.038	0.058	0.058	0.026	0.026	0.000					
27	Yemen	0.038	0.038	0.038	0.022	0.022	0.067	0.067	0.042	0.042	0.038	0.038				
28	Fianarantsoa1	0.196	0.201	0.179	0.212	0.212	0.184	0.184	0.207	0.207	0.190	0.190	0.213			
29	Mahajanga2	0.191	0.196	0.174	0.207	0.207	0.180	0.180	0.190	0.190	0.174	0.174	0.207	0.026		
30	Mahajanga3	0.191	0.196	0.174	0.207	0.207	0.180	0.180	0.190	0.190	0.174	0.174	0.207	0.026	0.000	
31	Mahajanga4	0.197	0.202	0.180	0.213	0.213	0.186	0.186	0.196	0.196	0.179	0.179	0.213	0.030	0.018	0.018
32	Antsiranana5	0.190	0.195	0.174	0.206	0.206	0.179	0.179	0.190	0.190	0.173	0.173	0.207	0.019	0.007	0.007
33	Toliara6	0.196	0.201	0.179	0.212	0.212	0.184	0.184	0.195	0.195	0.179	0.179	0.213	0.022	0.011	0.011
34	Toliara7	0.201	0.206	0.184	0.218	0.218	0.190	0.190	0.201	0.201	0.184	0.184	0.218	0.026	0.015	0.015
35	Toliara8	0.190	0.195	0.174	0.206	0.206	0.179	0.179	0.190	0.190	0.173	0.173	0.206	0.030	0.011	0.011

Table 6.8 continued.

	Sample	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
36	Toliara9	0.201	0.206	0.184	0.206	0.206	0.190	0.190	0.201	0.201	0.184	0.184	0.218	0.026	0.015	0.015
37	Toliara10	0.190	0.195	0.174	0.206	0.206	0.190	0.190	0.201	0.201	0.173	0.173	0.207	0.026	0.022	0.022
38	Toliara11	0.190	0.195	0.174	0.206	0.206	0.179	0.179	0.190	0.190	0.173	0.173	0.207	0.019	0.007	0.007
39	Toliara12	0.190	0.195	0.174	0.206	0.206	0.179	0.179	0.190	0.190	0.173	0.173	0.207	0.019	0.007	0.007
40	Toliara13	0.179	0.184	0.163	0.195	0.195	0.168	0.168	0.179	0.179	0.163	0.163	0.196	0.034	0.022	0.022
41	Toliara14	0.158	0.164	0.143	0.163	0.163	0.158	0.158	0.169	0.169	0.153	0.153	0.174	0.050	0.054	0.054
42	Antsiranana15	0.179	0.184	0.163	0.195	0.195	0.179	0.179	0.179	0.179	0.163	0.163	0.195	0.034	0.022	0.022
43	Antsiranana16	0.179	0.184	0.163	0.195	0.195	0.179	0.179	0.179	0.179	0.163	0.163	0.195	0.034	0.022	0.022
44	Antsiranana17	0.196	0.201	0.179	0.212	0.212	0.184	0.184	0.195	0.195	0.179	0.179	0.213	0.022	0.011	0.011
45	Antsiranana18	0.185	0.190	0.168	0.201	0.201	0.196	0.196	0.184	0.184	0.168	0.168	0.201	0.030	0.018	0.018
46	Fianarantsoa19	0.207	0.212	0.189	0.212	0.212	0.195	0.195	0.206	0.206	0.189	0.189	0.224	0.030	0.018	0.018
47	Antsiranana20	0.185	0.190	0.169	0.201	0.201	0.185	0.185	0.185	0.185	0.180	0.180	0.201	0.022	0.011	0.011
48	Antsiranana21	0.214	0.220	0.197	0.231	0.231	0.179	0.179	0.213	0.213	0.196	0.196	0.219	0.042	0.015	0.015
49	Antsiranana22	0.196	0.201	0.179	0.212	0.212	0.184	0.184	0.207	0.207	0.190	0.190	0.202	0.022	0.034	0.034
50	Antsiranana25	0.196	0.201	0.179	0.212	0.212	0.184	0.184	0.195	0.195	0.179	0.179	0.213	0.022	0.011	0.011
51	O.formosus	0.282	0.288	0.272	0.288	0.288	0.319	0.319	0.295	0.295	0.286	0.286	0.311	0.250	0.230	0.230
52	O.wroughtoni	0.297	0.290	0.291	0.308	0.308	0.288	0.288	0.283	0.283	0.289	0.289	0.301	0.257	0.219	0.219
53	Mormopterus_jugularisI1P	0.592	0.594	0.582	0.629	0.629	0.626	0.626	0.593	0.593	0.593	0.593	0.616	0.498	0.512	0.512

Table 6.9. Pairwise genetic distances of D-loop samples against samples 31 to 45.

	Sample	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
31	Mahajanga4															
32	Antsiranana5	0.011														
33	Toliara6	0.015	0.004													
34	Toliara7	0.018	0.007	0.004												
35	Toliara8	0.022	0.011	0.015	0.018											
36	Toliara9	0.018	0.007	0.004	0.007	0.018										
37	Toliara10	0.026	0.015	0.018	0.022	0.018	0.022									
38	Toliara11	0.011	0.000	0.004	0.007	0.011	0.007	0.015								
39	Toliara12	0.011	0.000	0.004	0.007	0.011	0.007	0.015	0.000							
40	Toliara13	0.026	0.015	0.011	0.015	0.026	0.015	0.030	0.015	0.015						
41	Toliara14	0.067	0.054	0.058	0.062	0.050	0.054	0.054	0.054	0.054	0.054					
42	Antsiranana15	0.018	0.015	0.011	0.015	0.026	0.015	0.022	0.015	0.015	0.022	0.062				
43	Antsiranana16	0.018	0.015	0.011	0.015	0.026	0.015	0.022	0.015	0.015	0.022	0.062	0.000			
44	Antsiranana17	0.015	0.004	0.000	0.004	0.015	0.004	0.018	0.004	0.004	0.011	0.058	0.011	0.011		
45	Antsiranana18	0.022	0.011	0.007	0.011	0.022	0.011	0.018	0.011	0.011	0.018	0.058	0.011	0.011	0.007	
46	Fianarantsoa19	0.022	0.011	0.007	0.011	0.022	0.004	0.026	0.011	0.011	0.018	0.058	0.018	0.018	0.007	0.015
47	Antsiranana20	0.022	0.011	0.015	0.018	0.022	0.018	0.019	0.011	0.011	0.026	0.050	0.018	0.018	0.015	0.015
48	Antsiranana21	0.034	0.022	0.019	0.022	0.026	0.022	0.038	0.022	0.022	0.030	0.071	0.030	0.030	0.019	0.026
49	Antsiranana22	0.038	0.026	0.022	0.018	0.038	0.026	0.034	0.026	0.026	0.034	0.058	0.034	0.034	0.022	0.030
50	Antsiranana25	0.015	0.004	0.000	0.004	0.015	0.004	0.018	0.004	0.004	0.011	0.058	0.011	0.011	0.000	0.007

Table 6.9 continued.

	Sample	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
51	O.formosus	0.242	0.243	0.250	0.256	0.230	0.243	0.237	0.243	0.243	0.257	0.206	0.248	0.248	0.25	0.242
52	O.wroughtoni	0.231	0.230	0.236	0.230	0.220	0.242	0.237	0.230	0.230	0.230	0.220	0.236	0.236	0.236	0.230
53	Mormopterus_jugularisI1P	0.485	0.488	0.489	0.496	0.512	0.482	0.497	0.488	0.488	0.486	0.520	0.479	0.479	0.489	0.488

Table 6.10. Pairwise genetic distances of D-loop samples against samples 46 to 52.

	Sample	46	47	48	49	50	51	52
46	Fianarantsoa19							
47	Antsiranana20	0.022						
48	Antsiranana21	0.026	0.026					
49	Antsiranana22	0.030	0.030	0.034				
50	Antsiranana25	0.007	0.015	0.019	0.022			
51	O.formosus	0.235	0.224	0.257	0.250	0.250		
52	O.wroughtoni	0.236	0.226	0.230	0.249	0.236	0.176	
53	Mormopterus_jugularisI1P	0.468	0.495	0.513	0.496	0.489	0.512	0.584

Appendix 5. Informative sites from haplotypes

```
                      10        20        30        40        50        60
Haplotype1   GCATCACCTCGCAGGCCGCTTCATACATTTGCGATACATGCGCATATTCTCGAATCCCTT
Haplotype2   ............................A.T.T........T..C..C.....T......
Haplotype3   ..............ATT..A...A......T.T........TT.....TC.ATTCT.T..
Haplotype4   ..............................T.T.......AT..C......ATTC.....
Haplotype5   ..............................T.T........T.........ATTC.....
Haplotype6   ..............A...............T.T........T.......C...TC.....
Haplotype7   ..............................T.T........T...........TC.....
Haplotype8   ..........................G...T.T..G.....TTC..........TC.....
Haplotype9   ..............A...............T.T..G.G...TT......C...TC.....
Haplotype10  ..........................G...T.T..G.....T...........TC.....
Haplotype11  ...A..........................T.T.A......T...........TC.....
Haplotype12  ..............................T.T........T...........TC.....
Haplotype13  ..............A...T........G..T.T..G.....T...........TC.....
Haplotype14  ..............A...............T.T..G.G...TT......C...TC.....
Haplotype15  ..............A...T...........T.T..G.....T...........TC.....
Haplotype16  ..............................T.T..G...A.T...........TC.....
Haplotype17  ..............................T.T..G.....T...........TC.....
Haplotype18  .............TA...............T.T..G.G...TT......C...TC....C
Haplotype19  ..............A...............T.T..G.G...TT......C...TC.....
Haplotype20  ..............A...............T.T..G.G...TT......C...TC.....
Haplotype21  ATG.TGTTA..A...TT....AG......CTTT...T.A..T..CGC......T..T...
Haplotype22  ATG.TGTTA..A...TT...CAG......CTTT...T.A..T..CGC......T..T...
Haplotype23  A.G..GTTA..A...TT...CAG......CTTT...T.A..T..CGC......T..T...
Haplotype24  A.G..GTTA..A...TT...CA.......CTTT...T.A..T..CGC......T..T...
Haplotype25  A.G..GTTA..A...TT....A..TT...CTTT...T.A..T..CGC......T..T...
Haplotype26  A.G..GTTA..A...TT....A..TT...CTTT...T.A..T..CGC......T..T...
Haplotype27  A.G..GTTA..AC..TT...CGG......CTTTG..T.A..T..CG.......T..T...
Haplotype28  A.G..GTTA..A...TT...CAG......CTTT...T.A..T..CGC......T..T...
Haplotype29  .........TAA..ATT....G........T.TG......AT..C.....T..T....C.
Haplotype30  .........TAA..ATT....G........T.TG.......T..C.....T..T......
Haplotype31  .........TAA..ATT....G........T.TG.......T..C.....T..T......
Haplotype32  .........TAA..ATTA...G........T.TG.......T..C.....T..T......
Haplotype33  .........TAA..ATT....G........T.TG.......T..C.....T..T......
Haplotype34  .........TAA..ATT....G........T.TG......AT..C.....T..T....C.
Haplotype35  .........TAA..ATTA...G........T.TG.......T..C.....T..T....C.
Haplotype36  .........TAA..ATT....G...T....T.TG.......T..C.....T..T......

                      70        80        90       100
Haplotype1   TAATATCGCACCAAATTTCACTTTTTCTCCAATCTTTCCTCCACCC
Haplotype2   ...........................................T..
Haplotype3   .............G............T................T..
Haplotype4   ..................................C.CT.....T..
Haplotype5   ...........................................T..
Haplotype6   .............G............T................T..
Haplotype7   ...........................................T..
Haplotype8   ...........................................T..
Haplotype9   .............G.............................T..
Haplotype10  .............G.............................T..
Haplotype11  ...........................................T..
Haplotype12  .....A.....................................T..
Haplotype13  .................CT.T..CC..AT....T...TT..A.TTT
Haplotype14  .............G............T................T..
Haplotype15  .................C.................C.......T.T
Haplotype16  C............G..................G..........T..
Haplotype17  C............G...C..T......................T..
Haplotype18  .............G...C........T.....G..........T..
Haplotype19  .............G...C........T.....G..........T..
Haplotype20  ............CG..................G..........T..
Haplotype21  ...C..T.T.TT.GGC..............G...C.....T.GT..
Haplotype22  ...C..T.T.TT.GGC..............G...C.....T.GT..
Haplotype23  ...C..T.T.TT.GGC..............G...C.....T.GT..
Haplotype24  ...C..T.T.TT.GGC..............G...C.....T.GT..
Haplotype25  ...C..T.T.TT..G..C.....C......G...C.....T.GT..
Haplotype26  ...C..T.T.TT..G..C.....C...................T..
Haplotype27  ...CT.TCTGTT..GCA.............GT..C.....T.GT..
Haplotype28  .C.C..T.TGTT.GGCA........C....GT..C.....T.GT..
Haplotype29  ..GC.........G.....G..C....C.T....C..T..T.....
Haplotype30  ..GC.........G.....G.CC....C.T....C..T..T.....
Haplotype31  ..GC.........G.....G..C....C.T....C.CT..T.....
Haplotype32  ..GC.........G.....G.CC....C.T....C..T..T.....
Haplotype33  ..GC.........G.....G..C....C.T....C..T..T.....
Haplotype34  ..GC.........G.....G..G....C.T....CCCTTC.A...A
Haplotype35  ..GC.........G.....G.CC....C.T....C..T..T.....
Haplotype36  ..GC.........G.....G.......C.T................
```

Figure 6.1. Informative (variable) sites from cytochrome *b* haplotype sequences. Haplotypes are defined in Table 3.8.

```
                      10        20        30        40        50
             ....|....|....|....|....|....|....|....|....|....|
Haplotype1   TATACCAAC-GTTCTCCCCCTCCTCCCATCAGTATTGATC-ATAGTTTTA
Haplotype2   .............................T.....C..........C...
Haplotype3   ...............T...................C..C.......CCC.
Haplotype4   ...............T......T.........C........G.......G
Haplotype5   ...............T......T.........C........G.......G
Haplotype6   ...............TT.....T.........C........G.......G
Haplotype7   .....T.........T..T...T.........C........G.......G
Haplotype8   ............C..T..T.C........T.........T.....C..C.
Haplotype9   CT............C....T......TC.T...G....CTG.C.ACA.A.
Haplotype10  CT............C....T......TT.T.........TG.CGACA.A.
Haplotype11  CT............C....T......TT.T.........TG.CGACA.A.
Haplotype12  CT............C....T......TT.T.........TG.CGACA.A.
Haplotype13  CT............C....T......TT.T.........TG.C.ACA.A.
Haplotype14  CT.G.......C..C....T......TC.T.A.G.....TA.CGACA.A.
Haplotype15  .TC....G......C....T.......T.T.........TG.CGACA.A.
Haplotype16  CT............C....T...C..TT.T.........TG.CGACA.A.
Haplotype17  CT............C....T......TT.T...G....CTG.C.ACA.A.
Haplotype18  .T.G..........C....T......TC.....G.....TA.CGACA.A.
Haplotype19  ....T.GGTTA..TCTTT...TT.AA...T.A..CCA.........C.C.
Haplotype20  ......GGTTA..TCTTT...TTCAA...T.A..C.A.C.......C.C.
Haplotype21  ......GGTTA..TCTTT...TTCAA..CT.A..C.A.C.......C.C.
Haplotype22  ......GGTTA..TCTTT...TTCAA...T.A..C.A.C.......C.C.
Haplotype23  ......GGTTA..TCTTT...TTCAA...T.A..C.AGC.......C.C.
Haplotype24  ......GGTTA..TCTTT...TTCAA...T.A..C.AGC.......C.C.
Haplotype25  ......GGTTA..TCTTT...TTCAA...T.A..C.A.C.......CC..
Haplotype26  ......GGTTAC.TCTTT...TTCAA...T.A..C.AGC.......C.C.
Haplotype27  ......G.TTA..TCTTT...TT.AA...T.A..C.A.C.......CCC.
Haplotype28  ......GGTTA..TCTT....TTCAA...T.A....AGC.......C.C.
Haplotype29  ......G..TAC.TCTT..T.TT..A...TGA..C.A.............
Haplotype30  ......G.TTA..TCTTT...TTCAA...T.A..C.AGC...C...C.C.
Haplotype31  ......G.TTA..TCTTT...TTCAAT..T.A..C.AGC.......C.C.
Haplotype32  ......GGTTAC.TCTTT...TTCAA...T.A..C.AGC.......C.CG
Haplotype33  ......G.TTA..TCTTT...TTCAA...T.A..C.A.........C.C.
Haplotype34  ..C...GGTTA..TCTTT...TTCAA.....A..C.AGC.......C.C.
Haplotype35  ......GGTTA..TCTTT...TT.AA.....A..C.AG........C.C.

                      60        70        80
             ....|....|....|....|....|....|....|....
Haplotype1   CGAAACTTGCGATAACGTCTAATAAACAGGTAATGCCTT
Haplotype2   TA......A........C..............G......
Haplotype3   T...............................G......
Haplotype4   TA..G...........A...GG.......A.........
Haplotype5   TA..G...........A...GG.......A.......C.
Haplotype6   TA..G...........A...GG.......A.........
Haplotype7   TA..G....T......A...GG.......A.........
Haplotype8   TA...T..A.......AC...........A.......C.
Haplotype9   .A...T.CA..GC...A.TC..G...T.A....CATTC.
Haplotype10  .AG..T.CA..GC...A.TC..G...TGA....CATTCC
Haplotype11  .AG..T.CA..GC...A.TC.GA...TGA....CATTCC
Haplotype12  .AG..T.CA..GC...A.TC..G...T.A....CAT.CC
Haplotype13  .A...T.CA..GC...A.TC..G...T.A....CATTC.
Haplotype14  .A...T.CA..GC...A.TC..G...TGA..G.CATTCC
Haplotype15  .A...TC.A.AGC.G.A.TC..AG..T.A....CA.TCC
Haplotype16  .AG..T.CA..GC...A.TC..G...TGA....CATTCC
Haplotype17  .A...T.CA..GC...A.TC..G...T.A....CATTC.
Haplotype18  .A...T.CA..GC...A.TC..G...T.AA.G.CATTCC
Haplotype19  .A.G.T.CA...C..TA.T..GAGGGT......C..AC.
Haplotype20  .A.G.T.CA.......TA.T..GAGG.T.A....C..AC.
Haplotype21  .A.G.T.CA...C..TA.T...AGGGT.A.......AC.
Haplotype22  .A.G.T.CA...C..TA.T..GAGGGT.A....C..AC.
Haplotype23  .A.G.T.CA...C..TA.T..GAGGGT.A....C..AC.
Haplotype24  .A.G.T.CA...C..TA.T..GAGGGT.A.C..C..AC.
Haplotype25  .A.G.T.CA...C..TA.T..GAGG.T.A....C..AC.
Haplotype26  .A.G.T.CA...C..TA.T..GAGGGT.A....C..AC.
Haplotype27  .A.G.T.CA...CG.TA.T..GAGGGT.A....C..AC.
Haplotype28  .A.G.T.CA...C..TA.T..GAG.GT.A....C..AC.
Haplotype29  .A.G.T.CA...C..TA.T..GAG..T......C..AC.
Haplotype30  .A.G.T.CA...C..TA.T...AGGGT.A....C..AC.
Haplotype31  .A.G.T.CA...C..TA.T..GAGGGT.A....C..AC.
Haplotype32  .A.G.T.CA...C..TA.T..GAGGGT.A....C..AC.
Haplotype33  .A.G.T.CA.......TA.T..GAGGGT.A....C..AC.
Haplotype34  .A.G.T..A.......TA.T..GAGG.T.A....C..AC.
Haplotype35  .A...T.CA...C..TA.T..GAGGGT...C..C..AC.
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Figure 6.2.Informative (variable) sites of D-loop haplotype sequences. Haplotypes are defined in Table 3.16.

Appendix 6. Dating of nodes as estimated by Lamb et al. (2008)

Table 6.11. Estimate absolute ages of nodes (Bayesian analysis – BEAST program) for uncorrelated relaxed clock models with log-normal distribution of branch rates and two fossil calibration dates: earliest divergence of *Mops* (11.2 – 16.4 MYA) and known dates of the earliest known African molossid (*Tadarida rusingae*: 17.5 – 18 MYA) (Lamb *et al*., 2008).

Node	Relaxed molecular clock		
	MYA	95% confidence limits	
		Lower	Upper
Otomops	7.73	4.65	11.20
Oriental *Otomops*	3.57	1.49	5.75
Africa + Madagascar	3.10	1.62	4.86
Africa (Clades 1 & 2)	2.38	1.20	3.80
Mops spp.	13.10	11.54	14.63
Tadarida-Mops-Otomops	17.76	17.40	18.16

CHAPTER THREE:

Investigation into the Molecular Phylogeny of Molossidae (Chiroptera) from Africa and Madagascar

ABSTRACT

Phylogenetic relationships among predominantly Afro-Malagasy genera of Molossidae were assessed using combined nuclear RAG2 and mitochondrial cytochrome *b* sequence data. Analyses were based on a sample of 48 molossid taxa represting 9 named genera and 21 species.*Mormopterus* spp. held a basal position within a monophyletic molossid clade which was sister to all other taxa, from which it diverged 36.56 MYA. *Tadarida* spp. do not exhibit monophyly and thus do not appear to form a natural grouping, suggesting that further studies are needed to define the phylogenetic associations of members of this genus. *Sauromys* is basal to a moderately-supported clade comprising *Tadarida fulminans* and members of the genera *Chaerephon* and *Mops*. The strongly-supported monophyletic *Chaerephon* / *Mops* clade diverged from the other molossid genera 19.07 MYA; within this, species of *Mops* are not monophyletic and are ancestral to a more derived monophyletic *Chaerephon* clade. Afro-Malagasy *Otomops* spp. form a well-supported discrete clade and exhibit no clearly-defined associations with other genera included in this study (divergence date: 21.31 MYA). The observed phylogenetic structure is consistent with that proposed by Simmons (2005), in which members of the Molossidae are divided into two subfamilies: Molossinae and Tomopeatinae, although *Chaerephon* and *Mops* are presented as separate genera by Simmons (2005), whereas present results suggest that *Mops* may not be a valid genus.

1. INTRODUCTION

1.1 Overview

Although a number of phylogeographic studies have been carried out on various molossid species, including those highlighted in the section below (1.2.2), the phylogenetic relationships among molossid genera, including *Otomops*, are not well resolved. This study is a report on a molecular investigation of phylogenetic relationships among Molossidae of the Afro-Malagasy region, with a particular focus on the relationship of *Otomops* to other genera of the Molossidae. The results of this chapter were published as part of Lamb *et al*. (2011). Ammerman *et al*. (2012) subsequently published on this topic as well.

Taxonomic sampling in systematic studies of the Molossidae has been poor, leading to systematic disagreements and contradicting taxonomic structures (Ammerman *et al*., 2012). A contributing factor is the lack of specimens available for analysis. It is well known that the capture of molossids is particularly challenging, reason for this being their natural aptitude for high and fast flight, owing to their wing structure (Norberg, 1981). The ability of molossids to elude capture means that these genera are usually poorly represented in museum and field collections compared with other families of bats.

Initially, higher-level classifications within the Molossidae were based primarily on traditional morphological data (Freeman, 1981; Legendre, 1984; Simmons, 2005). Freeman (1981) employed a multivariate approach, classifying the Molossidae through use of morphometric measurements. Results suggested the existence of two major groups, one comprising the genera *Mormopterus*, *Myopterus*, *Cheiromeles* and *Molossops* and the other including *Tadarida*, *Chaerephon*, *Mops*, *Otomops*, *Nyctinomops*, *Promops*, *Molossus* and *Eumops*. Through the use of genetic data we aim to test the validity of groupings hypothesised by Freeman (1981).

Legendre's (1984) study of the dental morphology of various molossids suggested the presence of three subfamilies: Molossinae, Tadarinae and Cheiromelinae. *Molossus*, *Eumops*, *Molossops*, *Cynomops*, *Neoplatymops*, *Myopterus* and *Promops* constituted the Molossinae; *Tadarida*, *Mormopterus*, *Nyctinomops*, *Otomops* and *Rhizomops* comprised the Tadarinae; and *Cheiromeles* represented the Cheiromelinae. One of the aims of this work was to test the validity of the groupings proposed by Legendre (1984).

Simmons (2005) used a combination of molecular and morphological data from various sources (Simmons and Geisler, 1998; Hulva and Horáček, 2002; Teeling *et al*., 2002, 2003, Hoofer *et al*., 2003) to re-classify families within Chiroptera. This study divided the Molossidae into 2 subfamilies: Molossinae (*Chaerephon*, *Cheiromeles*, *Cynomops*, *Eumops*, *Molossops*, *Molossus*, *Mops*, *Mormopterus*, *Myopterus*, *Nyctinomops*, *Otomops*, *Platymops*, *Promops*, *Sauromys* and *Tadarida*) and Tomopeatinae (*Tomopeas*). Molossidae groupings allocated by Simmons (2005) will also be compared against the findings of this study.

Gregorin and Cirranello (2015) used morphological data (characters from skull, dentition, postcrania, external morphology, tongue and penis) which divded the family into two clades comprising: (1) *Mormopterus*, *Platymops*, *Sauromys*, *Neoplatymops*, *Molossops*, *Cynomops*, *Cheiromeles*, *Molossus* and *Promops*; and (2) *Tadarida*, *Otomops*, *Nyctinomops*, *Eumops*, *Chaerephon* and *Mops*. Clades defined here will be compared against those found in this study.

1.2 Genera belonging to the family Molossidae

1.2.1 Current taxonomy

The superfamily Vespertilionoidea (Chiroptera) comprises a number of families, including the Molossidae, Cistugidae, Vespertilionidae, Natalidae and Miniopteridae (Eick *et al*., 2005; Lack *et al*., 2010). The name Molossidae is derived from the Greek word *molossus*, for a type of dog (Taylor, 2005). The Molossids (order Chiroptera; suborder Vespertilioniformes) (Hutcheon and Kirsch, 2006) are commonly known as either free-tailed bats, due to the portion of the tail that projects beyond the posterior margin of the uropatagium, or mastiff bats, owing to their bulldog-like appearance (Taylor, 2005). Their most distinctive features are their particularly large ears and the wrinkling of the thick upper lips (Freeman, 1981). They are also notably robust, strong fliers with long, narrow wings (Norberg, 1981). The Molossidae currently comprise 16 genera and approximately 100 species (Simmons, 2005). Members of this family have a wide range, being found on every continent sans Antarctica, in both the New World and Old World (Taylor, 2005).

Genera within the Molossidae include *Chaerephon* Dobson, 1872; *Cheiromeles* Horsfield, 1824; *Cynomops* Thomas 1920; *Eumops* Miller, 1906; *Molossops* Peters, 1865; *Molossus* Geoffroy, 1805; *Mops* Lesson, 1842; *Mormopterus* Peters, 1865; *Myopterus* Geoffroy, 1818; *Nyctinomops* Miller, 1902; *Otomops* Thomas, 1913; *Platymops* Thomas, 1906; *Promops* Gervais, 1855; *Sauromys* Robert, 1917; *Tadarida* Rafinesque, 1814 and *Tomopeas* Miller, 1900 (Simmons, 2005). Of the 16 genera within the Molossidae, eight can be found in the Afro-Malagasy region (including the Arabian

Peninsula) as well as the islands of Zanzibar and Pemba off the African coast, and islands from the Malagasy region, i.e. Mayotte, Anjouan, Grande Comore, Moheli, Aldabra, La Reunion and Mauritius. Genera endemic to the African mainland include *Myopterus*, *Platymops* and *Sauromys*. The genera forming part of this study include *Chaerephon*, *Molossus*, *Mops*, *Mormopterus*, *Nyctinomops*, *Otomops*, *Sauromys* and *Tadarida*.

The genus *Chaerephon* was initially included as a subgenus of *Tadarida* but was eventually classified as a separate genus by Koopman (1993). There are currently 21 species within the genus, 10 of which can be found within the Afro-Malagasy region (Simmons, 2005; Goodman and Cardiff, 2004; Goodman *et al*., 2010; Monadjem *et al*., 2010). Certain *Chaerephon* species exhibit physical characteristics which are similar to those found in both *Mops* and *Tadarida*, e.g. skull characters (Bouchard, 1998). This commonality between genera makes identification of true *Chaerephon* species more challenging, thereby necessitating the need for alternative diagnostic characters, e.g. the degree of palatial emargination, as well as genetic differentiation (Jacobs *et al*., 2004).

Systematics of the genus *Molossus* has been reviewed by a number of authors, all of whom found little consensus with regard to the number of species said to exist within the genus (González -Ruiz *et al*., 2010). Miller Jr. (1913) recognised 19 species, Goodwin (1959, 1969) added two further species, Dolan (1989) recognized seven species, Jennings *et al*. (2000) recognized five and Simmons (2005) recognized eight. This variation in the number of *Molossus* species is as a result of their morphological similarity, and because species diagnosis has been based predominantly on size (López-González and Presley, 2001; González -Ruiz *et al*., 2010).

The genus *Mops*, much like *Chaerephon*, was historically treated as a subgenus of *Tadarida* (Freeman, 1981), however this taxon has been raised to full generic status (Simmons, 2005). There are two subgenera within *Mops* (*Mops* and *Xiphonycteris*). According to Simmons (2005), there were 15 species of *Mops*, although the description of an additional species by Stanley (2008) has brought the current total to 16 species.

Mormopterus was also once considered to be part of *Tadarida* (Freeman, 1981), but is now recognised as a distinct genus that contains 11 described species (Jacobs and Fenton, 2002; Simmons, 2005).

There are four species within the genus *Nyctinomops* which, like *Mops*, *Chaerephon* and *Mormopterus*, was once classified as part of the genus *Tadarida* (Milner *et al*., 1990; Simmons, 2005).

Sauromys is a genus that contains only one species, *S. petrophilus*, characterised by its flattened skull (Freeman, 1981). This genus was initially described under the name *Platymops petrophilus* but this

was changed when Peterson (1965) restricted the distribution of *Platymops* to East Africa. Those individuals from the southern Africa subregion were classified as *Sauromys* (Taylor, 2005). Both *Platymops* and *Sauromys* were both once classified as subgenera of *Mormopterus* (Freeman, 1981; Koopman 1993, 1994) however authors such as Peterson (1965), Corbet and Hill (1992) and Jacobs and Fenton (2002) recognise these taxa as distinct genera (Simmons 2005; Taylor, 2005).

The genus *Tadarida* has had a dynamic history, and once included a number of now-distinct genera, namely *Chaerephon*, *Mops*, *Mormopterus*, *Nyctinomops*, *Platymops* and *Sauromys* (Simmons, 2005). There are currently 10 recognised species of *Tadarida* (Simmons, 2005).

The taxonomic history of *Otomops* has been discussed in Chapter 2, Section 1.1.1.

1.2.2 Phylogenetic and taxonomic studies of the Molossidae from the Afro-Malagasy region

The Afro-Malagasy region comprises various localities within Africa, Madagascar and their surrounding islands. Some of the genera constituting the Molossidae can be found in countries from southern, western and eastern Africa, e.g. South Africa, Mozambique, Ivory Coast and Kenya, and offshore islands such as Zanzibar and Pemba are also included as part of the African region. The Malagasy region includes Madagascar, islands of the Comoro Archipelago (Grand Comore, Mohéli, Anjouan and Mayotte), La Réunion and Mauritius of the Mascarene Islands and Aldabra in the western Seychelles.

Goodman and Cardiff (2004) described a new species from Madagascar, *Chaerephon jobimena*, based on morphological measurements, pelage and wing colouration and cranial and dental characters. Ratrimomanarivo *et al*. (2009a) found differences in size variation and the degree of sexual dimorphism between three *C. leucogaster* populations from Madagascar, Mayotte and Pemba. However, genetic analysis did not show clear correspondance with morphological findings and distances were not high enough to warrant the classification of new taxonomic units. The taxonomy and genetic similarity within the *C. pumilus* complex from both southern Africa and the Malagasy region has also been investigated by various authors, e.g. Jacobs *et al*. (2004) evaluated the possibility of a cryptic *C. pumilus* species in southern Africa on the basis of the existence of light- and dark-winged varieties, however analyses revealed that these two types are genetically similar. Goodman and Ratrimomanarivo (2007) investigated the taxonomic status of a bat that was initially classified as *C. pusillus* but subsequently renamed as *C. pumilus*. Morphologically, this bat from the Seychelles is distinctly smaller than its *C. pumilus* counterparts from Kenya, the Comoros Archipelago and Madagascar, thereby prompting the resurrection of the name *C. pusillus* for this bat. An mtDNA and craniometric study by Taylor *et al*. (2009) revealed that *C. leucogaster* from Madagascar is positioned

within the *C. pumilus* group, where Malagasy *C. pumilus* forms a sister group to African *C. pumilus* and Malagasy *C. leucogaster*. Goodman *et al*. (2010) established that *C. leucogaster* and *C. pumilus* from Madagascar represent two lineages but that the bat considered *C. pumilus* is distinct from those found on Africa, the Arabian Peninsula and the Comoros Archipelago, resulting in the naming of a new species, *C. atsinanana*, in this region. Findings from these studies show that the taxonomy of the *C. pumilus* complex is still unclear and yet to be completely elucidated. Ratrimomanarivo *et al*. (2007, 2008) investigated morphological and molecular variation in *Mops midas* and *M. leucostigma* from Madagascar and the WIO islands, and Stanley (2008) described a new species, *M. bakarii*, from Pemba. Ratrimomanarivo *et al*. (2007) found that morphological and genetic differences between African *M. m. midas* and Malagasy *M. m. miarensis* were not sufficient to uphold this sub-specific classification. Ratrimomanarivo *et al*. (2008) found that *M. leucostigma* from east and west Madagascar exhibit size differentiation however this distinction is not genetically supported. Additionally, *M. leucostigma* is not endemic to Madagascar since it is found on two islands from the Comoros Archipelago, i.e. Moheli and Anjouan, however it is morphologically and genetically distinct from African *M. condylurus* and Malagasy *M. midas*, each species exhibiting monophyly. Ratrimomanarivo *et al*. (2009b) investigated morphological and molecular variation in *Mormopterus jugularis*, revealing notable sexual dimorphism but a lack of genetic structure, where grouping according to latitude, longitude or altitude was non-existent and *M. jugularis* formed a single population currently ungergoing expansion. Goodman *et al*. (2008) described the species *M. francoismoutoui*. The phylogeography of *Otomops martiensseni* and *O. madagascariensis* with reference to the Asian varieties *O. wroughtoni* and *O. formosus* has also been explored (Lamb *et al*., 2008). *Otomops* from the Afro-Malagasy region separates into three species clades/clusters: *O. martiensseni* from southern and western Africa, *O. martiensseni* from north eastern Africa and *O. madagascariensis* from Madagascar (Lamb *et al*., 2006, 2008).

More broadly, both Freeman (1981) and Legendre (1984) used external skeletal measurements and dental morphology, respectively, in order to determine natural groupings, thereby inferring phylogenies based on phenetic similarity. Freeman (1981) found the existance of two natural groupings, whereas Legendre (1984) found that the molossids divided into three sub-families: Molossinae, Tadarinae and Cheiromelinae. Eick *et al*. (2005) used a nuclear perspective in their report on the historical biogeography of the Chiroptera and found that molossids, together with vespertilionids, miniopterids and natalids, all form part of the Vespertilionoidea where Molossidae appear as a sister taxon to Miniopteridae and Vespertilionidae. Analysis of nuclear and mitochondrial data used by Lamb *et al*. (2011) to investigate the phylogeny of Afro-Malagasy Molossidae showed strong support for the monophyly of the family based on the species included in the study. Some genera displayed monophyly while others created geographically based clades, as seen with the New World species, or showed associations with species from other genera, in some instances creating

combined clades, e.g. *Chaerephon*/*Mops*. Findings by Ammerman *et al*. (2012) based on nuclear and mitochondrial data on the phylogenetics of the Molossinae showed similarities to those of Lamb *et al*., (2011), supporting the monophyly of most genera, as well as the existence of both a New World and *Chaerephon*/*Mops* clade. Extant relationships revealed using molecular data appear to reflect the biogeographic proximity of species and do not support those hypothesized on the basis of morphological data.

1.2.3 Distribution and ecology

Molossid bats are distributed in the tropical and subtropical regions of the world (Ammerman *et al*., 2012). *Chaerephon* and *Mops* have an Old World distribution, with species found in the African, Arabian and Oriental regions. Bats of the genus *Molossus* are found within Neotropical America from northern Mexico to southern Argentina (López-González and Presley, 2001). Members of *Mormopterus* have a global range that extends from Australia and Asia through to South America, western and southern parts of Africa and the Western Indian Ocean (WIO) islands, including Madagascar. *Nyctinomops* has a New World distribution with a range that extending from SW British Columbia to NE Argentina (Milner *et al*., 1990; Simmons, 2005). *Sauromys* species can be found within the region of southern Africa, i.e. South Africa, Zimbabwe, Botswana, Namibia, Mozambique and possibly Ghana. *Tadarida* have a global distribution, occurring in Asia, Australia, Africa and the Americas.

1.3 Methods used in investigating the phylogenetic position of Otomops *within the Molossidae*

1.3.1 Rationale for methods used in this study

Although mitochondrial DNA (mtDNA) is well suited to molecular systematic studies, these regions are too fast-evolving to provide the resolution needed to examine genus-level relationships (Moore, 1995). The disadvantages of mtDNA use stems from its maternal inheritance and factors such as incomplete lineage sorting, retention of ancestral polymorphisms and the possibility of amplifying nuclear pseudogenes (Van Den Bussche *et al*., 2003; Capelli *et al*., 2006; Ruedi and McCracken, 2009). Use of a maternally-inherited gene results in a gene tree which may not reflect the true species tree therefore nuclear data, which reflects both male and female contributions to gene flow, may provide a more accurate representation of the tree (Dor *et al*., 2012). The use of bi-parentally inherited nuclear DNA (ncDNA) (eg. Lewis-Oritt *et al*., 2001) helps circumvent these problems whilst allowing for male gene flow and mating systems to be investigated (Ruedi and McCracken, 2009).

Additionally, due to their slow-evolving nature and lower levels of sequence divergence, nuclear genes are utilised when needing resolution in either more ancient divergences, at higher taxonomic levels, e.g. family, class and/or order level (Lovejoy and Collette, 2001; Springer *et al*., 2001; Steppan *et al*., 2004). Both cytochrome *b* (mtDNA) and RAG (ncDNA) were used in analysis which may help elucidate a more accurate species tree.

1.3.2. Recombination-activating gene (RAG)

The recombination-activating gene (RAG) region comprises two tandemly paired genes (RAG1 and RAG2) that are responsible for the encoding of a site-specific recombinase (recombination-activating protein) (Sadofsky *et al*. 1994; Steen *et al*., 1999; Baker *et al*. 2000; Fugmann, 2011). RAG2 appears to be a more popular choice of nuclear marker since insertion-deletion events are rare and therefore do not cause problems in aligning sequences (Van Den Bussche *et al*., 2003; Steppan *et al*. 2004).

Baker *et al*. (2000) and Roehrs *et al*. (2010) used RAG2 to elucidate phylogenetic relationships in the Phyllostomidae family and Vespertilioninae subfamily. Almeida *et al*. (2009) described the phylogenetics of the sub-family Cynopterinae based 6 genes, including nuclear RAG2. Similar studies involving genus to family-level phylogenetics have been conducted by Davalos (2005), Velazco and Patterson (2008), Lewis-Oritt *et al*. (2001) and Stadelmann *et al*. (2007). More recently, Lamb *et al*. (2011) and Ammerman *et al*. (2012) incorporated the use of RAG2 to investigate the molecular phylogeny of Molossidae.

1.3.3 Dating

Molecular methods of determining divergence dates vary and can include: use of a molecular clock and one global substitution rate, e.g. tree-based maximum likelihood clock optimization (Langley and Fitch, 1974; Sanderson, 2003); character-based maximum likelihood clock optimisation (Felsenstein, 1981; Swofford *et al*., 1996); and use of a relaxed clock or rate heterogeneity, e.g. linearized trees (Li and Tanimura, 1987; Takezaki *et al*., 1995; Hedges *et al*., 1996), or Bayesian implementation of rate variation in BEAST (Drummond *et al*., 2006).

The method chosen for analysis was the Bayesian implementation of rate variation in BEAST (Drummond *et al*., 2006), which uses allows for the simultaneous analysis of multiple data sets with various substitution models whilst providing Bayesian credibility intervals (Rutschmann, 2006). Both mitochondrial and nuclear gene regions can provide a good representation of the phylogenetic relationships between various organisms, nuclear genes such as RAG2 in particular having been

favoured for dating purposes due to their ability to provide greater resolution in the deeper nodes of phylogenies (Douzery *et al*., 2003).

1.4 Aims and Objectives

Most molecular phylogenetic and phylogeographic work pertaining to Molossidae has focused on particular genera whilst higher-level relationships among genera were somewhat unclear, including the phylogenetic position of *Otomops*. The first family-level study to be published was Lamb *et al*. (2011), focusing on the phylogeny of the Molossidae of the WIO region. This was followed by the more inclusive study of Ammerman *et al*. (2012).

The aim of this chapter is to report on the evolution and phylogenetic position of *Otomops* within the molossid family and, more generally, phylogenetic associations between other members of the Molossidae.

The specific objectives of this chapter are:

(1) To assess phylogenetic structure using two molecular markers

- (a) The nuclear Recombination Activating Gene 2 (RAG2)
- (b) The mitochondrial cytochrome *b* gene.

(2) To analyse phylogenetic relationships among representative samples of the Molossidae family using phenetic, cladistic and Bayesian inference methods and to look for patterns of association among genera. Mitochondrial cytochrome *b* sequences of *Otomops* were those presented in Chapter 2. Cytochrome *b* sequences from other molossid genera were obtained from the research outputs of fellow students and the NCBI Genbank. Nuclear RAG2 sequences of species of *Otomops*, *Chaerephon*, *Mops*, *Mormopterus*, *Sauromys* and *Tadarida* were generated for the purposes of this study. Additional RAG2 sequences were sourced from research outputs of fellow students and Genbank.

(3) To assess the position of *Otomops* within the Molossidae family and its association with other members of the molossid family.

(4) To estimate divergence dates of the major supported clades within the Molossidae and to investigate the evolutionary history of these clades, especially *Otomops*.

(5) To provide evidence relating to the hypothesis of Freeman (1981), that the Molossidae comprise two major groups, with the genera *Mormopterus* (including *Sauromys* and *Platymops*), *Myopterus*, *Cheiromeles* and *Molossops* (including *Cynomops* and *Neoplatymops*) in one group and *Tadarida*, *Chaerephon*, *Mops*, *Otomops*, *Nyctinomops*, *Promops*, *Molossus* and *Eumops* in the other.

(6) To provide evidence relating to the hypothesis of Legendre (1984), that the Molossidae comprise 3 subfamilies: Molossinae (*Molossus*, *Eumops*, *Molossops*, *Cynomops*, *Neoplatymops*, *Myopterus* and *Promops)*, Tadarinae (*Tadarida*, including *Chaerephon* and *Mops*, *Mormopterus*, including *Sauromys*, *Platymops* and *Micronomus*, *Nyctinomops*, *Otomops* and *Rhizomops*) and Cheiromelinae (*Cheiromeles*).

(7) To provide evidence relating to the hypothesis of Simmons (2005), that the Molossidae comprise 2 subfamilies: Molossinae (*Chaerephon*, *Cheiromeles*, *Cynomops*, *Eumops*, *Molossops*, *Molossus*, *Mops*, *Mormopterus*, *Myopterus*, *Nyctinomops*, *Otomops*, *Platymops*, *Promops*, *Sauromys* and *Tadarida*) and Tomopeatinae (*Tomopeas*).

(8) To provide evidence relating to the hypothesis of Gregorin and Cirranello (2015), that the Molossidae comprise 2 clades: (1) *Mormopterus*, *Platymops*, *Sauromys*, *Neoplatymops*, *Molossops*, *Cynomops*, *Cheiromeles*, *Molossus* and *Promops*; and (2) *Tadarida*, *Otomops*, *Nyctinomops*, *Eumops*, *Chaerephon* and *Mops*.

2. MATERIALS AND METHODS

2.1 Sample collection

The phylogenetic position of *Otomops* among other members of the family Molossidae was investigated via sequencing of the nuclear RAG2 gene (n=48), as well as through the use of a concatenated data set comprising cytochrome *b* and RAG2 sequences. A total of 21 molossid species were used in the analysis. Samples from the range of molossid bat genera were obtained from a variety of sources, including the Durban Natural Science Museum, Field Museum of Natural History, National Museum of Kenya, National Museum of Prague, Senckenberg Museum and the Université d'Antananarivo. Samples were obtained and preserved as described in Chapter 2, Section 2.1. Sample details are summarized in Table 2.1.

Table 2.1. Locality, specimen details and Genbank accession numbers of sampled individuals of Molossidae and outgroup taxa.

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	RAG2	RAG2 + Cyt *b*	RAG2 (dated tree)	GenBank #	
				Fig. 3.3	Fig. 3.4	Fig. 3.5	Cyt *b*	RAG2
Chaerephon atsinanana	Madagascar: Vohipeno, Fianarantsoa Province	22.354° S, 47.840° E	FMNH185294	1	1	1	HQ 384479	HQ 384487
“	Madagascar: Vangaindrano, Fianarantsoa Province	23.355° S, 47.596° E	FMNH185229	1	2	1	HQ 384480	HQ 384488
Chaerephon leucogaster	Madagascar: Toliara, Toliara Province	23.395° S, 43.720° E	FMNH184237	1	6	1	HM802905	HM631634
“	Madagascar: Toliara, Toliara Province	23.395° S, 43.720° E	FMNH184239	1	6	1	EU 716036	HM631635
“	Madagascar: Toliara, Toliara Province	23.395° S, 43.720° E	FMNH184240	1	6	1	EU 716037	HM631636
“	Madagascar: Toliara, Toliara Province	23.395° S, 43.720° E	FMNH184245	1	6	1	HM802900	HM631629
“	Madagascar: Mahajanga, Mahajanga Province	15.713° S, 46.312° E	FMNH184608	1	5	1	HM802901	HM631630
“	Madagascar: Antanimbary, Mahajanga Province	17.185° S, 46.855° E	FMNH184899	1	5	1	HM802902	HM631631

Table 2.1 continued

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	RAG2	RAG2 + Cyt *b*	RAG2 (dated tree)	GenBank #	
				Fig. 3.3	Fig. 3.4	Fig. 3.5	Cyt *b*	RAG2
Chaerephon leucogaster	Madagascar: Nosy Be, Antsiranana Province	13.367° S, 48.315° E	FMNH187756	1	6	1	HM802903	HM631632
"	Tanzania: Pemba Island	4.970° S, 39.715° E	FMNH192887	1	6	1	HM802904	HM631633
Chaerephon pusillus	Seychelles: Aldabra	9.389° S, 46.202° E	FMNH205318	1	12	1	GQ 489134	HM631643
"	France: Mayotte	5.997° S, 39.391° E	FMNH194031	4	13	4	HQ 384481	HM631644
"	France: Mayotte	5.997° S, 39.391° E	FMNH194032	5	14	5	HQ 384482	HM631645
Chaerephon pumilus	Tanzania: Pemba Island	5.130° S, 39.440° E	FMNH192823	3	11	3	HQ 384483	
"	RSA: Athlone Park, KwaZulu-Natal	30.050° S, 30.883° E	DM7377	1	7	1	HM802906	HM631637
"	RSA: Athlone Park, KwaZulu-Natal	30.050° S, 30.883° E	DM7401	1	8	1	HM802907	HM631639

Table 2.1 continued

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	RAG2	RAG2 + Cyt *b*	RAG2 (dated tree)	GenBank #	
				Fig. 3.3	Fig. 3.4	Fig. 3.5	Cyt *b*	RAG2
Chaerephon pumilus	RSA: Mkuze Game Reserve, KwaZulu-Natal	27.583° S, 32.217° E	DM7373	1	8	1	FJ 415815	HM631641
"	RSA: Mkuze Game Reserve, KwaZulu-Natal	27.583° S, 32.217° E	DM7374	1	10	1	FJ 415816	HM631642
"	RSA: Hell's Gate, KwaZulu-Natal	28.000° S, 32.300° E	DM7367	1	8	1	FJ 415814	HM631638
"	RSA: Hell's Gate, KwaZulu-Natal	28.067° S, 32.421° E	DM7371	1	9	1	HM802908	HM631640
Chaerephon jobimena	Madagascar: Isalo National Park, Fianarantsoa Province	45.380° S, 22.540° E	FMNH175992	2	3	2	HM802932	HM631627
"	Madagascar: Isalo National Park, Fianarantsoa Province	45.380° S, 22.540° E	FMNH175993	2	4	2	HM802933	HM631628
Mops bakarii	Tanzania: Pemba Island	4.970° S, 39.715° E	FMNH192898	6	15	6	HM802911	HM631646
Mops condylurus	RSA: Hell's Gate, KwaZulu-Natal	26.003° S, 32.917° E	DM6291	7	16	7	HM802912	HM631647

Table 2.1 continued.

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	RAG2	RAG2 + Cyt *b*	RAG2 (dated tree)	GenBank #	
				Fig. 3.3	Fig. 3.4	Fig. 3.5	Cyt *b*	RAG2
Mops condylurus	RSA: Phinda Game Reserve, KwaZulu-Natal	27.856° S, 32.308° E	DM6332	7	16	7	HM802913	HM631648
Mops leucostigma	Madagascar: Mahajanga, Mahajanga Province	15.708° S, 46.311° E	FMNH184698	8	17	8	HM802914	HM631649
"	Madagascar: Ampitabe, Toamasina Province	19.002° S, 48.530° E	FMNH188009	8	17	8	HQ 384484	HQ 384489
Mops midas	Madagascar: Sakaraha, Toliara Province	22.907° S, 44.530° E	FMNH184306	9	18	9	HM802915	HM631650
"	Madagascar: Ankazomborona, Mahajanga Province	16.116° S, 46.073° E	FMNH185187	9	19	9	HM802916	HM631652
Mormopterus francoismoutoui	La Réunion: Saint Clotilde	20.918° S, 55.483° E	FMNH194015	10	20	10	HM802917	HM631653
"	La Réunion: Saint Clotilde	20.918° S, 55.483° E	FMNH194016	10	20	10	HM802918	HM631654
Mormopterus jugularis	Madagascar: Sakaraha, Toliara Province	22.907° S, 44.530° E	FMNH184347	11	21	11	HM802919	HM631655

Table 2.1 continued.

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	RAG2	RAG2 + Cyt *b*	RAG2 (dated tree)	GenBank #	
				Fig. 3.3	Fig. 3.4	Fig. 3.5	Cyt *b*	RAG2
Mormopterus jugularis	Madagascar: Andasibe, Toamasina Province	18.896° S, 48.415° E	FMNH184576	11	22	11	HM802920	HM631656
"	Madagascar: Fianarantsoa, Fianarantsoa Province	21.461° S, 46.077° E	FMNH184445	12	23	12	HM802921	HM631657
Otomops madagascariensis	Madagascar: Bisihiko Cave, Toliara Province	23.540° S, 43.770° E	FMNH172944	13	24	13	HM802922	HM631658
"	Madagascar: Isalo National Park, Fianarantsoa Province	22.540° S, 45.380° E	UADBA SMG10996	13	25	13	HQ 384485	HQ 384490
Otomops martiensseni	RSA: Brynderyn Flats, Morningside, KwaZulu-Natal	29.864° S, 31.040° E	DM7909	14	26	14	HM802923	HM631659
"	RSA: Kingsway School, Amanzimtoti, KwaZulu-Natal	30.039° S, 30.894° E	DM7914	14	27	14	HM802924	HM631660
"	Ivory Coast: Comoe National Park	8.733° S, 3.389° E	SMF92049	14	28	14	HM802925	HM631661
"	Kenya: Ithundu Caves, Makuena District	2.358° S, 37.717° E	NMK15461	14	29	14	HM802927	HM631663

Table 2.1 continued.

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	RAG2	RAG2 + Cyt *b*	RAG2 (dated tree)	GenBank #	
				Fig. 3.3	Fig. 3.4	Fig. 3.5	Cyt *b*	RAG2
Otomops martiensseni	Kenya: Ithundu Caves, Makuena District	2.358° S, 37.717° E	NMK15462	14	30	14	HM802926	HM631662
Sauromys petrophilus	RSA: Cedarberg	32.136° S 19.003° E	DM8613	21	-	-		HM631664
Tadarida aegyptiaca	Mozambique: 40 km west of Ribaue	NA	DM8617	20	-	15		HM631668
Tadarida fulminans	Mozambique: 40 km west of Ribaue	NA	DM8619	22	-	-		HM631667
Equus caballus	NA	NA	NA	-	-	25		AF447533.1
Eumops auripendulus	NA	NA	NA	-	-	17		AY834668
Molossus molossus	NA	NA	NA	17	-	18		AY141017
Myotis daubentoni	NA	NA	NA	18	-	20		AM265653.1
M. welwitschii	NA	NA	NA	-	-	21		AM265698.1
Natalus micropus	NA	NA	NA	-	-	22		AY141023

Table 2.1 continued.

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	RAG2	RAG2 + Cyt *b*	RAG2 (dated tree)	GenBank #	
				Fig. 3.3	Fig. 3.4	Fig. 3.5	Cyt *b*	RAG2
N. saturatus	NA	NA	NA	-	-	23		AY604467.1
N. stramineus	NA	NA	NA	19	32	-	AY621019.1	AY141024.1
Nyctiellus lepidus	NA	NA	NA	-	-	24		AY604463.1
Nyctinomops macrotis	NA	NA	NA	16	-	19		AY141018.1
Tadarida brasiliensis	NA	NA	NA	15	-	16		AY141019.1
Mormoops blainvilii	NA	NA	NA	-	31	-	AY604462.1	AF338701

RSA = Republic of South Africa; DM: Durban Natural Science Museum; FMNH: Field Museum of Natural History, Chicago; NMK: National Museum of Kenya, Nairobi; NMP: National Museum of the Czech Republic, Prague; SMF: Senckenberg Museum, Frankfurt; UADBA: Université d'Antananarivo, Département de Biologie Animale, Antananarivo. Uncatalogued specimens are denoted with collector numbers which include PB = Petr Benda, SMG = Steven M. Goodman. NA = not available.

2.2. DNA isolation

DNA was isolated from samples using the QIAGEN DNeasy® Tissue Kit according to the protocol(s) described in Chapter 2, Section 2.2.

2.3 DNA Quantification

2.3.1 Evaluation of DNA integrity

DNA integrity was assessed visually via gel electrophoresis according to the protocol(s) described in Chapter 2, Section 2.3.1.

2.3.2 Measurement of DNA concentration

Once DNA isolation was complete, concentrations were measured using the ND-1000 Spectrophotometer V3.3 (NanoDrop Technologies Inc.) as per manufacturer's instructions, i.e. 1 µl of sample was placed in the spectrophotometer and DNA concentration then determined.

2.4 Polymerase Chain Reaction (PCR) amplification and sequencing

2.4.1 Nuclear Recombination Activating Gene 2 (RAG2) amplification

Due to the relatively long length of the RAG2 gene (1581 bp), it was PCR-amplified as two overlapping fragments (Saiki *et al*., 1988). These fragments were amplified by two primer pairs (Baker *et al*., 2000; Stadelmann *et al*., 2007): 179F (5'- CAG TTT TCT CTA AGG AYT CCT GC -3') and 968R (5'- CCC ATG TTG CTT CCA AAC CAT A - 3') for the 5' portion of the sequence; and F1 (5'- TTT GTT ATT GTT GGT GGC TAT CAG -3') and R2 (5'- GRA AGG ATT TCT TGG CAG GAG T -3') for the 3' portion. Amplifications were performed in 25 µl reactions containing 30 – 60 ng template DNA (totalling 9 µl when mixed with sterile water), 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Roche Diagnostics), 0.2 µl 5 U/µl *Taq* polymerase (Super-Therm) and 4 µl of 6 µM primer dilution (forward and reverse) per reaction. The thermal cycling parameters used were: 95 °C for 1 min; followed by 39 cycles of (95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min); followed by 72 °C for 10 min.

2.4.2 Mitochondrial cytochrome *b* amplification

Amplification of the mitochondrial cytochrome *b* was carried out as in Chapter 2, Section 2.4.1 for all additional molossid samples.

2.4.3 DNA recovery and concentration measurement

Amplified fragments were separated via electrophoresis and excised from the agarose gel according to the protocol(s) described in Chapter 2, Section 2.4.3. Amplified fragments were removed and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp.) according to manufacturers' instructions. Fragments were then quantified and concentrations checked (as described in Chapter 2, Section 2.4.3) before sequencing.

2.4.4 DNA sequencing

Sequences were obtained directly from purified PCR products using the primers used for the initial amplifications. Sequencing was carried out as described in Chapter 2, Section 2.4.4. Reactions were carried out in the facilities of Inqaba Biotec, Pretoria, Gauteng, South Africa. All fragments were sequenced in both directions. Sequences were deposited in GenBank (Accession numbers: HQ384487 – HQ 384490, HM631627 – HM631668).

2.5 General data analyses

2.5.1 Construction of consensus sequences

A consensus sequence for each sample was constructed as described in Chapter 2, Section 2.5.1. The resulting RAG2 sequence alignment was trimmed to 1262 nucleotides in length. Included in the alignment were sequences from various other Molossidae, which were obtained from the National Center for Biotechnology Information (NCBI) GenBank (Table 2.1). These included samples of *Chaerephon ansorgei*, *C. chapini*, *C. jobensis*, *C. nigeriae*, *Eumops glaucinus*, *E. perotis*, *Molossus molossus*, *Mormopterus kalinowski*, *Nyctinomops aurispinosis*, *N. laticaudatus*, *N. macrotis*, *Promops centralis*, *Tadarida brasiliensis*, *T. fulminans* and *T. teniotis*. Outgroups included in the analyses were also obtained from GenBank and comprised samples from the Vespertilionidae (*Myotis daubentoni*), Natalidae (*Natalus stramineus*) and Mormoopidae (*Mormoops blainvilii*) families.

2.5.2 Concatenation of sequence data sets

In this study, both RAG2 alone, and concatenated RAG2 + cytochrome *b* data sets were analysed. This allowed for the inclusion of additional molossid samples, for which cytochrome *b* data was available but only part of the RAG2 data (5' end or 3' end) was available. Cytochrome *b* sequences for inclusion were obtained from GenBank. Before concatenation could be carried out, incongruence between data sets was evaluated by determining whether there were any nodes that were consistently strongly supported (≥ 70% bootstrap support, ≥ 95% Bayesian posterior probability) in one data set that may conflict with strongly-supported nodes in another (DeQueiroz, 1993; Eick *et al*., 2005).

2.5.3 Sequence analysis

Analyses of conserved, variable, parsimony informative and singleton sites were carried out using PAUP 4.0b10 (Swofford, 1993).

jModelTest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008), applying the AKAIKE information criterion, was used to determine the most suitable evolutionary model for use with a particular dataset. It was found that the General Time Reversible (GTR) model fit both the RAG2 and concatenated RAG2 + cytochrome *b* data sets best (refer to Chapter 2, Section 1.5.1) and analyses were then performed according to the assumptions of this model.

Genetic distances were calculated using the GTR model in PAUP 4.0b10 (Swofford, 1993). Individual pairwise distances were calculated for each data set. In addition, net between-group distances were calculated for defined groups. Groups were defined at both inter-species and inter-genus level, i.e. *Chaerephon* (excluding *C. jobimena*), *C. jobimena*, *Mops*, *Mormopterus*, *Otomops*, *Tadarida*, *Nyctinomops*, *Molossus*, *Natalus* and *Myotis*.

Neighbour joining trees were generated in PAUP 4.0b10 (Swofford, 1993) under the assumptions of the GTR model. The reliability of nodes was estimated using bootstrap resampling analysis (1000 iterations).

Bayesian inference analysis was performed using MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001). Bayesian analysis was run under the GTR + I + G model as determined in jModeltest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008). Analysis was run using four Markov chains for five million generations each (standard deviation of split frequencies <0.01), or until the standard deviation of the split frequencies was less then 0.01, sampling every 100 generations. The chains

were heated with the temperature scaling factor T = 0.02. The first 50 000 trees were discarded as burn-in, having checked that this was more than sufficient to achieve stationarity in preliminary runs. A 50% majority rule consensus tree was constructed from the remaining trees.

For maximum likelihood analysis, trees were initially obtained by neighbour-joining followed by TBR branch swapping. Maximum likelihood analysis was carried out in PAUP 4.0b10 (Swofford, 1993). The reliability of nodes was estimated using bootstrap resampling analysis (1000 iterations).

Parsimony analysis was carried out in PAUP 4.0b10 (Swofford, 1993), with starting trees obtained by step-wise addition. The addition sequence was random, with one tree held at each step and with 10 replicates. The tree-bisection-reconnection branch-swapping algorithm was used and 1000 bootstrap replicates were carried out using a heuristic search.

2.6 Dating

Six hundred nucleotides of the 5' end of the RAG2 data were used to estimate nodal dates via use of programs within the BEAST v. 1.6.1 package (Drummond and Rambaut, 2010). The RAG2 dataset was used as it provided good node support at the deeper nodes of interest within Molossidae. The HKY + G substitution model, as determined in jModelTest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008), was used with estimated base frequencies. Bayesian analysis was used to estimate mean and 95% confidence limits of specified nodal dates within Molossidae via Markov Chain Monte Carlo [MCMC] algorithm, with a chain length of 5 million, sampled every 1000 iterations with burn-in of 500 000. The uncorrelated relaxed clock model was used, which assumes that branch-specific rates followed a log-normal distribution. Analysis was carried out using BEAST v. 1.6.1 (Drummond and Rambaut, 2010) in conjunction with BEAUti v.1.6.1 (Drummond and Rambaut, 2009) and Tracer v. 1.5 (Rambaut and Drummond, 2009). The suggested time of the crown divergence of bats (estimate of 65 MYA) and the suggested crown divergence dates for Molossidae and Vespertilionidae (35 – 38 MYA and 47 – 49 MYA, respectively) were used in order to estimate the divergence dates of major supported clades within the Molossidae (Teeling *et al*., 2005; Jones *et al.,* 2005). Based on the stipulated estimated crown divergence dates, the Chiroptera, Molossidae and Vespertilionidae nodes were calibrated using normal distribution priors, with means for Chiroptera set to 65 MYA and the midpoints of ranges given for Molossidae (35 – 38 MYA) and Vespertilionidae (47 – 49 MYA). The standard deviation was set at 0.5 for Chiroptera, Molossidae and Vespertilionidae. Tree Annotator v. 1.6.1 (part of the BEAST v. 1.6.1 package (Drummond and Rambaut, 2010)) was used to get an estimate of the phylogenetic trees and to find the best supported tree. The dated tree was then viewed and edited in FigTree v.1.3.1 (Rambaut, 2006).

3. RESULTS

3.1 Polymerase Chain Reaction (PCR) amplification and sequencing of the nuclear Recombination Activating Gene 2 (RAG2)

PCR amplification of the RAG2 (~ 1581 bp) gene was successfully completed in 2 parts using the stipulated primer pairs (Section 2.4.1) (Figs. 3.1, 3.2).

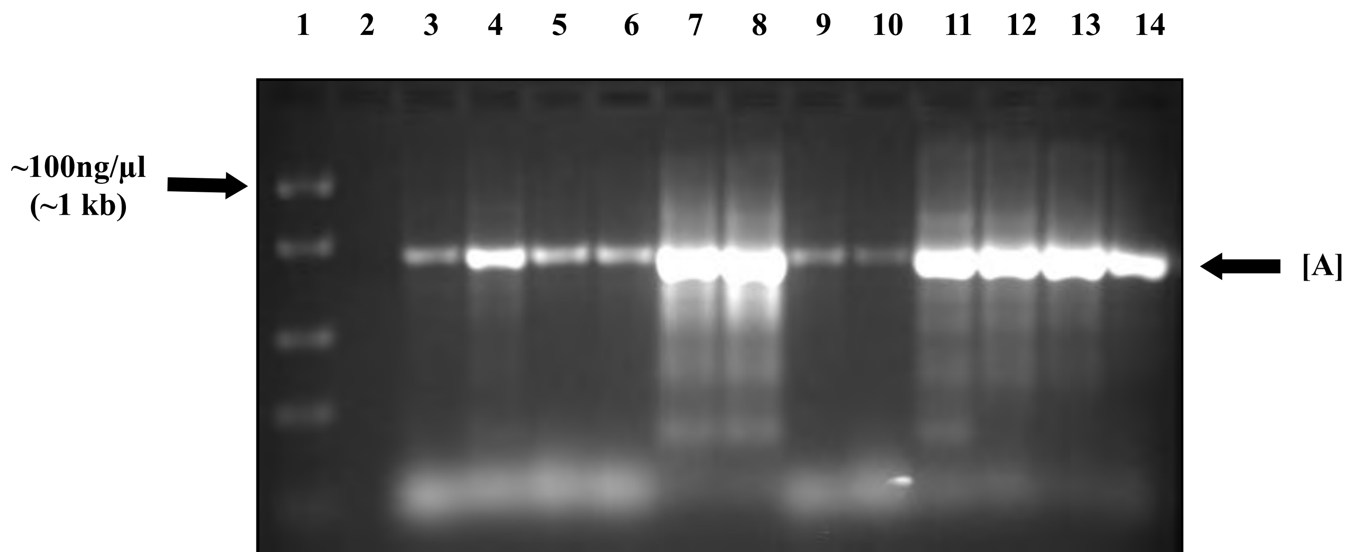

Figure 3.1. Agarose gel electrophoretic separation of the 5' end of the RAG2 gene primed using 179F and 968R. Lane 1: O'GeneRuler™ 100 bp DNA Ladder (Fermentas Life Sciences). Lanes 3 – 10: *Otomops madagascariensis* (Madagascar); lanes 11 and 12: *O. martiensseni* (Kenya); lanes 13 and 14: *O. martiensseni* (South Africa). [A: target fragments].

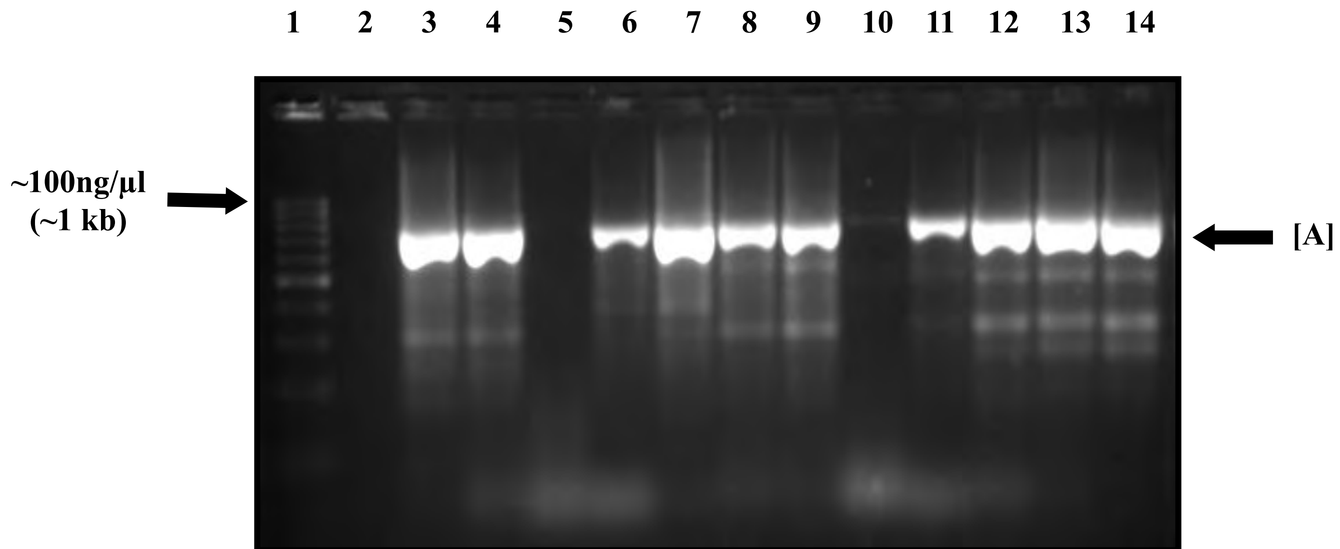

Figure 3.2. Agarose gel electrophoretic separation of the 3' end of the RAG2 gene primed using F2 and R2. Lane 1: O'GeneRuler™ 100 bp DNA Ladder (Fermentas Life Sciences). Lane 3: *C. leucogaster*; lanes 4: *Chaerephon pumilus*; lanes 6 and 7: *Mops midas*; lanes 8 and 9: *M. leucostigma*; lanes 10 and 11: *Mormopterus jugularis*; lane 12: *Tadarida jugularis*; lanes 13 and 14: *M. francoismoutoui*. All samples were collected from various provinces within Madagascar. Lane 5 contained a failed reaction. [A: target fragments].

Variation in concentration of template DNA between 30 and 60 ng per reaction did not affect target amplification. In some reactions, non-target regions were co-amplified with the region of interest. In these instances, the region of interest was excised from the gel and purified to remove any non-target DNA before sequencing. DNA from excised gel slices was, in certain instances (based on sequencing facility requirements), recovered using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp.) before being sent for sequencing.

3.2 Analysis of RAG2 sequence data

3.2.1 Analyses of sequence data

Characteristics of the datasets that were used in the analyses are listed in Table 3.1. These include the length of the sequence, the substitution model used, the number of variable characters, parsimony-informative characters, as well as the homoplasy index and the retention index. Retention indices and homoplasy indices were calculated for both RAG2 and cytochrome *b* data (Table 3.1).

Table 3.1. Dataset characteristics for the RAG2 gene and the RAG2 + cytochrome *b* gene.

Analysis type	Dataset	RAG2	RAG2 + cytochrome *b*
	Length (nucleotides)	1262	2031
	Model of substitution	GTR + I + G	GTR + I + G
Maximum parsimony parameters	Variable characters	178	412
	Parsimony-informative characters	95	302
	MP tree length	211	907
	Equally-parsimonious trees	2	1
	Homoplasy index (HI)	0.128	0.406
	Retention index (RI)	0.908	0.828
Maximum likelihood	Number of ML trees	1	1
	-log likelihood of most likely tree(s)	3056	7357

Higher retention indices (RI) and lower homoplasy indices (HI) suggest that there is a lack of homoplasy for both the RAG2 and RAG2 + cytochrome *b* data sets. The RAG2 dataset has an RI of 0.908 and an HI of 0.128, and the RAG2 + cytochrome *b* dataset has an RI of 0.828 and an HI or 0.406.

Numbers of conserved, variable, parsimony informative and singleton sites for each of the genus and species groups are presented in Table 3.2.

Table 3.2. Number of conserved, variable, parsimony informative and singleton sites out of 1262 nucleotides for four molossid genus groups using RAG2 gene sequence data.

Variables	*Chaerephon*	*Mops*	*Mormopterus*	*Otomops*
Conserved sites	1224	1240	1260	1261
Variable sites	38	22	2	1
Parsimony informative sites	33	13	1	1
Singleton sites	5	9	1	0

3.2.2 Genetic distance

All genetic distances were calculated using the GTR model in PAUP 4.0b10 (Swofford, 1993). Pairwise genetic distances between haplotypes were calculated and are given in Appendix 1. Net between-group distances are presented in Table 3.3 and 3.4.

Table 3.3. Net between-group GTR genetic distances for molossid genera groups and outgroups for RAG2 gene (1262 nucleotides) (standard deviation above the diagonal).

Taxon	1	2	3	4	5	6	7	8	9	10
[1] *Chaerephon*		0.005	0.002	0.005	0.005	0.004	0.005	0.005	0.007	0.007
[2] *C. jobimena*	0.020		0.004	0.004	0.004	0.003	0.004	0.005	0.006	0.006
[3] *Mops*	0.007	0.018		0.004	0.004	0.003	0.003	0.005	0.007	0.006
[4] *Mormopterus*	0.026	0.020	0.024		0.004	0.004	0.004	0.005	0.006	0.006
[5] *Otomops*	0.024	0.018	0.023	0.023		0.004	0.004	0.005	0.006	0.006
[6] *Tadarida*	0.015	0.004	0.012	0.015	0.013		0.003	0.004	0.006	0.006
[7] *Nyctinomops macrotis*	0.021	0.015	0.017	0.018	0.019	0.009		0.004	0.006	0.006
[8] *Molossus molossus*	0.026	0.023	0.026	0.025	0.027	0.017	0.016		0.007	0.007
[9] *Natalus stramineus*	0.054	0.047	0.051	0.051	0.052	0.044	0.049	0.057		0.003
[10] *Myotis daubentonii*	0.056	0.051	0.054	0.049	0.056	0.044	0.050	0.057	0.058	

Between-genus group genetic distances show a high divergence between *Myotis daubentonii* and *Natalus stramineus* with a RAG2 distance value of 5.8% between them (Table 3.3). In terms of the molossid genera, the greatest divergences appear to be between *Molossus* and *Otomops* (2.7% - RAG2). Among *Chaerephon*, *Mops*, *Mormopterus, Otomops* and *Tadarida* the greatest divergence appears to be between *Mormopterus* and *Chaerephon* (2.6% - RAG2). *Otomops* is approximately equidistant from *Chaerephon*, *Mops* and *Mormopterus*, separated from them by RAG2 distances of

2.4%, 2.3% and 2.3%, respectively. By comparison, the distance between *Mops* and *Chaerephon* is relatively much lower (0.7% - RAG2). Distances between the *Tadarida* genus and *Chaerephon*, *Mops*, *Mormopterus* and *Otomops* have a range between 1.2% and 1.5% (Table 3.3). *Chaerephon jobimena* was not placed in the *Chaerephon* grouping since these samples do not appear to be phylogenetically associated with other species of the genus, instead forming a clade with the *Tadarida* genus (Fig. 3.3 and 3.4). *Chaerephon jobimena* is separated from *Chaerephon*, *Mops* and *Tadarida* by RAG2 distances of 2.0%, 1.8% and 0.4%, respectively.

3.2.3 RAG2 gene tree

As neighbour-joining, Bayesian and maximum parsimony analyses were congruent, only one tree is presented (Fig. 3.3). Two maximum parsimony trees were recovered from the complete RAG2 dataset (1262 nt). In the case of the additional maximum parsimony tree, it was found that the differences between the trees were in the unresolved and unsupported terminal branches.

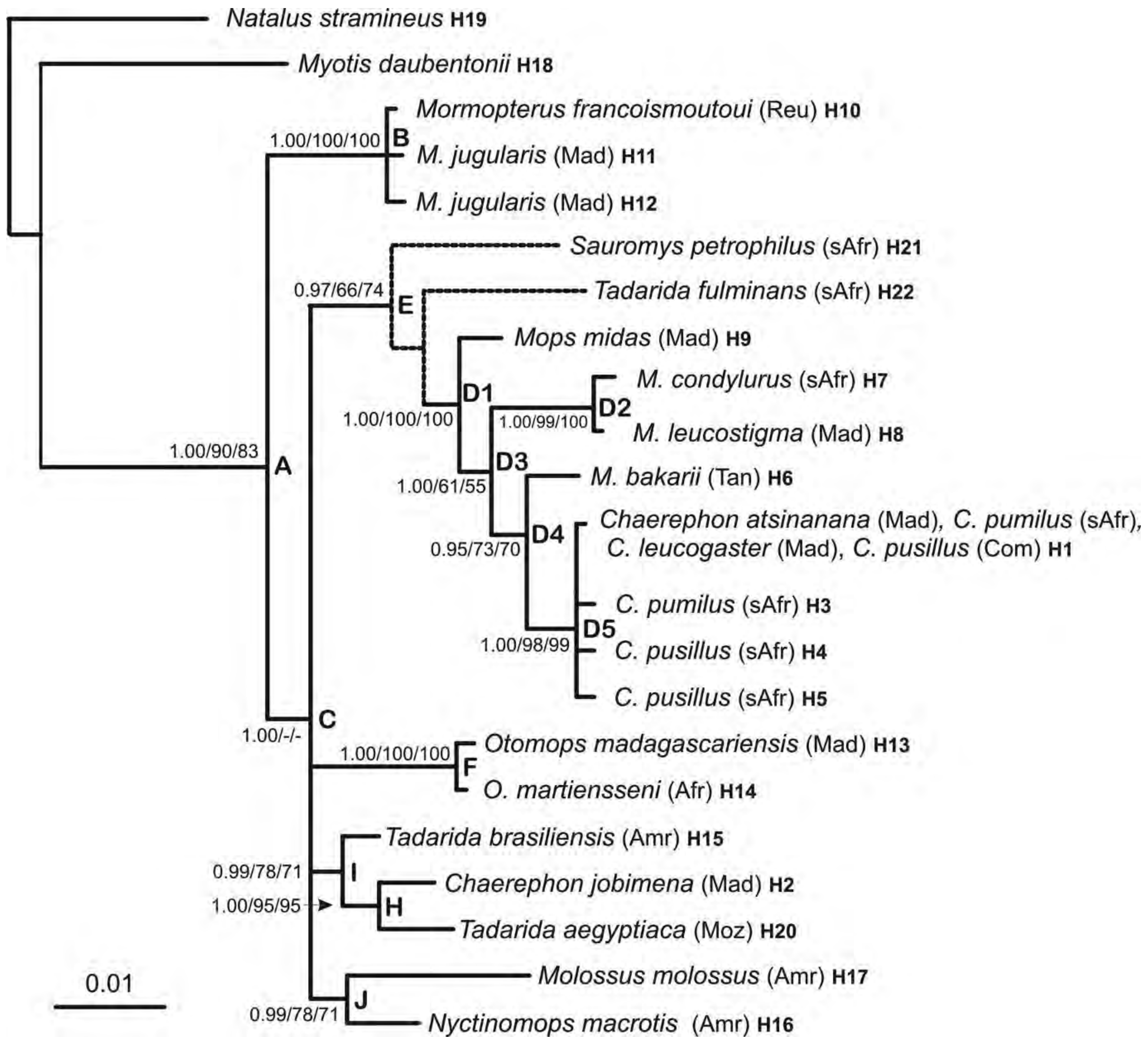

Figure 3.3. Bayesian phylogram based on 1262 nt of the RAG2 gene region showing relationships between molossid bat haplotypes with reference to non-molossid outgroups (support: Bayesian posterior probabilities/ maximum parsimony bootstrap percent/ neighbour-joining bootstrap percent). Dotted lines indicate the position of *Sauromys petrophilus* and *Tadarida fulminans* based on analysis of 634 nt of the 3' end of the RAG2 gene.

H1 – 22: haplotypes 1 through 22 (Table 2.1); Afr: Africa; Amr: Americas; Com: Comoros; Mad: Madagascar; Moz: Mozambique; Reu: La Reunion; sAfr: southern Africa; Tan: Tanzania.

3.2.4 RAG2/cytochrome *b* gene tree

As neighbour-joining, Bayesian and maximum parsimony analyses were congruent, only one tree is presented (Fig. 3.4). A single maximum parsimony tree was recovered from the concatenated RAG2/cytochrome *b* dataset (2031 nt).

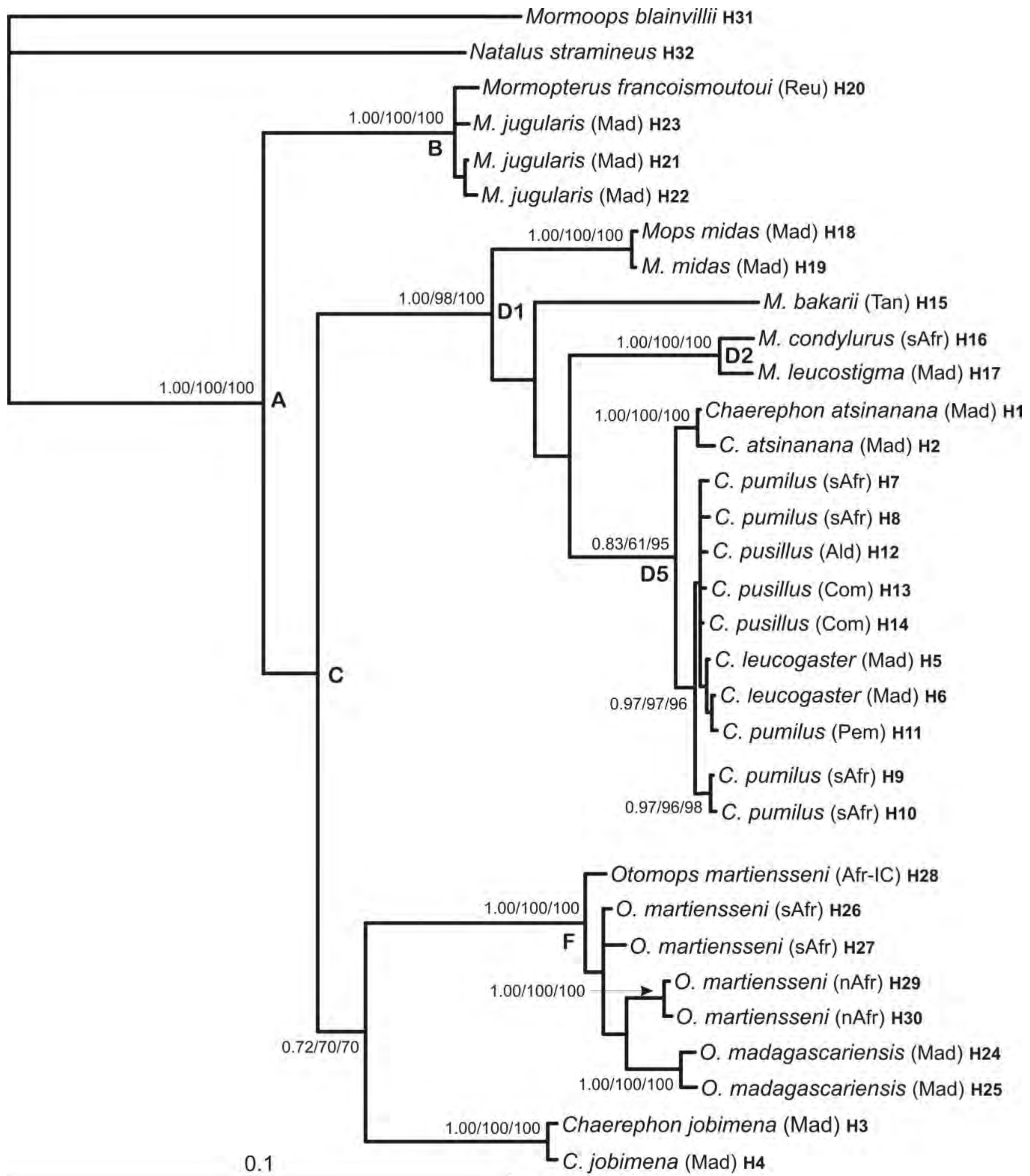

Figure 3.4. Bayesian phylogram based on 2031 nt of the concatenated RAG2/cytochrome *b* gene region showing relationships between molossid bat haplotypes with reference to non-molossid outgroups (support: Bayesian posterior probabilities/ maximum parsimony bootstrap percent/ neighbour-joining bootstrap percent).

H1 – 33: haplotypes 1 through 33 (Table 2.1); Afr-IC: Africa – Ivory Coast; Ald: Aldabra; Com: Comoros; Mad: Madagascar; nAfr: northern Africa; Pem: Pemba; Reu: La Reunion; sAfr: southern Africa; Tan: Tanzania.

Of the two trees created (Figs 3.3 and 3.4), the RAG2/cytochrome *b* tree resolved nodes within and between genera with greater support values. In both the RAG2 and RAG2/cytochrome *b* trees, it can be seen that the molossids included in this study form a very strongly- to moderately-supported monophyletic group (node A: 1.00 pp and bootstrap support >80%) with respect to outgroups *Natalus* and *Myotis* in the RAG2 tree and *Natalus* and *Mormoops* in the RAG2/cytochrome *b* tree (Figs. 3.3, 3.4). There is also moderate support in the RAG2 gene tree for the basal position of the well-supported *Mormopterus* genus (node/clade B: 1.00 pp and 100% bootstrap support), which is sister to all other represented molossid genera, descended from common ancestor C (Fig. 3.3). Genetic distance between the 3 *Mormopterus* haplotypes was low (p-distance 0.08%).

Chaerephon and *Mops* formed a very strongly-supported monophyletic group (node D1: 1.00 pp and ~100% bootstrap support) in both the RAG2 and RAG2/cytochrome *b* gene trees (Figs. 3.3, 3.4). Unlike all other *Chaerephon* samples, *C. jobimena* is not found within this grouping. In the RAG2 tree, *C. jobimena* groups with *Tadarida aegyptiaca* (Fig. 3.3) (well-supported at node H), whereas in the RAG2/cytochrome *b* tree it is found in a separate well-supported clade, sister to the *Otomops* clade F (Fig. 3.4). Within the *Chaerephon*/*Mops* clade it appears that *Mops* maintains the more ancestral position (node D1), relative to the more derived *Chaerephon* samples (node D5) (Figs. 3.3, 3.4).

Grouping of *Otomops* samples (clade F) in both the RAG2 and RAG2/cytochrome *b* trees is very strongly-supported (1.00 pp and 100% bootstrap support) and separation into geographically-circumscribed clades (from southern Africa, northern Africa and Madagascar) can be seen in Chapter 2, Fig. 3.11. There is no support for any higher-level association between *Otomops* and other members of the Molossidae.

Tadarida aegyptiaca is grouped with *Chaerephon jobimena* in clade H, which is then associated with *T. brasiliensis* (clade I) in the RAG2 tree (Fig. 3.3). *Sauromys petrophilus* and *T. fulminans* (clade E) appear basal to the moderately-supported clades D1 through D5, which comprise samples of *Chaerephon* and *Mops*. The RAG2 tree shows the moderate to well-supported association of *Molossis molossus* and *Nyctinimops macrotis* in clade J (0.99 pp and >70% bootstrap support).

3.2.5 Dating divergences within the Molossidae

A dated tree was created using the BEAST v.1.6.1 package (Drummond and Rambaut, 2010) including BEAUTi, BEAST, Tree Annotator, as well as FigTree v.1.5.1 (Rambaut, 2006).

Table 3.4. Estimated divergence dates (MYA), calculated using BEAST v1.6.1, for selected nodes from Fig 3.5. Crown divergence dates for Chiroptera, Vespertilionidae and Molossidae were obtained from Jones *et al*. (2005) and Teeling *et al*. (2005).

Taxon-set	Mean node date	95% confidence interval	Node	Comment
Chiroptera	64	58 – 71		Crown divergence date (Jones *et al*., 2005)
Natalidae	50.5	50 – 51		Crown divergence date (Jones *et al*., 2005)
Vespertilionidae	50	45 – 56		Crown divergence date (Jones *et al*., 2005)
Molossidae	36	35 – 38		Crown divergence date (Teeling *et al*., 2005)
Mormopterus	36.56	35.56 – 37.5	A	Node splitting *Mormopterus* from other molossids
Otomops	21.31	11.02 – 30.57	F	Node dividing *Otomops* from *T. brasiliensis* - *C. jobimena*
Chaerephon/Mops	19.07	10.69 – 27.86	D1	Node including all *Mops* and *Chaerephon* (with exception of *C. jobimena*)
Tadarida	14.18	5.36 – 23.88	I	Node separating *T. brasiliensis* from *C. jobimena*

Jones *et al*. (2005) estimates the crown divergence dates for the Chiroptera, Vespertilionidae and Molossidae at 64 MYA (95% confidence interval: 58 – 71 MYA), 50 MYA (95% confidence interval: 45 – 56 MYA) and 36 MYA (95% confidence interval: 35 – 38 MYA), respectively.

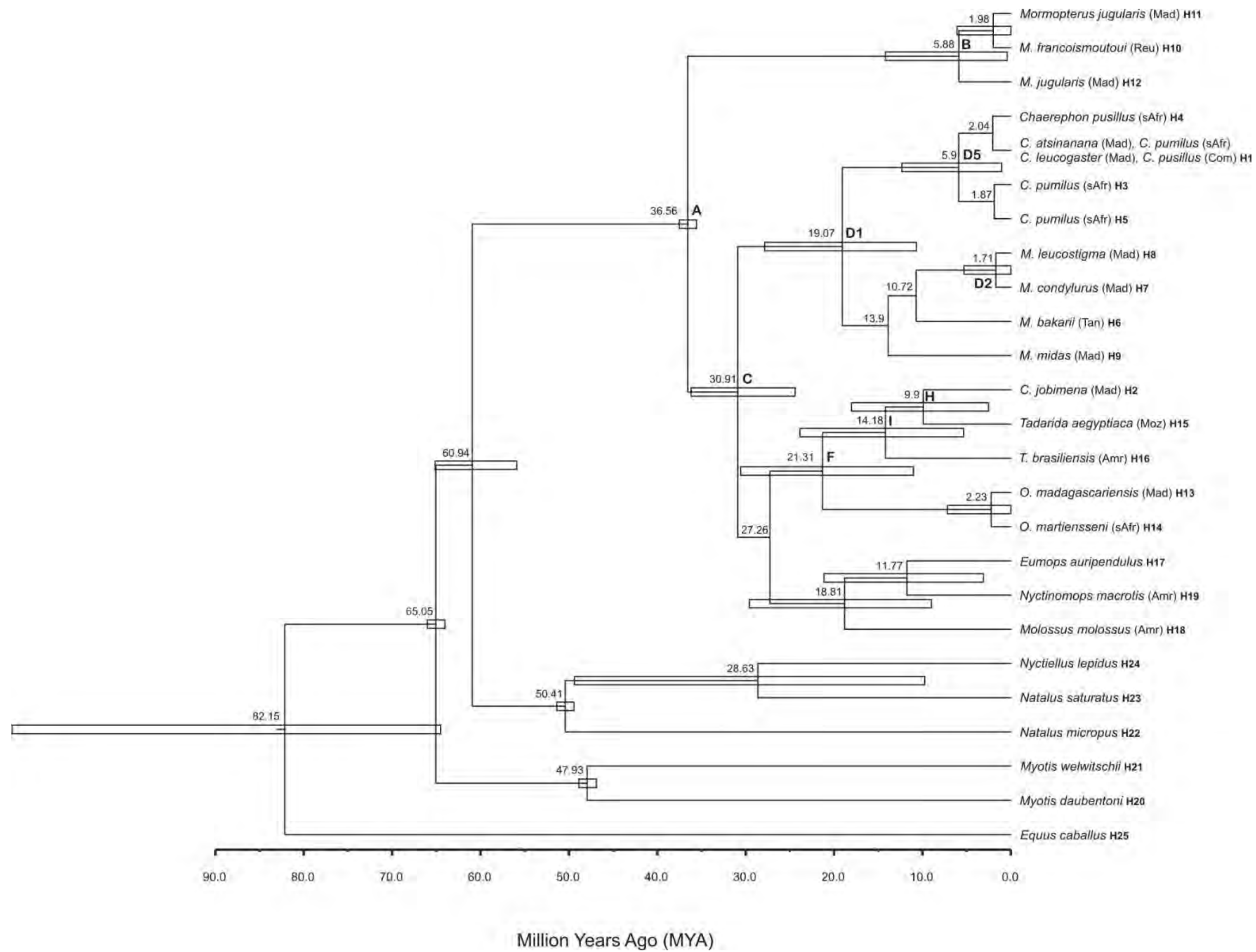

Figure 3.5. Dated tree based on 600 nt of the 5' end of RAG2 gene showing relationships between molossid bat haplotypes with reference to non-molossid outgroups and divergence dates of nodes in millions of years.

The split between members of *Mormopterus* and the other molossids included in this study is dated at 36.56 MYA (node A; 95% confidence interval: 35.56 – 37.7 MYA). The split between the groups comprising most *Chaerephon*/*Mops* samples and the other Molossidae included in this study is dated at 19.07 MYA (node D1; 95% confidence interval: 10.69 – 27.86 MYA). The split between the *Otomops* clade and clade I comprising *Tadarida brasiliensis*, *Chaerephon jobimena* and *T. aegyptiaca* is dated at 21.31 MYA (95% confidence interval: 11.02 – 30.57 MYA). Clade I is estimated at 14.18 MYA (95% confidence interval: 5.36 – 23.88 MYA). The age of the clade containing *Eumops auripendulus*, *Molossus molossus* and *Nyctinomops macrotis* has been estimated at approximately 18.81 MYA (95% confidence interval: 8.99 – 29.59 MYA).

4. DISCUSSION

The aim of this study was to use the nuclear RAG2 and mitochondrial cytochrome *b* sequence markers to assess phylogenetic relationships among genera of the Molossidae, assess the position of *Otomops* within the Molossidae and estimate the divergence dates of the major, supported clades.

4.1 Phylogenetic relationships within the Molossidae

According to previous studies, the Vespertilionoidea and Molossidae are estimated to have originated 52 to 50 MYA and 38 to 35 MYA, respectively (Jones *et al*., 2005; Teeling *et al*., 2005). Based on the RAG2 tree generated in this study, the splitting of the family into the various genera appears to have occurred during the Oligocene (34 – 23 MYA) and Miocene (23 – 5.3 MYA) epochs (Fig. 3.5 and Table 3.4).

The results of this study indicate that the Molossidae form a monophyletic clade, based on the use of both nuclear RAG2 data and a concatenated mitochondrial cytochrome *b* and nuclear RAG2 data set (Figs. 3.3 and 3.4). Ammerman *et al*. (2012) used a variety of markers, including nuclear RAG2, dentin matrix protein 1, beta fibrinogen 7 and mitochondrial nicotinamide adenine dinucleotide dehydrogenase 1, to elucidate the phylogeny of the Molossidae and found that RAG2 yielded lower genetic distances than the other nuclear markers that were employed, ranging from 1.2% between *Tadarida* and *Sauromys* and 4.97% between *Cheiromeles* and *Mops*. Distances found in this study were of similar values and will be used to infer distinctions between the various genera of the Molossidae.

Taylor *et al*. (2012) report on cytochrome *b* genetic distances between Molossidae. Genetic distances calculated at the intra-specific level revealed the mean distance between haplotypes within species to be 0.68%, with distances ranging from a minimum of ~0.2% in *Mops midas* to a maximum of approximately 5.8% in *Otomops martiensseni*. *Otomops martiensseni* appears to have the highest mean genetic intra-specific distance of 2%, which is consistent with findings in Chapter 2. The mean inter-specific genetic distance is given as 11.1%. *Mormopterus* appears to have the widest range of inter-specific genetic distances. A distance of 17.9% was found between the genera used in these analyses.

4.1.1 *Mormopterus*

Species of the genus *Mormopterus*, i.e. *M. francoismoutoui* and *M. jugularis*, appear basal within the Molossidae, with respect to the genera that formed part of this study, i.e. *Otomops*, *Chaerephon*, *Mops*, *Sauromys* and *Tadarida* (Figs. 3.3 and 3.4). Freeman (1981) suggested that *Mormopterus* may be phenetically similar to *T. aegyptiaca* and *T. brasiliensis*, however neither the RAG2 nor the RAG2/cytochrome *b* data support an association between these taxa and the *Mormopterus* clade. Further, genetic distance data does not reveal low genetic distances between *Mormopterus* and *Tadarida* (1.5% - RAG2) (Table 3.3).

Dating analysis places the split of *Mormopterus* from the other molossid genera at 36.56 MYA (Table 3.4), similar to the reports of Lamb *et al*. (2011) (31.18 MYA) and Ammerman *et al*. (2012) (33.3 MYA). This is reflected in the relatively large genetic distances separating *Mormopterus* from other molossid genera, ranging from 2.0% to 2.6% (RAG2) (Table 3.3). In contrast, genetic distances among the *Mormopterus* species range from 0.1% to 0.7% across both data sets (Tables 6.1 and 6.5) and 0.94% to 1.26% using cytochrome *b* data (Lamb *et al*. 2011).

4.1.2 *Tadarida*

Tadarida aegyptiaca, *T. brasiliensis* and *T. fulminans* are not monophyletic based on RAG2 data and do not appear to form a natural grouping as would be expected of congeneric taxa (Fig. 3.3).

4.1.2.1 *Tadarida* and *Chaerephon jobimena*

Tadarida aegyptiaca and *Chaerephon jobimena* form a strongly-supported clade (1.00 pp and 95% bootstrap support) which forms a moderate to well-supported association with a more basal *T. brasiliensis* (0.99 pp; 78% and 71% bootstrap support) (Fig. 3.3). *T. aegyptiaca* and *C. jobimena* are separated by a RAG2 genetic distance of 0.9% (Table 6.1), which is relatively low for an inter-generic distance and suggestive of an interspecific distance, similar to that between *Tadarida aegyptiaca* and *T. brasiliensis* (1.0%) (Table 6.2). Inter-generic molossid-molossid RAG2 distances average 1.8% whereas inter-specific molossid-molossid RAG2 distances average 0.3%. Lamb *et al*. (2011) also report a closer affinity of *T. aegyptiaca* to *C. jobimena* (cytochrome *b* distance = 11.15%) than to *T. brasiliensis* (cytochrome *b* distance = 15.07%). One implication of the above is that *Chaerephon jobimena* is a member of *Tadarida* (*T. jobimena*). These results also suggest that the Old World *T. aegyptiaca* and *C. jobimena* are congeners and split from the New World *T. brasiliensis* 14.18 MYA (Fig. 3.5; Table 3.4).

4.1.2.2 *Tadarida* and *Sauromys*

Analysis based on 634 nucleotides of the 3' end of RAG2 revealed that both *Tadarida fulminans* and *Sauromys petrophilus* are basal in a moderately-supported clade also containing all species of *Chaerephon* and *Mops* included in this study with the exception of *C. jobimena* (clades D1 to D5) (Fig. 3.3). *T. fulminans* appears to be most closely associated with members of the *Chaerephon* genus (mean 3' end RAG 2 distance = 0.8%), and, in contrast, is more distant from the two other members of the genus *Tadarida* (mean 3' end RAG 2 distance = 1.1%), with which it would have been expected to form a monophyletic clade. Both these results and those of Ammerman *et al.* (2012) show a phylogenetic affinity between *Tadarida fulminans* and *S. petrophilus*; in this study they are included in the same supported clade, and are separated from each other by a distance of 1.0% (Table 6.4), a value similar to that found by Ammerman *et al*. (1.2%).

The RAG2 data used in this study suggest that *Tadarida* may not be a natural genus, and that its taxonomic status needs further study.

4.1.3 *Sauromys*

Studies by Freeman (1981) and Legendre (1984) both propose that *Sauromys* is a subgenus of *Mormopterus*, however phylogenetic patterns (Fig. 3.3) and genetic distances (Table 6.3) do not support this. Genetic distances average 1.8% between *Sauromys* and *Mormopterus* species (Table 6.3) and the RAG2 tree indicates that *Mormopterus* is monophyletic and does not include *Sauromys* as part of this genus. Analysis of the 3' end of RAG2 shows that *Sauromys* is basal to a moderately-supported clade (E), which comprises *Tadarida fulminans* and members of the genera *Chaerephon* and *Mops* (clades D1 to D5) (Fig. 3.3).

4.1.4 *Chaerephon* and *Mops*

Description of the *Chaerephon*/*Mops* clade in relation to other Molossidae will not include *C. jobimena* since this species has been shown to be more closely associated with a member of the *Tadarida* genus (Chapter 3, Section 4.1.2.1). Genetic distances between *Chaerephon* and *C. jobimena* (RAG2 distance = 2.0%) are equivalent to those displayed at genus level, e.g. *Otomops* and *Mormopterus* (RAG2 distance = 2.3) (Table 3.3). Phylograms show that *C. jobimena* is not closely associated with other members of the *Chaerephon* genus, and that it forms a clade with *Tadarida* (Fig. 3.3) or a clade by itself (Fig. 3.4), thereby revealing paraphyly within *Chaerephon*. Cytochrome *b* distances display a similar result, showing *Chaerephon* and *C. jobimena* separated by genus-level

differences (14.62% to 18.74%) and *C. jobimena* and *T. aegyptiaca* separated by lower, possibly species-level differences (11.15%) (Lamb *et al*., 2011). The mean distance observed between currently established molossid genera is 17.9% (approximately 12% to 25%) and between molossid species is 11.1% (1.1% to 16.4%), respectively (Taylor *et al*. 2012).

All other *Chaerephon* and *Mops* species included in this study form a strongly-supported monophyletic clade (D1) (Figs. 3.3. and 3.4). Dating analysis places the split of the *Chaerephon*/*Mops* clade from the other molossids at 19.07 MYA, a value similar to that found in Lamb *et al*. (2011) (Table 3.4). Species of *Mops* appear ancestral to a more derived *Chaerephon* clade, but genetic distances between *Chaerephon* and *Mops* species (RAG2 = 0.8% to 1.2%) are similar to those between the various *Mops* species (RAG2 = 1.0% to 1.2%) (Table 6.1). The various *Chaerephon* species (excluding *C. jobimena*) are separated by relatively lower genetic distances (RAG2 = 0%) (Table 6.1). *Mops condylurus* and *M. leucostigma* form a well-supported clade (D2) within the nested set of moderate- to well-supported clades (D1 to D5) consistent with their status as sister species (Ratrimomanarivo *et al*., 2008). Moderate support is given to clade D5, which is found in both phylograms and contains *Chaerephon* species *C. atsinanana*, *C. leucogaster*, *C. pumilus* and *C. pusillus*. In Figure 3.4, *C. atsinanana* is in a supported position as sister to the other *Chaerephon* taxa. The presence of paraphyly in *Chaerephon* and a lack of clear distinction among *Chaerephon* and *Mops* species suggest that these two genera (excluding *C. jobimena*) should be combined into a single genus. This suggestion is supported by cytochrome *b* distances between *Chaerephon* and *Mops* species (ranging from 8.62% to 15.58%), which are in line with distances between molossid species (Lamb *et al*., 2011, Taylor *et al*. 2012), as well as the results of Ammerman *et al*. (2012), which show similar phylogenetic patterns to those found in this study. Results from this study highlight the need for more comprehensive taxonomic sampling in order to fully resolve the phylogenetic positions of these two genera.

4.2 The position of Otomops *within the phylogeny of the Afro-Malagasy Molossidae*

Sister species *Otomops martiensseni* and *O. madagascariensis*, appear as a well-supported (1.00 pp and 100% bootstrap support), discrete clade within the Molossidae with no clearly-defined associations with other genera included in this study.

Genetic distances separating *Otomops* from the other molossid genera range from 1.3% to 2.4% (RAG2) (Table 3.3). These distances are equivalent to genus-level separation seen in other genus-genus comparisons in this study, e.g. RAG2 distance of 2.4% between *Mops* and *Mormopterus*, and those indicated in Ammerman *et al*. (2012). Genetic distances within the *Otomops* genus (RAG2 =

0.1%) reveal low genetic diversity among haplotypes, especially with regards to RAG2 data. These relatively lower genetic distances among *Otomops* species are indicative of its status as a discrete genus that has been evolving separately for the past ~21 million years. *Otomops* species from the Afrotropical region appear geographically circumscribed due to the existence of barriers to dispersal (Chapter 2, Sections 4.1 and 4.2.2). These barriers have allowed for the subsequent speciation of these samples into their respective species.

Otomops appears to have diverged from the other molossids 21.31 MYA during the Miocene epoch. The Miocene was characterised by major long-term climatic cooling, interspersed with warmer periods reaching the middle Miocene Climatic Optimum (MCO) (Kürschner *et al*., 2008; Finarelli and Badgley, 2010). These climatic shifts may have resulted in contraction or expansion in the geographical range of organisms (Casanovas-Vilar *et al*., 2010; Finarelli and Badgley, 2010) and may have been associated with isolation of the first *Otomops* species, thought to have come from Southeast Asia (Lamb *et al*., 2008). Consistent with this, cytochrome *b* results of Lamb *et al*. (2011) show strong support for a moderate- to well-supported, monophyletic clade of Asian *Otomops*, *O. wroughtonii* and *O. formosus*, basal to the Afro-Malagasy species. Similar phylogenetic structure among Asian and Afro-Malagasy *Otomops* has been observed in the cytochrome *b* results of Chapter 2 (Section 3.3).

4.3 Genetic structure within Molossidae in relation to the structure hypothesized by Freeman (1981)

Freeman (1981) proposed that genera of the Molossidae are divided into two major groups, with the genera *Mormopterus* (including *Sauromys* and *Platymops*), *Myopterus*, *Cheiromeles* and *Molossops* (including *Cynomops* and *Neoplatymops*) in Group 1 and *Tadarida*, *Chaerephon*, *Mops*, *Otomops*, *Nyctinomops*, *Promops*, *Molossus* and *Eumops* in Group 2. Phylograms based on RAG2 and RAG2/cytochrome *b* data do not support these hypothesized groupings, as the Molossidae appear as one monophyletic group, including *Mormopterus*, *Otomops*, *Tadarida*, *Molossus*, *Nyctinomops*, *Sauromys*, *Chaerephon* and *Mops* (Figs. 3.3 and 3.4). Freeman (1981) placed *Sauromys* and *Mormopterus* in Group 1 whereas results from this study would suggest that these would fall under Freeman's proposed Group 2. Additionally, Freeman (1981) cited traits such as the flattening of the skull and associated morphological characteristics as a reason for designating *Sauromys* as a subgenus of *Mormopterus* (Peterson, 1965). Results from this study however place these two genera within a single Molossidae clade and as separate genera (Chapter 3, Section 4.1.3). Freeman (1981) acknowledged that the use of morphological data to distinguish between genera may not be ideally suited for elucidating phylogenetic patterns since characteristics such as palatial emargination may be

a result of functional morphology and convergence in morphological characteristics rather than a reflection of true phylogenetic separation.

4.4 Genetic structure within Molossidae in relation to the structure hypothesized by Legendre (1984)

Legendre (1984) hypothesized that members of Molossidae comprise three subfamilies: Molossinae (*Molossus*, *Eumops*, *Molossops*, *Cynomops*, *Neoplatymops*, *Myopterus* and *Promops)*, Tadarinae (*Tadarida* (including *Mops* and *Chaerephon* as subgenera), *Mormopterus*, *Nyctinomops*, *Otomops* and *Rhizomops*) and Cheiromelinae (*Cheiromeles*). The name *Rhizomops* was put forward by Legendre (1984) for *T. brasiliensis* however this classification has since been rejected (Owen *et al*. 1990). The present study includes only one representative of the proposed subfamily Molossinae (*Molossus molossus*), does not include *Cheiromeles*, and contains all taxa included in the proposed group Tadarinae.

The present results show *Mormopterus* as sister to a clade comprising *Tadarida*, *Nyctinomops*, *Otomops*, *Chaerephon*, *Mops*, *Sauromys* and *Molossus*. The presence of *Molossus* in this clade is contrary to expectation under the hypothesis of Legendre (1984), and fails to support the proposed composition of the Molossinae or Tadarinae.

Further *Chaerephon* and *Mops* form a monophyletic clade, which includes *Tadarida fulminans* in a basal position. They are not phylogenetically associated with *Tadarida brasiliensis* or *Tadarida aegyptiaca*, however. Thus the classification of *Chaerephon* and *Mops* as subgenera of *Tadarida* is not supported, both because *Tadarida* itself is not supported as a monophyletic genus, and because *Chaerephon* and *Mops* are distinct from two of the three members of *Tadarida* represented in this study.

Present results also fail to support the Legendre (1984) classification of *Sauromys* as a subgenus of *Mormopterus*, as *Mormopterus* is monophyletic and *Sauromys* is supported as basal to the *Mops/ Chaerephon* clade.

4.5 Genetic structure within Molossidae in relation to the structure hypothesized by Simmons (2005)

Findings by Simmons (2005) divided members of the Molossidae into two subfamilies: Molossinae (*Chaerephon*, *Cheiromeles*, *Cynomops*, *Eumops*, *Molossops*, *Molossus*, *Mops*, *Mormopterus*,

Myopterus, *Nyctinomops*, *Otomops*, *Platymops*, *Promops*, *Sauromys* and *Tadarida*) and Tomopeatinae (*Tomopeas*). Although the phylogenetic structure seen in Figs. 3.3 and 3.4 appears to follow the groupings proposed by Simmons (2005), this study has suggested that *Chaerephon* and *Mops* be classified as one genus, in contrast to the classification of Simmons (2005), in which *Chaerephon* and *Mops* are presented as separate genera. Apart from the above, the structure seen in the phylograms (Figs. 3.3 and 3.4) is consistent with that proposed by Simmons (2005), placing the genera used in this study into one phylogenetic group, as well as classifying *Sauromys* as a separate genus from *Mormopterus*. It must be noted that without the inclusion of additional genera, e.g. *Cheiromeles*, *Cynomops* and *Promops*, as well as a *Tomopeas* representative, the validity of these proposed subfamilies, Molossinae and Tomopeatinae, cannot be confirmed.

4.6 Genetic structure within Molossidae in relation to the structure hypothesized by Gregorin and Cirranello (2015)

Findings by Gregorin and Cirranello (2015) separate the molossid genera into two groups which equate to the Molossinae and Tomopeatinae. It is within the Molossinae that there is further subdivision into two clades: *Mormopterus*-like (*Mormopterus*, *Platymops*, *Sauromys*, *Neoplatymops*, *Molossops*, *Cynomops*, *Cheiromeles*, *Molossus* and *Promops*) and *Tadarida*-like (*Tadarida*, *Otomops*, *Nyctinomops*, *Eumops*, *Chaerephon* and *Mops*). The phylogenetic structure found in Figs. 3.3 and 3.4 shows some similarity to the clades suggested by Gregorin and Cirranello (2015) with the exception of the *Molossus molossus* and *Sauromys petrophilus* sample which is placed with member of the *Tadarida*-like clade using RAG2 data only. The *Mormopterus*-like clade appears to correspond to those genera maintaining a more basal position within the family with the more derived genera forming the *Tadarida*-like clade (Gregorin and Cirranello, 2015). Results based on combined RAG2 and cytochrome *b* data appear to support the hypothesis of two clades within the Molossinae however it should be noted that *Molossus* and *Sauromys* samples were not included in this analysis.

4.7 General conclusions and future research

Mormopterus appears as the most basal genus among the Molossidae used in this study. Both *Mormopterus* and *Otomops* appear as well-supported, monophyletic, discrete clades (Figs. 3.3 3.4), consistent with their status as distinct genera within the Molossidae. In addition, *Sauromys* also appears as a distinct genus. *Tadarida* does not exhibit monophyly and the status of this genus warrants further investigation with a more extensive taxonomic and genetic sample. The genus *Chaerephon* exhibits some paraphyly, with *C. jobimena* a member of a clade including *T. brasiliensis* and *T. aegyptiaca*, rather than that containing the other *Chaerephon* species, suggesting that *C.*

jobimena may belong to *Tadarida*, if *Tadarida*, which is not monophyletic, is indeed a valid genus. Other members of *Chaerephon* (excluding *C. jobimena*) occupy a derived position with respect to members of *Mops* in a strongly supported clade, suggesting that these may be a better referred to a single genus.

In order to gain a clearer understanding of the relationships within the Molossidae, it would be most beneficial to increase the number of samples taken from each of the genera studied. Use of additional molecular markers may also contribute to the current body of work and may help in elucidating those relationships and lineages which prove problematic at present, especially at the deeper nodes in the phylogeny. Use of appropriate additional markers may also allow, as was done in this study, for the concatenation of the data sets. In addition, an increased number of species from each of the genera, including both Old World and New World specimens would also allow for the future study to be more comprehensive and thus allow researchers to be able to make more confident assessments as to the true phylogeny of the family Molossidae on a global scale.

Findings of this study highlight the need for further taxonomic sampling, as well as the need for a variety of taxonomic methodologies and/or approaches comprising both morphological and molecular data. This will help provide a more comprehensive picture regarding the phylogenetic structure within the Molossidae.

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6. APPENDICES

Appendix 1. Haplotype pairwise genetic distances

Table 6.1. Pairwise genetic distances of RAG2 haplotypes against haplotypes 1 – 14*. Full haplotype details in Table 2.1.

	Haplotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	H1 (*C. atsinanana* etc.)														
2	H2 (*C. jobimena*)	0.020													
3	H3 (*C. pumilus*)	0.000	0.020												
4	H4 (*C. pusillus*)	0.000	0.020	0.000											
5	H5 (*C. pusillus*)	0.000	0.020	0.000	0.000										
6	H6 (*M. bakarii*)	0.008	0.023	0.008	0.008	0.008									
7	H7 (*M. condylurus*)	0.012	0.023	0.012	0.012	0.012	0.011								
8	H8 (*M. leucostigma*)	0.011	0.022	0.011	0.011	0.011	0.010	0.001							
9	H9 (*M. midas*)	0.010	0.019	0.010	0.010	0.010	0.011	0.011	0.010						
10	H10 (*M. francoismoutoui*)	0.026	0.020	0.026	0.026	0.026	0.029	0.029	0.028	0.025					
11	H11 (*M. jugularis*)	0.026	0.020	0.026	0.026	0.026	0.029	0.029	0.028	0.025	0.001				
12	H12 (*M. jugularis*)	0.027	0.021	0.027	0.027	0.027	0.030	0.030	0.029	0.026	0.001	0.002			
13	H13 (*O. madagascariensis*)	0.024	0.019	0.024	0.024	0.024	0.029	0.029	0.028	0.024	0.024	0.024	0.024		
14	H14 (*O. martiensseni*)	0.024	0.018	0.024	0.024	0.024	0.028	0.028	0.027	0.023	0.023	0.023	0.024	0.001	
15	H 20 (*T. aegyptiaca*)	0.022	0.009	0.022	0.022	0.022	0.024	0.025	0.024	0.021	0.022	0.022	0.023	0.021	0.020

Table 6.1 continued.

	Haplotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
16	H15 (*T. brasiliensis*)	0.017	0.008	0.017	0.017	0.017	0.020	0.020	0.019	0.016	0.018	0.018	0.019	0.016	0.015
17	H16 (*N. macrotis*)	0.021	0.015	0.021	0.021	0.021	0.024	0.022	0.021	0.018	0.018	0.018	0.019	0.020	0.019
18	H17 (*M. molossus*)	0.026	0.023	0.026	0.026	0.026	0.031	0.031	0.030	0.027	0.025	0.025	0.026	0.027	0.026
19	H19 (*N. stramineus*)	0.055	0.048	0.055	0.055	0.055	0.058	0.056	0.057	0.054	0.052	0.052	0.053	0.054	0.053
20	H18 (*M. daubentonii*)	0.057	0.052	0.057	0.057	0.057	0.060	0.060	0.059	0.056	0.050	0.050	0.051	0.058	0.057

Table 6.2. Pairwise genetic distances of RAG2 haplotypes against haplotypes 15 – 20*. Full haplotypes details in Table 2.1.

	Haplotypes	15	16	17	18	19
15	H20 (*T. aegyptiaca*)					
16	H15 (*T. brasiliensis*)	0.010				
17	H16 (*N. macrotis*)	0.015	0.013			
18	H17 (*M. molossus*)	0.024	0.020	0.016		
19	H19 (*N. stramineus*)	0.051	0.048	0.050	0.058	
20	H18 (*M. daubentonii*)	0.052	0.048	0.051	0.058	0.060

*Haplotypes 21 and 22 excluded from analysis due to missing data.

Table 6.3. Pairwise genetic distances of 3' end of RAG2 (634 nt) haplotypes against haplotypes 1 – 14. Full haplotype details in Table 2.1.

	Haplotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	H1 (*C. atsinanana* etc.)														
2	H2 (*C. jobimena*)	0.014													
3	H3 (*C. pumilus*)	0.000	0.014												
4	H4 (*C. pusillus*)	0.000	0.014	0.000											
5	H5 (*C. pusillus*)	0.000	0.014	0.000	0.000										
6	H6 (*M. bakarii*)	0.000	0.014	0.000	0.000	0.000									
7	H7 (*M. condylurus*)	0.008	0.018	0.008	0.008	0.008	0.008								
8	H8 (*M. leucostigma*)	0.006	0.016	0.006	0.006	0.006	0.006	0.002							
9	H9 (*M. midas*)	0.012	0.018	0.012	0.012	0.012	0.012	0.012	0.010						
10	H10 (*M. francoismoutoui*)	0.020	0.014	0.020	0.020	0.020	0.020	0.024	0.022	0.024					
11	H11 (*M. jugularis*)	0.020	0.014	0.020	0.020	0.020	0.020	0.024	0.022	0.024	0.002				
12	H12 (*M. jugularis*)	0.020	0.014	0.020	0.020	0.020	0.020	0.024	0.022	0.024	0.000	0.002			
13	H13 (*O. madagascariensis*)	0.026	0.020	0.026	0.026	0.026	0.026	0.030	0.028	0.026	0.022	0.022	0.022		
14	H14 (*O. martiensseni*)	0.024	0.018	0.024	0.024	0.024	0.024	0.028	0.026	0.024	0.020	0.020	0.020	0.001	
15	H 15 (*T. brasiliensis*)	0.016	0.010	0.016	0.016	0.016	0.016	0.020	0.018	0.020	0.016	0.016	0.016	0.022	0.020
16	H17 (*M. molossus*)	0.028	0.022	0.028	0.028	0.028	0.028	0.032	0.030	0.032	0.020	0.020	0.020	0.030	0.028
17	H16 (*N. macrotis*)	0.018	0.012	0.018	0.018	0.018	0.018	0.018	0.016	0.018	0.010	0.010	0.010	0.020	0.018

Table 6.3 continued.

	Haplotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
18	H21 (*S. petrophilus*)	0.014	0.012	0.014	0.014	0.014	0.014	0.022	0.020	0.022	0.018	0.018	0.018	0.020	0.021
19	H20 (*T. aegyptiaca*)	0.014	0.004	0.014	0.014	0.014	0.014	0.018	0.016	0.018	0.014	0.014	0.014	0.020	0.018
20	H22 (*T. fulminans*)	0.008	0.010	0.008	0.008	0.008	0.008	0.016	0.014	0.016	0.016	0.016	0.016	0.022	0.020
21	H19 (*N. stramineus*)	0.051	0.041	0.051	0.051	0.051	0.051	0.051	0.053	0.055	0.047	0.047	0.047	0.053	0.051
22	H18 (*M. daubentoni*)	0.060	0.053	0.060	0.060	0.060	0.060	0.064	0.062	0.064	0.055	0.055	0.055	0.062	0.060

Table 6.4. Pairwise genetic distances of 3' end of RAG2 (634 nt) haplotypes against haplotypes 15 – 22. Full haplotype details in Table 2.1.

	Haplotypes	15	16	17	18	19	20	21
15	H 15 (*T. brasiliensis*)							
16	H17 (*M. molossus*)	0.024						
17	H16 (*N. macrotis*)	0.014	0.018					
18	H21 (*S. petrophilus*)	0.014	0.026	0.016				
19	H20 (*T. aegyptiaca*)	0.010	0.022	0.012	0.012			
20	H22 (*T. fulminans*)	0.012	0.024	0.014	0.010	0.010		
21	H19 (*N. stramineus*)	0.047	0.055	0.045	0.049	0.041	0.047	
22	H18 (*M. daubentoni*)	0.051	0.062	0.053	0.058	0.053	0.056	0.062

CHAPTER FOUR:

Cross-genus Amplification and Characterisation of Microsatellite Loci in the Large-Eared Free Tailed Bat, *Otomops* (Chiroptera: Molossidae) from Africa and Madagascar

academicJournals

Vol. 12(27), pp. 4233-4237, 3 July, 2013
DOI: 10.5897/AJB2013.12239
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Cross-genus amplification and characterisation of microsatellite loci in the large-eared free tailed bat, *Otomops* (Chiroptera: Molossidae) from Africa and Madagascar

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Accepted 1 July, 2013

Primers developed for the Brazilian free-tailed bat, *Tadarida brasiliensis*, were successfully used to cross-amplify microsatellite loci in two Afro-tropical *Otomops* species. Seventy one (71) bats from two species were genotyped for two dinucleotide and four tetranucleotide loci, yielding 1 to 15 alleles per locus. For the combined sample, the observed and expected heterozygosities ranged from 0.125 to 1.000 and 0.125 to 0.919, respectively. The polymorphism information content (PIC) values were 0.295 to 0.905 (mean 0.687) for *Otomops martiensseni* and 0.110 to 0.797 (mean 0.442) for *Otomops madagascariensis*. Five *O. martiensseni* loci deviated significantly from Hardy-Weinberg equilibrium. These six loci provide genetic markers that will be useful in investigating the population genetic structure of Afro-Arabian *O. martiensseni* and Malagasy *O. madagascariensis*, with potential application to Asian species of *Otomops* and possibly other genera within the Molossidae.

Key words: Bats, *Otomops*, Chiroptera, microsatellites, Molossidae, cross-genus amplification.

INTRODUCTION

Microsatellites or short tandem repeats are popular markers in population genetic studies as they show high levels of polymorphism and are useful for estimating parameters such as gene flow, inbreeding, migration rates, population size and kinship (Selkoe and Toonen, 2006; Barker, 2002; Zane et al., 2002. As development of new microsatellite markers is relatively expensive and time-consuming (Zane et al., 2002; Abdelkrim et al., 2009), cross-amplification of microsatellites using primers developed for another species is considered a cost-effective and viable option (Barbará et al., 2007). Cross-species amplification of microsatellites has been utilised in various taxa, including plants (Datta et al., 2010; Eliott et al., 2013), insects (Chen and Dorn, 2010), fish (Dubut et al., 2010) and mammals (Kaňuch et al., 2007; Kretschmer et al., 2009; Sanvito et al., 2013). This method does, however, have limitations since the primers work best for the species for which they were developed. Loci are less likely to amplify successfully as the genetic distance between the original and target species increases, and those which amplify usually exhibit lower levels of polymorphism than in the original species (Primmer et al., 2005). Projects based on cross-species amplification should therefore be preceded by a preliminary study which assesses the ability of candidate primers to amplify suitably variable microsatellites in the target species (Schlötterer, 2000; Scribner and Pearce, 2000).

The Molossidae are one of the less studied families

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Table 1. Primer sequences from Russell et al. (2005) used in the cross-amplification of microsatellites in *O. martiensseni* and *O. madagascariensis*.

Locus	Primer sequences (5' – 3')
TabrA10	F: AAG TGG TTG GGC GTT GTC
	R: GCG ATG CAC TGC CTT GAG A ϖ
TabrA30	F: AGT CGC GGG TTT GAT TCC AGT TA
	R: ACC CCT TCC CTT TGT TCC TTC AG ω
TabrD10	F: CCC CAC TCA TTT ATC CAT CCA CA ϖ
	R: ATC TCG CAG CTA TTG AAG TA
TabrD15	F: AGT CCT GGC TCC TAT TCT CAT TG
	R: CTA TCC GTC TAC CTG TCC GTC TAT ω
TabrH6	F: ATC TCT CCA GTC CTT ACC A ϖ
	R: TTT ACC CTC CAC AGT CTC A
TabrH12	F: CCA TGT GAG CCA ATT CCT A ω
	R: GTC AGG ACT CTC CAG AGA

F = forward primer; R = reverse primer. Primer labels are indicated: ϖ6-FAM, ωNED.

within the Chiroptera, and phylogenetic and population genetic studies on this family have been based primarily on mitochondrial and nuclear sequence data (for example, Lamb et al., 2011; Ammerman et al., 2012). However, Russell et al. (2005) used microsatellites to study the population genetics of the American species, *Tadarida brasiliensis*, and recently Naidoo et al. (2013) reported on the utility of the primers of Russell et al. (2005) to cross-amplify polymorphic microsatellites in the molossid species, *Chaerephon pumilus* sensu lato from south eastern Africa.

Afro-tropical members of the Old World genus *Otomops*, *Otomops martiensseni* from Africa (including the Arabian Peninsula) and *Otomops madagascariensis* from Madagascar, have a wide but somewhat sparse distribution throughout the region (Peterson et al., 1995; Simmons, 2005; Lamb et al., 2008). According to the 2008 IUCN (The World Conservation Union) Red List of Threatened Species, *O. martiensseni* has been classified globally as having a "Near Threatened" status (Mickleburgh et al., 2008). Although species-level phylogenetic and phylogeographic investigations of *Otomops* have been undertaken (Lamb et al., 2006, 2008), fine-scale genetic investigations within the genus have been limited, leaving many unanswered questions including the number of species and taxonomic status of Afro-tropical individuals.

Our aim was to test the ability of primers developed to amplify hypervariable nuclear microsatellites in the American genus *T. brasiliensis* (Molossidae) (Russell et al., 2005) to cross-amplify and reveal polymorphism in two Afro-Malagasy species of the molossid genus *Otomops*, namely *O. martiensseni* and *O. madagascariensis*. If successful, these primers may also be useful for population genetics studies on Asian species of *Otomops*, and possibly other genera within this pan-tropical bat family. Consequent studies on gene-tic variation, gene flow and kinship in *Otomops* may prove useful in the amendment of current legislations used for the protection and conservation of this genus.

MATERIALS AND METHODS

A total of 71 individuals from two *Otomops* species, *O. martiensseni* (n=63) and *O. madagascariensis* (n=8), were tested for successful genotyping of the loci TabrA10, TabrA30, TabrD10, TabrD15, TabrE9, TabrH2, TabrH3, TabrH6 and TabrH12 (Russell et al., 2005). Genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (QIAGEN Inc.). The optimised polymerase chain reaction (PCR) amplifications were performed in 25 µl reactions containing 30 to 60 ng template DNA, 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Fermentas), 0.2 µl 5 U/µl *Taq* polymerase (Super-Therm) and 4 µl of 6 µM primer dilution (forward and reverse) per reaction (primer sequences in Table 1). The thermal cycling parameters used were as follows: 95°C for 1 min; followed by 39 cycles of (95°C for 30 s, primer-specific annealing temperature for 30 s and 72°C for 2 min); followed by 72°C for 10 min. Annealing temperatures for each primer pair were optimised using gradient PCR (Table 2). Genotyping was performed on an ABI 3500 Genetic Analyzer (Applied Biosystems) at the South African Sugar Research Institute, Mount Edgecombe, South Africa. Each reaction comprised 1 µl of prediluted PCR product, labelled with dyes 5' 6-FAM or 5' NED (Applied Biosystems), 0.5 µl of LIZ® 600 Size Standard (Applied Biosystems) and 8.5 µl of Hi-Di™ Formamide (Applied Biosystems).

Raw allelic data were analysed and called using STRand v.2.4.59 (Toonen and Hughes, 2001; Hughes, 2006) and 1000 randomizations were performed in Micro-Checker v. 2.2.3 (Van Oosterhout et al., 2004) to check the *O. martiensseni* and *O. madagascariensis* data separately for null alleles, stuttering and large allele dropout. Additionally, FreeNA software (Chapuis and Estoup, 2007) was used to determine whether null alleles detected in the data were introducing bias in the analyses, where pairwise F_{ST} values were calculated between *O. martiensseni* and *O. madagascariensis* with and without the excluding null alleles (ENA) method applied. The ENA method corrects for the presence of null alleles. GenAlEx 6.5b4 (Peakall and Smouse, 2006, 2012) was used to calculate the number of alleles and the observed (H_O) and expected (H_E) heterozygosities. Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010) was used to determine deviation from Hardy-Weinberg equilibrium (HWE) and Cervus v.3.0 (Kalinowski et al., 2007) was used to calculate polymorphism information content (PIC) values.

RESULTS AND DISCUSSION

Six of nine *T. brasiliensis* primer pairs successfully cross-amplified microsatellites in *O. martiensseni* and *O. madagascariensis*, namely TabrA10, TabrA30, TabrD10, TabrD15, TabrH6 and TabrH12. The remaining loci were not useable due to the presence of null alleles (TabrH3) or ambiguity in the peak data (TabrH2 and TabrE9) which rendered us unable to score these loci with confidence. Russell et al. (2005) also reported difficulty in amplifying TabrH2 across all Tadarida populations tested. Naidoo et al. (2013), who successfully cross-amplified six of the above loci in C. pumilus, were successful with TabrA10, TabrA30, TabrD10, TabrD15, TabrH6 and TabrE9. Repeat motifs of all loci were the same in Otomops and

Table 2. Characteristics of six microsatellite loci cross-amplified in *Otomops martiensseni*, *O. madagascariensis* and *Chaerephon pumilus* sensu lato using primers developed for *Tadarida brasiliensis* (Russell et al., 2005).

Locus	Specie	Genbank accession number	Repeat motif	T_a (°C)	Allele size range	Number of alleles	H_O	H_E	PIC
TabrA10	*O. martiensseni*	KC701453	GA	65	230 – 290	5	0.286	0.668*	0.610
	O. madagascariensis	KF112058	GA	65	270 – 280	2	0.500	0.400	0.305
	C. pumilus	KC896691	TAGA/TGGA	60	178 – 254	9	0.51	0.69	0.69
	T. brasiliensis	AY954900	GA	65	226 – 268	20	0.756	0.934*	-
TabrA30	*O. martiensseni*	KC701454	GA	65	282 – 314	9	0.143	0.303*	0.295
	O. madagascariensis	KF112059	GA	65	284 – 300	3	0.125	0.342	0.294
	C. pumilus	KC896690	GA	65	240 – 296	9	0.78	0.64	0.61
	T. brasiliensis	AY954901	GA	57	193 – 281	27	0.326	0.333	-
TabrD10	*O. martiensseni*	KC701455	GATA	60	330 – 374	13	0.545	0.866*	0.845
	O. madagascariensis	KF112060	GATA	60	340 – 348	2	0.125	0.125	0.110
	C. pumilus	KC896693	GATA	60	331 – 379	13	0.81	0.81	0.80
	T. brasiliensis	AY954902	GATA	50	308 – 376	15	0.773	0.818	-
TabrD15	*O. martiensseni*	KC701456	GATA	60	350 – 414	16	0.857	0.919	0.905
	O. madagascariensis	KF112061	GATA	60	350 – 382	7	1.000	0.875	0.797
	C. pumilus	KC896692	GATA	60	148 – 284	10	0.06	0.54	0.51
	T. brasiliensis	AY954903	GATA	58	235 – 395	29	0.872	0.945*	-
TabrH6	*O. martiensseni*	KC701457	TAGA	60	210 – 258	13	0.683	0.905*	0.889
	O. madagascariensis	KF112062	TAGA	60	256 – 284	6	0.750	0.783	0.702
	C. pumilus	KC896695	TAGA	60	139 – 318	14	0.46	0.64	0.61
	T. brasiliensis	AY954907	TAGA	55	187 – 357	47	0.936	0.952	-
TabrH12	*O. martiensseni*	KC701458	TAGA	60	330 – 374	6	0.492	0.640*	0.577
	O. madagascariensis	KF112063	TAGA	60	260 – 260	1	-	-	-
	T. brasiliensis	AY954908	TAGA	57	126 – 366	54	0.846	0.959	-
TabrE9	*C. pumilus*	KC896694	GA	60	329 – 365	15	0.84	0.80	0.79
	T. brasiliensis	AY954904	GA	52	349 – 461	52	0.814	0.952*	-

T_a, Optimised annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphism information content. Significant deviations from Hardy-Weinberg equilibrium for each locus are indicated (*, $P < 0.05$ after sequential Bonferroni correction). Data for *Chaerephon pumilus* sensu lato (Naidoo et al., 2013) and the Argentinean population of *Tadarida brasiliensis* (Russell et al., 2005) are included for comparative purposes.

T. brasiliensis (Russell et al., 2005), whereas *C. pumilus* showed a different repeat motif for marker TabrA10 (Naidoo et al., 2013) (Table 2). Analysis of *O. martiensseni* data in micro-checker detected possible scoring error due to stuttering in 2 loci (TabrA10 and TabrH12) and the presence null alleles in 5 of the 6 loci tested (TabrA10, TabrA30, TabrD10, TabrH6 and TabrH12). *O. madagascariensis* data showed no null alleles or scoring error due to stuttering. None of the loci from either species showed any large allele dropout. To determine whether any substantial bias was introduced through the presence of the null alleles, pairwise F_{ST} values were calculated between *O. martiensseni* and *O. madagascariensis* with (0.248) and without (0.249) the ENA algorithm. As the difference between the corrected and uncorrected estimates of genetic differentiation was not substantial, we report analyses performed on uncorrected data only. Null alleles, stuttering and large allele dropout were not reported for microsatellites cross-amplified in *C. pumilus* (Naidoo et al., 2013).

All of the *O. martiensseni* loci were polymorphic, with 5 to 16 (mean 10.33) alleles per locus. *O. madagascariensis*, however, showed lower levels of polymorphism; five of six loci were polymorphic, with polymorphism levels ranging from 1 to 7 (mean 3.5) alleles per locus. The lower level of polymorphism in *O. madagascariensis* is likely a reflection of the smaller sample size used for this species. Polymorphism levels in cross-amplified *C. pumilus* sensu lato microsatellites [9 to 15 (mean 11.7) alleles per locus] (Naidoo et al., 2013) were slightly higher than, but comparable to those of *O. martiensseni*. This is somewhat unexpected, as the higher divergence (RAG2 genetic distance) between *T. brasiliensis* and *C. pumilus* s.l. (4.6%) than between *T. brasiliensis* and *O. martiensseni* (3.2%) (Lamb et al., 2011) leads to an expectation of lower polymorphism in *C. pumilus* s.l. (Primmer et al., 2005).

Polymorphism levels in all cross-amplified microsatellites were considerably lower than those in *T. brasiliensis*, the species for which the primers were developed [15 to 54 (mean 36.67) alleles per locus]. This is to be expected as number of amplified loci and the level of polymorphism tends to decrease with increasing genetic distance between the original and cross-amplified taxa (Primmer et al., 2005), and we are dealing here with cross-genus rather than cross-species amplification. The lower levels of polymorphism in *Otomops* and *Chaerephon* species are likely to reflect divergence which has occurred since *Tadarida* and *Otomops* (24.7 MYA) and *Tadarida* and *Chaerephon* (26.1 MYA) last shared common ancestors (Ammerman et al., 2012).

There was considerable variability in observed (H_O) and expected (H_E) heterozygosities across *Otomops* samples (Table 2). Consistent with expectation, the expected fractions of polymorphic offspring, as indicted by PIC values, are a little lower than the expected heterozygosities. The PIC of the *O. martiensseni* microsatellites ranged from 0.295 to 0.905 (mean 0.687), comparable to that of the similarly-sized sample of cross-amplified *C. pumilus* s.l. microsatellites, 0.51 to 0.80 (mean 0.67) (Naidoo et al., 2013). The PIC of the *O. madagascariensis* samples was generally lower (0.110 to 0.797 (mean 0.442), possibly due to the smaller sample size. PIC values showed some markers to be more informative than others, for example, TabrD15 was the most informative, with values of 0.905 and 0.797 in *O. martiensseni* and *O. madagascariensis*, respectively.

Markers with PIC values > 0.4 are considered moderately informative and those with values > 0.7 are considered highly informative (Hildebrand et al., 1992; Xu, 2010). Thus most of the loci tested in *O. martiensseni* can be deemed informative for linkage analysis, as three markers had PIC values >0.7 and two had PIC values >0.4 (Table 2). Only two markers in *O. madagascariensis* can be considered highly informative, that is, TabrD15 and TabrH6, but this may be due to the low sample number used. By comparison, all markers tested in *C. pumilus* appear to be informative, with TabrD10 having the highest PIC value (0.80) (Naidoo et al., 2013).

Analyses revealed the existence of significant linkage disequilibrium among 7 pairs of loci in *O. martiensseni* (TabrA10 and TabrA30, TabrD10 TabrD15 and TabrH6; TabrD10 and TabrA30 and TabrH12; and TabrD15 and TabrH12) and 1 pair in *O. madagascariensis* (TabrA30 and TabrD15) after standard Bonferroni correction ($P < 0.001$). All *O. martiensseni* loci except TabrD15 showed a significant deviation from HWE; the possible presence of population stratification, migration, mutation, natural selection or assortative mating within this species (Wigginton et al., 2005) needs further investigation. Three *T. brasiliensis* loci (TabrA10, TabrD15 and TabrH12) exhibited deviation from HWE (Table 2); in contrast, no *O. madagascariensis* loci and one *C. pumilus* locus (Naidoo et al., 2013) deviated significantly from HWE.

In summary, six of nine microsatellite markers reported for *T. brasiliensis* (Russell et al., 2005) have been successfully cross-amplified in two species of the molossid genus *Otomops*. These nuclear markers do not have as high a level of polymorphism as in the originally-studied species, *T. brasiliensis*, but PIC values indicate that they are sufficiently polymorphic for use in population-, colony- and individual-level genetic studies. This will allow for future work on intra- and inter-colony relationships in *Otomops*. Additionally, these markers may also be useful in population genetic studies on the other *Otomops* species, such as *O. wroughtonii* from southern India and *O. formosus* from Java. Comparison of marker statistics in cross-amplified *Otomops* and *Chaerephon* microsatellites (Naidoo et al., 2013) revealed some similarities, for example, relatively lower allele numbers and PIC values, which may be attributed to mutations which have occurred in these lineages in the 24.7 to 26.1 million years since they last shared a common ancestor with *T. brasiliensis*. The markers developed by Russell et al. (2005) have been successfully cross-amplified in two other molossid genera, *Otomops* and *Chaerephon*, and may therefore have the

potential to be used for population genetic studies of not only *Otomops*, but also other poorly-studied molossid genera in the future.

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CHAPTER FIVE:

Examination of the Population Genetics of Afro-Malagasy *Otomops* (Chiroptera: Molossidae) via Microsatellite Analysis

ABSTRACT

We examined the population genetics of 10 colony-based groupings of Afro-Malagasy *Otomops*: *O. martiensseni* from eastern South Africa (6 colonies); *O. harrisoni* from northeast Africa (3 colonies); and *O. madagascariensis* from Madagascar (1 colony). Our aim was to compare genetic structure based on 6 nuclear microsatellites with that based on mitochondrial cytochrome *b* and D-loop sequence data, at both species and colony level. Further, we aimed to shed light on social structure in *Otomops* by analysing gene flow, migration, relatedness and kinship among and within colonies. Three major lineages were identified in analyses of nuclear and mitochondrial datasets, separated by significant ($p < 0.01$) pairwise F_{ST} values, consistent with *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. Pairwise F_{ST} and mean relatedness values between colonies from the same species lineage were lower than those between lineages. 70% of individuals sampled were part of either parent/offspring, full-sibling or half-sibling relationships within geographically-based species level lineages, whereas no kinship was observed across lineages. Two parent/offspring dyads were identified within colonies belonging to the *O. martiensseni* lineage and one within the *O. harrisoni* lineage, whereas no parent/offspring dyads were established between colonies. Full-sibling and half-sibling pairs were observed both within and between colonies within their respective lineages. However, most kinship within lineages took the form of half-sibling relationships, reinforcing the suggestion that *Otomops* engages in extra-colony mating. Our results suggest that individuals do not exhibit strict colony faithfulness, and that gene flow is maintained through extra-colony mating. We find little evidence to support the presence of a social system based on either female or male philopatry in Afro-Malagasy *Otomops*.

1. INTRODUCTION

There are currently three recognised species of Afro-Malagasy *Otomops* (family: Molossidae; suborder Vespertilioniformes); the near-threatened *O. martiensseni* (Matschie, 1897) from southeast and west Africa (Mickleburgh *et al*., 2008), *O. madagascariensis* Dorst, 1953 from Madagascar (Peterson *et al*., 1995; Simmons, 2005; Lamb *et al*., 2008), and the newly-described *O. harrisoni* from northeast Africa and the Arabian Peninsula (including Djibouti, Eritrea, Ethiopia, Kenya and Yemen) (Ralph *et al*., 2015). Support for this species circumscription has been based on molecular (mitochondrial and nuclear sequences and nuclear microsatellites) and craniometric data. Relatively little has been published on their biology, behavioural ecology and social structure, save for some studies based on observation and echolocation data (Mutere, 1973; Richardson and Taylor, 1995; Fenton *et al*., 2002; Kock and Zinner, 2004; Kock *et al*., 2005; Taylor, 2005; Taylor *et al*., 2005). Our aim here is to use microsatellite data to estimate levels of nuclear structure at a variety of scales, from species to colony level, and compare these with estimates based on mitochondrial sequence data. We further aim to use our nuclear data to estimate gene flow, relatedness and degree of kinship among and within colonies, and migration between colonies in order to shed light on the social structure of *Otomops*.

Population genetics is an important adjunct in investigations of animal biology as it allows investigators to gain insight into mating systems, gene flow, dispersal patterns, roosting biology and/or sex-biased dispersal without the use of direct observation methods (Selkoe and Toonen, 2006; Bryja *et al*., 2009). These methods can also be used to provide insight into social structure or mating systems such as harem breeding structures and philopatry. Harem structures are defined as single male: multi-female roosting groups where the resident male may have increased mating success with females from that roost; this has been observed in a number of bat species, including *Phyllostomus hastatus*, *Noctilio leporinus*, *Myotis bocagei* and *Chaerephon pumila* (McCracken and Wilkinson, 2000 and references therein). Population genetic methods are particularly useful in studies of the social behaviour of bats, since their roosting habits and nocturnal nature can make them difficult to observe directly (Burland *et al*., 2001; Bryja *et al*., 2009).

Roosting habitats and the number of individuals per roost appear to differ from region to region. *Otomops harrisoni* individuals from northeast Africa and the Arabian Peninsula tend to roost in dark and poorly-ventilated caves, where they occur in tightly-packed colonies of several hundred (Mutere, 1973; Richardson and Taylor, 1995; Kock *et al*., 2005). In contrast, *O. martiensseni* from the Durban metropolitan area of South Africa inhabit the roofs of homes and buildings whilst making use of a number of day and night roost sites (Richardson and Taylor, 1995; Taylor, 1999, 2005; Hutson *et al*.,

2001; Fenton *et al*., 2002, 2004). Fenton *et al*. (2002) showed that roosts *O. martiensseni* in Durban are comparatively smaller, with a mean colony size of 11.2 from 24 roosts sampled. The Malagasy species, *O. madagascariensis*, is generally associated with the drier, western parts of the island and can be found in eroded limestone and sandstone outcrops of this area, as well as sea caves and the hollows of trees (Peterson *et al*., 1995; Goodman *et al*., 2005; Andriafidison *et al*., 2007; Goodman and Ramasindrazana, 2014). Kofoky *et al*. (2007) reported the presence of *O. madagascariensis* in circular, vertical erosions in the ceiling of the Anjohikinakina Cave with numbers of individuals ranging from 88 (in July) to 114 (in October).

Male: female ratios in colonies of the three species of *Otomops* range from 1:1 to 1:4 in eastern African colonies, 1:2 to 1:11 in South African (Durban) colonies and 1:0 to 1:11 in Malagasy colonies (Mutere, 1973; Fenton *et al*., 2002; Andriafidison *et al*., 2007). Andriafidison *et al*. (2007) suggests that roosting habits of *O. madagascariensis* may be influenced by the relative size and suitability of each roost as well as the time of year, thus ratios may change during the mating season and after the birth of young. *Otomops* spp. females usually give birth to a single young during the austral spring/summer months (October to December) after a gestation period of approximately three months (Fenton *et al*., 2002; Taylor, 2005; Andriafidison *et al*., 2007). Sex ratios and colony size can also be influenced by the eviction of sub-adult or sub-dominant males once maturity is reached in the latter (Richardson and Taylor, 1995; Fenton *et al*., 2002, 2004; Heckel and von Helversen, 2003; Taylor, 2005). Taking these male: female ratios into account, it has been suggested that a harem social structure exists within colonies of *O. martiensseni*, most prominently displayed in the Durban area of South Africa (Fenton *et al*., 2002) and that this may promote female philopatry. However, phylogenetic studies based on mitochrondrial DNA have not supported this in any of the Afro-Malagasy *Otomops* spp., as individuals exhibited high haplotpe diversity, and haplotypes did not group according to colony, as might have been expected in a system based on female philopatry, where mothers and offspring would be expected to share mitochondrial haplotypes (Lamb *et al*., 2008). This suggests that *Otomops* spp. females do not exhibit strict colony faithfulness and do not group to form genetically stable harems. In addition to extra-colony mating, the dispersal capability of certain bats can contribute to the lack of genetic uniformity among colonies (Burland and Worthington Wilmer, 2001; Miller-Butterworth *et al*., 2003). Members of the genus have a high dispersal capability, possessing the ability for powerful flight and thus enabling individuals to cover long distances (Kingdon, 1974; Freeman, 1981).

In this study, we investigate genetic variation within and between members of *Otomops* colonies using nuclear microsatellite data. This approach has been used in a number of similar studies on Chiroptera: for example, Campbell *et al*. (2009) investigated genetic variation, relatedness and gene flow in *Myotis*; Chen *et al*. (2010) made inferences regarding the social organization and roosting ecology of

Rousettus leschenaulti and *Cynopterus sphinx*; Dixon (2011) studied population structure and natal philopatry in *Myotis lucifugus*; and Hua *et al*. (2011) investigated kin selection and paternity of *Tylonycteris pachypus*.

Our overall aim was to provide a nuclear microsatellite-based perspective on the extent and nature of genetic variation within and between *Otomops* spp. level lineages, colonies, and individuals, and to compare this with analyses of mitochondrial sequence data for the same sample set.

Our first objective was to determine whether our nuclear (microsatellite) and equivalent mitochondrial (concatenated cytochrome *b* and D-loop sequence) data support the existence of the following three species level lineages, currently defined based on morphological and primarily mitochondrial DNA sequence data; *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. Based on previous published analyses (Lamb *et al*., 2006, 2008), we would expect the mitochondrial dataset to be structured into the three major species groups, and hypothesise that the nuclear data will reflect this high-level structure.

Our second objective was to determine whether the nuclear microsatellite data reveal population subdivision within each of the regional species lineages, and if so, whether different colonies are genetically distinct from each other. As previous mitochondrial analyses have revealed little structure within species level lineages (Lamb *et al*., 2008; Ralph *et al*., 2015), we expect our mitochondrial dataset to reflect this. Low levels of mitochondrial genetic structure suggest the absence of a social system based on female philopatry and we expect to find a similar pattern based on nuclear microsatellite data. Low levels of nuclear genetic structure would support this and also indicate that male philopatry is unlikely. Because of the proposed harem structure within these colonies, characterised by the presence of a dominant male, we hypothesise that we may find evidence of male philopatry. Higher levels of nuclear genetic structure in the presence of little mitochondrial structure would indicate the possibility of male philopatry, although this would need to be confirmed by other analyses.

The third objective of this study was to determine levels of gene flow among and within colonies, and migration between colonies. Given the high genetic diversity and low levels of structure within species lineages observed in mtDNA studies, the use of day roosts, and the strong dispersal ability of *Otomops*, we hypothesize that we will observe migration and gene flow between colonies within a species, but not between species level lineages.

The fourth objective was to search for relationships between individuals among and within colonies at the following kinship levels; parent/offspring, full-sibling and half-sibling. High genetic variability

and low levels of within-species structure observed using mtDNA suggests that kin relationships between colonies will be observed.

2. MATERIALS & METHODS

2.1 Sampling

The sample comprised 71 individuals (48 female, 19 male, 4 sex unknown) from 10 colonies/localities from Madagascar, Ethiopia, Kenya, Yemen and South Africa (Fig. 2, Table 8.1). Tissue specimens were obtained from material deposited in museum collections or through live capture using mist nets or, where possible, direct capture within the roost. Direct capture allowed us to ensure that each bat colony was sampled once only. We cannot claim to have sampled all members of each colony-based grouping, as some members may not have been present in roosts at the time of sampling. When bats were released after capture, wing punches were taken using a 3 mm biopsy punch. All samples were preserved in 90% ethanol. The Ethiopian colony was an exception since samples from this colony comprised only pregnant females and their unborn offspring (no males were recorded or sampled at this locality). As collection of South African samples took place before the birth season (October to December), it was not possible to assign age-classes with certainty because definitive sampling was not carried out. However, based on visual observation, it was apparent that no juveniles were captured. Wing punches of individuals from Durban, South Africa, were collected under Ezemvelo KZN Wildlife and Threatened or Protected Species 'ToPS' permit numbers OP 853/2009 and OP 360/2013.

2.2 ncDNA isolation, microsatellite PCR and genotyping

DNA was isolated from samples using a QIAGEN DNeasy® Blood and Tissue Kit according to manufacturer's instructions. Nine primer pairs, originally developed for *Tadarida brasiliensis*, were selected and tested for microsatellite amplification (Russell *et al*., 2005). Of these, six which amplified consistently and displayed polymorphisms were chosen for use in this study (Ralph and Lamb, 2013). Optimal annealing temperatures, amplification reagents, primers and thermal cycling parameters for each individual locus are presented in in Ralph and Lamb (2013). Fluorescently labelled microsatellite PCR products were genotyped in 10 µl reactions containing 1 µl of the already-diluted PCR product, 0.5 µl of GeneScan™ 600 LIZ® Size Standard (Applied Biosystems) and 8.5 µl of Hi-Di™ Formamide (Applied Biosystems). Fragments were separated on an ABI3500 Genetic Analyser. Each sample animal was genotyped individually to avoid complications incurred by multiplex reactions, e.g. primer competition. All sequencing and fragment analysis reactions were carried out in the facilities of the South African Sugar Research Institute (SASRI) Biotechnology Unit, Mount Edgecombe, KwaZulu-Natal, South Africa. Alleles were viewed and scored using STRand v 2.4.59 (Toonen and Hughes, 2001; Hughes, 2006).

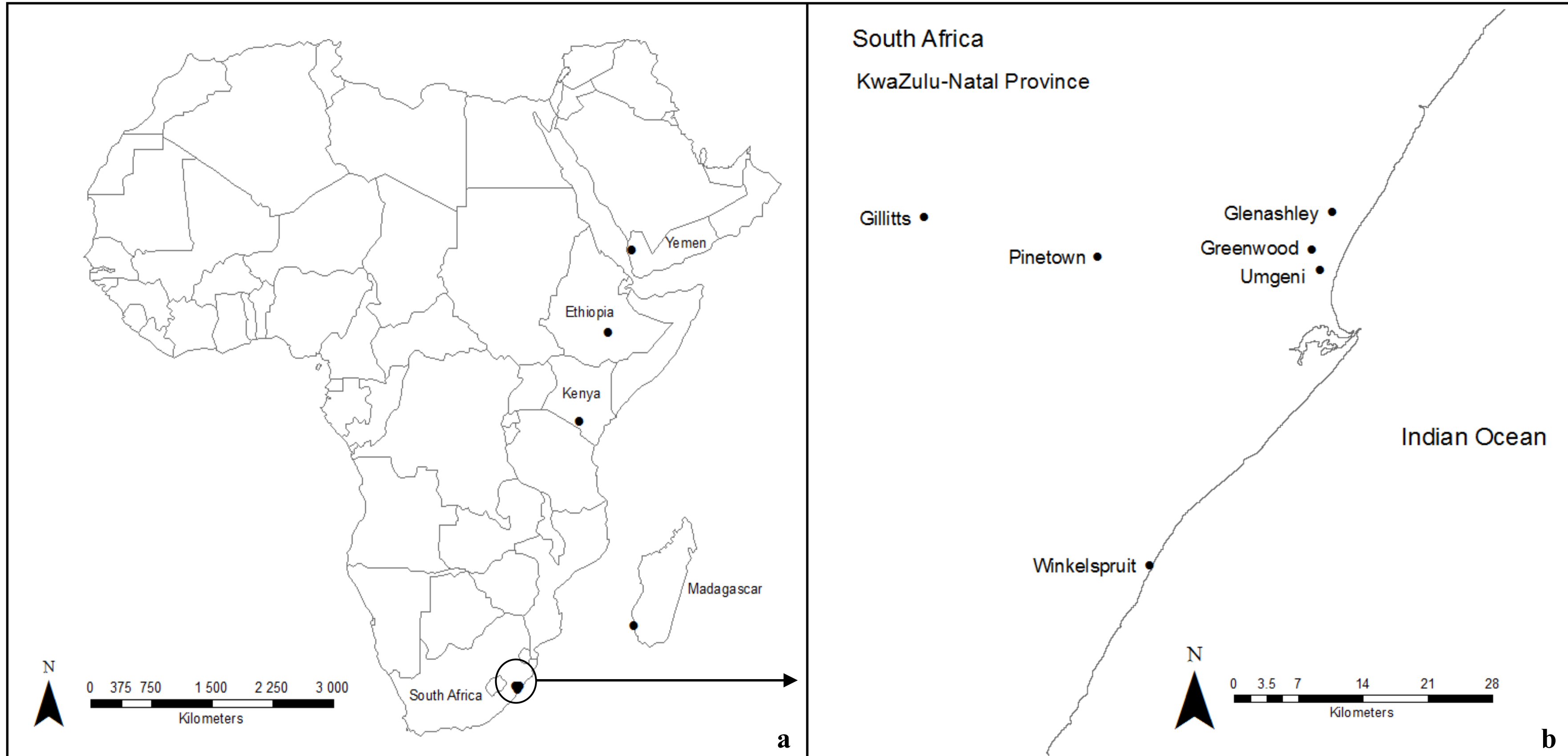

Figure 2. Sampling localities of colonies of the Afro-Malagasy *Otomops* spp. Figure 2a shows the sampling localities of *O. martiensseni* (southeastern Africa), *O. harrisoni* (northeastern Africa and the Arabian Peninsula) and *O. madagascariensis* (Madagascar). Figure 2b details the sampling localities of *O. martiensseni* colonies from the KwaZulu-Natal Province of South Africa.

2.3 Microsatellite analyses

Data was checked for null alleles, stuttering and large allele dropout by performing 1000 randomisations in Micro-Checker v. 2.2.3 (von Oosterhout *et al*., 2004). Additionally, FreeNA software (ENA method) (Chapuis and Estoup, 2007) was used to determine whether null alleles detected in the data were introducing bias in the analyses and to correct for the presence of null alleles.

Genetic variability statistics were generated for defined species level lineages (*O. martiensseni*, *O. harrisoni* and *O. madagascariensis*) as well as colonies (Fig. 2, Table 8.1). GenAlEx 6.5b4 (Peakall and Smouse, 2006, 2012) was used to calculate the number of alleles and the observed (H_O) and expected (H_E) heterozygosities. ARLEQUIN v.3.5.1.2 (Excoffier and Lischer, 2010) was used to test for deviation from Hardy-Weinberg equilibrium (HWE) and FSTAT v.2.9.3.2 (Goudet, 1995, 2001) to calculate allelic richness (AR) and the inbreeding coefficient (F_{IS}). Pairwise F_{ST} values and pairwise genotypic differentiation (1000 random permutations used to calculate *P*-values) were calculated using ARLEQUIN.

STRUCTURE v.2.3.4 (Pritchard *et al*., 2000) was used to generate genetic clustering patterns among individuals. Six runs for each *K* were implemented ranging from the minimum to maximum number of sites per lineage (1 to 6). Results defined the lineages to be analysed and allowed us to determine whether groupings of individuals corresponded to geographic locality at an inter- and intra-species level. STRUCTURE HARVESTER (Earl and vonHoldt, 2012) was used to visualise STRUCTURE results, and to create a Delta-K versus K plot to determine the most-likely number of groups within this region. An analysis of molecular variance (AMOVA) (16000 permutations) was conducted in ARLEQUIN to determine the percentage of variance among species level lineages, among colonies within lineages and among individuals. Isolation by distance was assessed between all species lineages using a Mantel test (999 permutations) implemented in GenAlEx.

A variety of analyses were carried out to provide insight into the social organization and breeding systems at the individual and colony level. Mean relatedness coefficients were calculated in COANCESTRY v.1.0.1.1 (Wang, 2011) using the TrioML (triadic likelihood) method. Mean relatedness was calculated for each colony and for all possible dyads at within-colony and within-lineage level. Mean relatedness values were also used to support the kinship associations produced in the parentage and sibship analyses, where only dyads with relatedness values (*r*) above 0.25 were considered to be supported.

Parentage analysis within each colony was performed using CERVUS v.3.0 (Kalinowski *et al*., 2007); this allowed assignments of maternity to a group female (paternity unknown) and paternity to a group male (maternity unknown). Within-group parentage simulations were run setting the number of candidate mothers (fathers) of offspring was 2 times the total number of female (male) genotypes to account for any unsampled candidate parents. It was assumed that only 50% of candidate parents had been sampled. The proportion of typed loci was set at 1.00 and the measured typing error set at 0.01%. The likelihood of each offspring and candidate parent pair relationship was calculated and parentage inferred only in pairs displaying over 80% confidence. To account for the possiblity of extra-colony mating, parentage analysis was also performed among individuals within defined species lineages. In these analyses the percentage of candidate parents was changed to 25% and all other parameters remained unchanged.

Sibship assignment was performed using COLONY v.2.0.4.0 (Wang, 2004; Jones and Wang, 2010); parent/offspring, full-sib and half-sib dyads were inferred for individuals within each colony as well as among individuals within defined species lineages. Probabilities of relationships allowed for a genotyping error of 1%, and were generated assuming that maternity/paternity is unknown and that males mate polygamously.

GENECLASS2 v.2.0 (Piry *et al*., 2004) was used to detect the presence of first generation migrants and assign/exclude individuals from their respective colonies. Both assignment/exclusion of individuals and detection of first generation migrants were performed using the predefined colonies under the Bayesian criterion of Rannala and Mountain (1997) and the Monte-Carlo resampling method of Paetkau *et al*. (2004). Assigning/excluding individuals to/from populations is best done using the Bayesian inference method in conjunction with the Paetkau *et al*. (2004) algorithm. This resampling method is thought to be better suited to these analyses since it takes into account the sample size of the reference population and thus the sampling variance associated with the dataset (Piry *et al*., 2004). An alpha of 0.01 was assumed and 10000 simulated individuals used. Detection of migrants was performed using the L_h/L_{max} likelihood computation, i.e. the population where individuals were sampled (L_h) over the highest likelihood value among all available population samples (L_{max}).

2.4 mtDNA PCR, sequencing and analysis

Methods for obtaining mitochondrial cytochrome *b* and D-loop region sequence data are presented in Lamb *et al*. (2008), including PCR amplification and sequencing protocols. This dataset comprised 68 sample animals from the various colonies (Table 8.1); cytochrome *b* and D-loop sequences were

placed into a concatenated dataset comprising 1322 nucleotides. DnaSP v. 5.10.01 (Librado and Rozas, 2009) was used to determine haplotypes and the dataset was then analysed using TCS v 1.21 (Clement *et al*., 2000) to create statistical parsimony haplotype networks.

3. RESULTS

3.1 Lineage-level structure

3.1.1 Nuclear structure

STRUCTURE analysis showed the division of the 71 samples into three groups (*K* = 3). These clusters corresponded to the lineages defined by Lamb *et al*. (2008) and Ralph *et al*. (2015) i.e. (1) *Otomops martiensseni* (n=43), (2) *O. harrisoni* (n=20) and (3) *O. madagascariensis* (n=8). The existence of three lineages was also supported by a Delta K versus K plot generated in STRUCTURE HARVESTER (Fig. 3.1), with separate Delta K versus K plots for *O. martiensseni* and *O. harrisoni* species (Fig. 3.2), as well as the parsimony networks (95% confidence) created using TCS (Fig. 3.3).

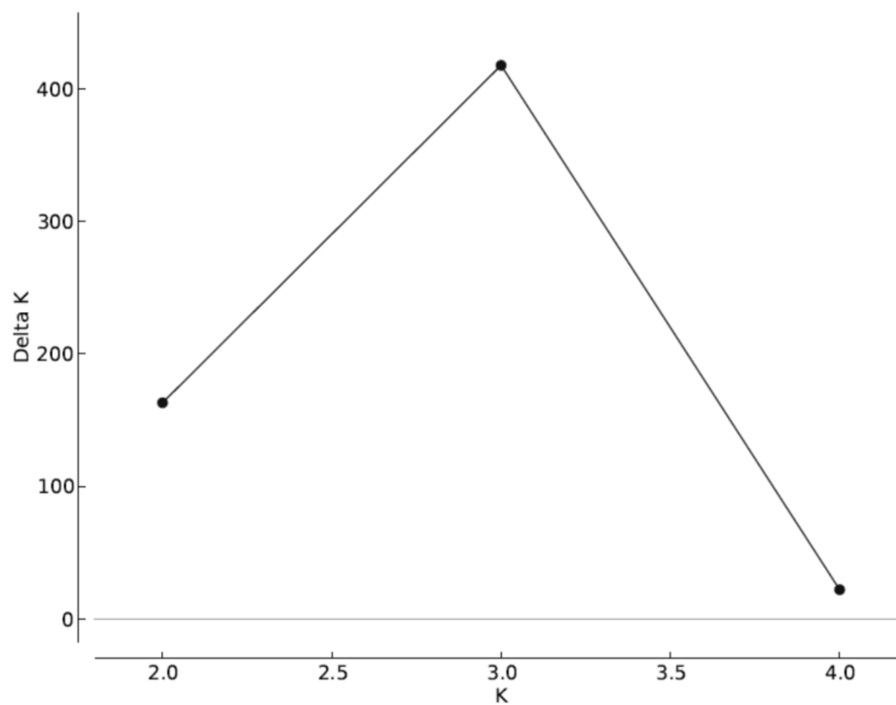

Figure 3.1. Delta-K versus K plot showing the best-supported number of Afro-Malagasy region *Otomops* groups, as defined by the definite peak in K. (DeltaK = mean(|L''(K)|) / sd(L(K)).

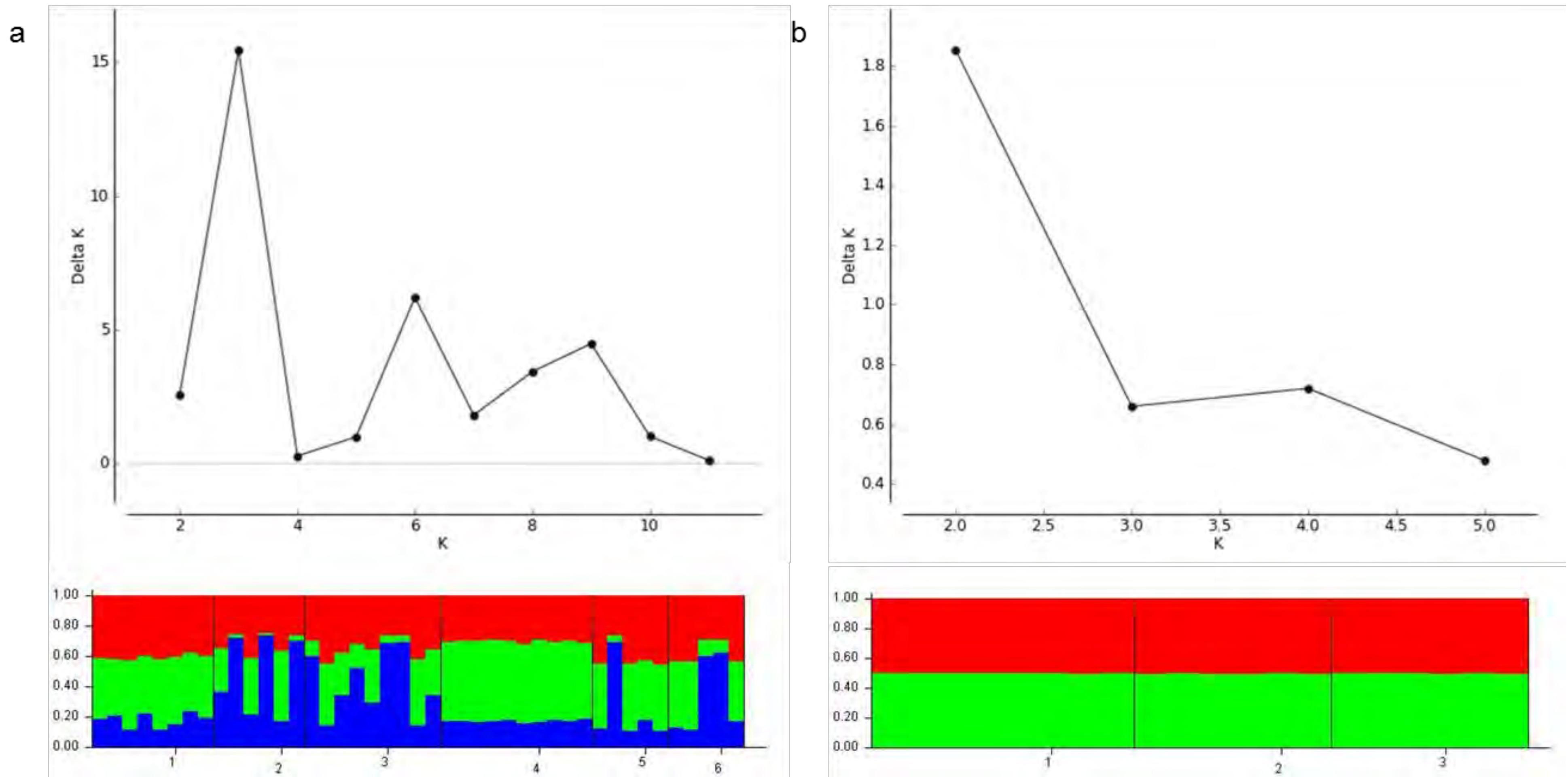

Figure 3.2. Delta-K versus K plot showing the best-supported number of groups in (a) *Otomops martiensseni* and (b) *Otomops harrisoni*, as defined by the definite peak in K. (DeltaK = mean(|L''(K)|) / sd(L(K)) with corresponding STRUCTURE bar plots.

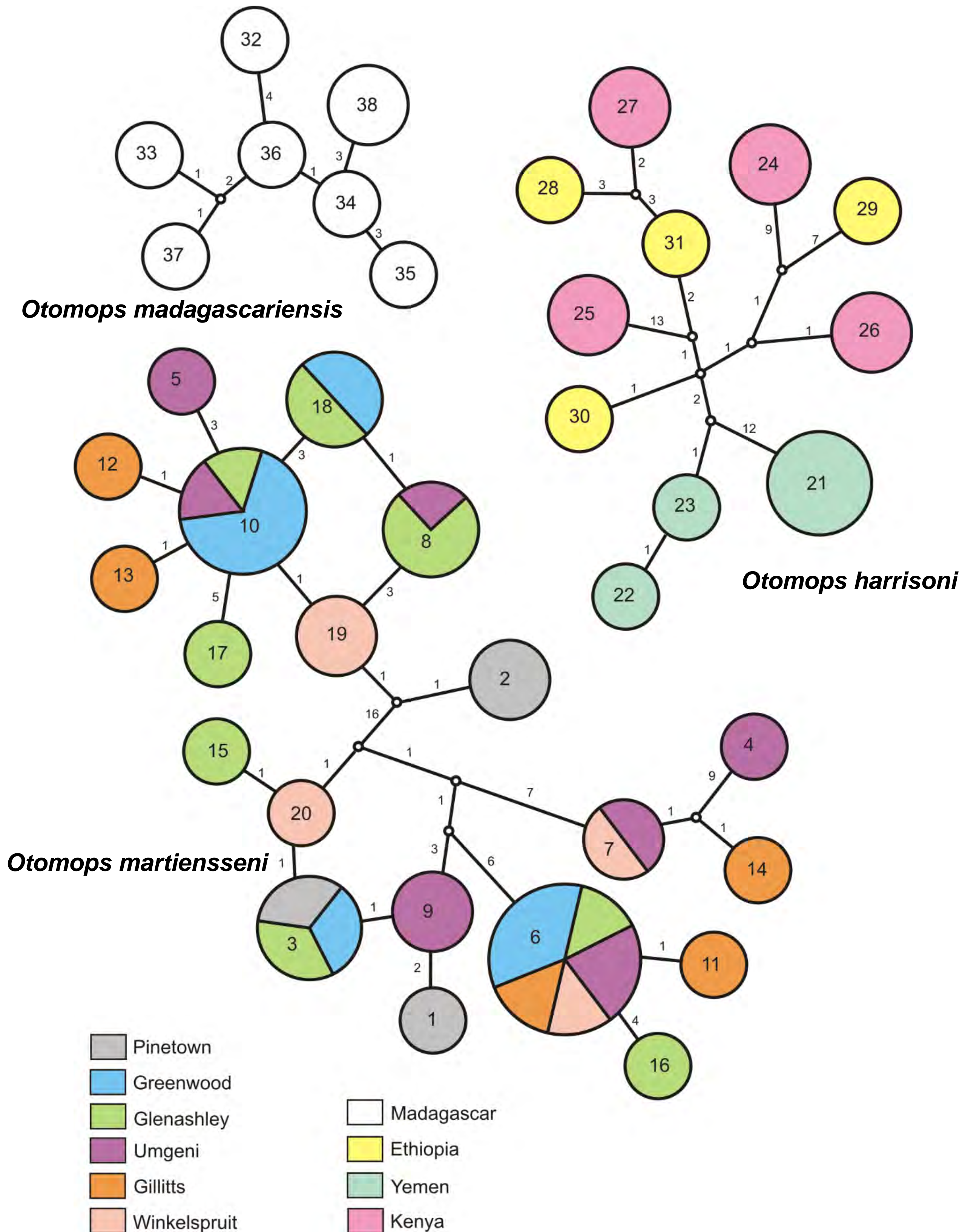

Figure 3.3. Statistical parsimony network (95% confidence; 18-step connection limit) of concatenated cytochrome *b* and D-loop data (1322 nucleotides) for Afro-Malagasy *Otomops* spp. Numbers next to connection branches indicate the number of mutational steps between haplotypes and circle size indicates the relative number of samples per haplotype. Haplotype samples are indicated in Table 8.1.

Analysis of *O. martiensseni* data in Micro-checker detected possible scoring error due to stuttering at two loci (TabrA10 and TabrH12) and the presence null alleles in five of the six loci used (TabrA10, TabrA30, TabrD10, TabrH6 and TabrH12). By comparison, null alleles were present in only one *O. harrisoni* locus (TabrD10) and were not present in *O. madagascariensis*. Large allele dropout was not detected. To determine whether substantial bias was introduced through the presence of the null alleles, pairwise F_{ST} values were calculated between the three lineages with and without the ENA algorithm. Additionally, global F_{ST} values with (0.248) and without (0.248) the ENA algorithm, were also calculated. The differences between the corrected and uncorrected estimates of genetic differentiation were not substantial, e.g. pairwise F_{ST} values between *O. martiensseni* and *O. madagascariensis* were 0.274 and 0.277 with and without the ENA adjustment, respectively, therefore we report analyses performed on uncorrected data only.

Analysis of genetic variability among species lineages revealed that most loci were polymorphic, comprising between two and 15 alleles per locus. Only marker TabrH12 in *O. madagascariensis* was monomorphic (Table 3.1). TabrD15 displayed the highest number of alleles per locus for each lineage (7 – 15), and the greatest H_O (0.84 – 1.00), whereas TabrA10 had the lowest number of alleles per locus (2 – 4) and TabrA30 showed the lowest H_O (0.10 – 0.16). Mean H_O values for *O. martiensseni*, *O. harrisoni* and *O. madagascariensis* were 0.47 (0.16 – 0.84), 0.57 (0.10 – 0.90) and 0.50 (0.13 – 1.00), respectively. F_{IS} values among lineages ranged from a minimum in *O. harrisoni* (-0.21) to a maximum in *O. martiensseni* (0.73) for marker TabrA10 (Table 3.1). These values were not significant, which may indicate a lack of inbreeding within these lineages, however it should be noted that greater populations numbers would be needed to completely discount the existence of inbreeding. The range of allelic richness values was similar across lineages, with means of 5.75, 4.66 and 3.50 for *O. martiensseni*, *O. harrisoni* and *O. madagascariensis* respectively. Marker TabrD15 displayed the highest number of alleles and allelic richness values for all three lineages.

Pairwise F_{ST} values among lineages were significantly different ($P < 0.05$) and revealed lower differentiation between *O. martiensseni* and *O. harrisoni* lineages (0.087) than between *O. madagascariensis* and *O. martiensseni* (0.274), and *O. madagascariensis* and *O. harrisoni* (0.276). Analysis of molecular variance (AMOVA) revealed that 62.82% of the variance occurred among individuals, 22.11% among colonies within species lineages, and 15.07% occurred among species lineages (Table 3.4). F_{ST} (0.15), F_{IS} (0.26) and F_{IT} (0.37) statistics generated across the full data set were each significant (P (random value >= observed value) = 0.001).

A Mantel test for isolation by distance revealed no significant correlation between pairwise genetic and geographical distances between the species lineages investigated in this study (correlation

coefficient (Rxy) = -0.026, probability of positive autocorrelation (one tailed) (P = 0.328)). The proportion of variance in genetic distance that can be explained by geographical distance (R^2) = 0.0007; the linear regression line (Y), reflecting the strength of the relationship between the two variables, = (-4^{-05}) x (11.666), is indicative of a more negative relationship.

3.1.2 Mitochondrial structure

The concatenated mitochondrial cytochrome *b*/ D-loop sequence alignment (1322 nucleotides; 68 samples) yielded 38 haplotypes with a haplotype diversity (*h*) of 0.970 and a nucleotide diversity (π) of 0.035. Twenty-three haplotypes were unique to individuals, whereas the remaining 15 were present in between two and eight individuals. Statistical parsimony analysis yielded three separate networks at a 95% connection limit (Fig. 3.3). These networks correspond to the lineages observed in the microsatellite analyses; (1) *O. martiensseni* (20 haplotypes), (2) *O. harrisoni* (11 haplotypes) and (3) *O. madagascariensis* (7 haplotypes).

3.2 Colony-level nuclear structure

The mean number of alleles across all loci ranged from 3.50, in the Yemen and Madagascar colonies, to 5.17, in the Umgeni colony in Kwa-Zulu Natal (Table 3.3). Inbreeding coefficient values (F_{IS}) were not significant but showed considerable range (Yemen = -0.12, Winkelspruit = 0.55). Allelic richness was similar across all colonies, ranging from 2.99 (Madagascar) to 4.50 (Winkelspruit) (Table 3.3). H_O ranged from 0.33 (Winkelspruit) to 0.70 (Ethiopia). There was no significant deviation from HWE in any of the colonies. Further analysis of the *O. martiensseni* and *O. harrisoni* populations showed the division of each species into 3 and 2 groupings, respectively according to STRUCTURE HARVESTER results, however STRUCTURE bar plots do not show clear division of samples where K = 3 (*O. martiensseni*) and K = 2 (*O. harrisoni*) (Fig. 3.2). *Otomops madagascariensis* was excluded from further analysis since this comprised only one population.

Table 3.1. Descriptive statistics for six microsatellite loci in three Afro-Malagasy species of *Otomops*: *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. Alleles = number of alleles, AR = allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, and F_{IS} = inbreeding coefficient.

	O. martiensseni (n=43)					*O. harrisoni* (n=20)					*O. madagascariensis* (n=8)				
Locus	Alleles	AR	H_O	H_E	F_{IS}	Alleles	AR	H_O	H_E	F_{IS}	Alleles	AR	H_O	H_E	F_{IS}
TabrA10	4	3.56	0.19	0.69	0.73	3	2.87	0.50	0.42	-0.21	2	2.00	0.50	0.40	-0.27
TabrA30	8	3.91	0.16	0.39	0.59	3	1.80	0.10	0.10	0.01	3	3.00	0.13	0.34	0.65
TabrD10	9	5.91	0.56	0.80	0.31	7	5.89	0.50	0.83	0.40	2	2.00	0.13	0.13	-
TabrD15	15	9.04	0.84	0.91	0.08	10	7.61	0.90	0.87	-0.04	7	7.00	1.00	0.88	-0.16
TabrH6	12	8.27	0.58	0.90	0.36	8	6.86	0.90	0.87	-0.03	6	6.00	0.75	0.78	0.05
TabrH12	5	3.82	0.49	0.67	0.27	3	2.93	0.50	0.55	0.08	1	1.00	-	-	-
Mean	8.83	5.75	0.47	0.73		5.67	4.66	0.57	0.61		7	3.50	0.50	0.51	

Table 3.2. Analysis of Molecular Variance among and within three Afro-Malagasy species of *Otomops*: *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. Fixation indices for the lineages were as follows: F_{IS} = 0.260, F_{ST} = 0.151, F_{IT} = 0.372. Based on 16000 permutations, all values were significant (P < 0.01).

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation
Among lineages	2	32.147	0.35306	15.07
Among populations (colonies) within lineages	68	170.543	0.51808	22.11
Among individuals within populations (colonies)	71	104.500	1.47183	62.82
Total	141	307.190	2.34297	

Table 3.3. Descriptive statistics averaged across all loci for three Afro-Malagasy species of *Otomops* (*O. martiensseni*, *O. harrisoni* and *O. madagascariensis*) based on a minimal sample size of five. *n* = number of individuals, *N* = mean number of alleles , AR = allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficient, HWE = deviation from Hardy-Weinberg equilibrium, NS = non significant deviation.

Species (Lineage)	Colony Site	*n*	*N*	AR	H_O	H_E	F_{IS}	HWE
O. martiensseni	Umgeni	8	5.17	4.42	0.50	0.74	0.34	NS
	Gillitts	6	4.00	3.76	0.40	0.70	0.45	NS
	Glenashley	9	4.83	4.01	0.57	0.69	0.18	NS
	Greenwood	10	4.83	3.87	0.58	0.71	0.19	NS
	Pinetown	5	3.83	3.83	0.50	0.64	0.24	NS
	Winkelspruit	5	4.50	4.50	0.33	0.62	0.55	NS
O. harrisoni	Yemen	6	3.50	3.27	0.67	0.60	-0.12	NS
	Ethiopia	8	4.33	3.80	0.70	0.75	0.07	NS
	Kenya	6	3.67	3.46	0.56	0.58	0.04	NS
O. madagascariensis	Madagascar	8	3.50	2.99	0.50	0.51	0.01	NS

Pairwise F_{ST}s between colonies within the same lineage were lower than those between colonies from different lineages. The lowest F_{ST} (0.065) was between the Kenya and Yemen colonies, whereas the highest F_{ST} values (0.288 – 0.403) occurred between Madagascar and each of the mainand Africa colonies (Table 3.4). Within the *O. martiensseni* lineage pairwise F_{ST}s ranged from 0.024 to 0.232. Pairwise F_{ST}s among the six *O. martiensseni* colonies were not significantly different, with the exception of the Greenwood colony, which was significantly different from all other colonies within that lineage. A lack of significance may be due to the lack of equal numbers sampled for each colony. Pairwise F_{ST} values between the three *O. harrisoni* colonies ranged from 0.065 to 0.088 and were all significantly different from each other (Table 3.4). These were separated by greater distances than the *O. martiensseni* colonies.

Mean relatedness values within colonies were relatively low and ranged from 0.011 to 0.078 in the *O. martiensseni* lineage, 0.032 to 0.086 in the *O. harrisoni* lineage and 0.056 in the *O. madagascariensis* lineage (Table 3.5). Levels of relatedness among the males within each colony were also relatively low, with a maximum value of 0.048 (*O. madagascariensis*). The mean relatedness of females within colonies (0.031) was greater than that of males (0.017), with a maximum of 0.053 (Greenwood). Overall relatedness values among all individuals within each lineage were comparable to those within colonies, all displaying values below 0.10.

Although potential paternal/maternal candidates were identified in CERVUS (males indicated in boldface font), the identity of the individual deemed the parent and that of the offspring in the dyad could not be clearly/confidently determined. For this reason we rely on the probabilities generated using COLONY to give us a better estimation of parent and offspring assignments. Parent/offspring (PO) dyads ($r \geq 0.50$) were assigned within three of the 10 defined colonies; i.e. Gillitts (two dyads), Greenwood (two dyads) and Ethiopia (four dyads) (Table 3.6). No individuals from the Madagascar colony were found to be in any PO associations. Within *O. martiensseni*, the four PO dyads accounted for 16.3% of individuals. Two females (DBN_28 and DBN_29) in the Greenwood colony were identified as the mothers of two individuals (**DBN_34** and **DBN_31**). No males were identified as candidate fathers. Within the Gillitts colony one male (**DBN_10**) was assigned as the parent of two individuals in the colony (**DBN_12** and DBN_14), however these individuals do not appear as half-siblings, as would be expected. This may be due to a problem with the data, eg. scoring error and/or null alleles (Jones *et al*., 2010). The absence of potential mother candidates may be due to the mother(s) not being physically present in the colony at the time of sampling. Within the *O. harrisoni* lineage, the four PO dyads identified in the Ethiopian colony were observed during sampling (mothers and their associated foetuses) and confirmed by the various analyses. Overall, 40% of all individuals from this lineage are in PO associations.

Table 3.4. Pairwise F_{ST} values for colonies of three Afro-Malagasy species of *Otomops*: *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. Statistically significant values ($P < 0.05$) are displayed in boldface font.

Species (Lineage)	Colony site	1	2	3	4	5	6	7	8	9
O. martiensseni	(1) Umgeni									
	(2) Gillitts	0.105								
	(3) Glenashley	0.041	0.054							
	(4) Greenwood	**0.159**	**0.232**	**0.179**						
	(5) Pinetown	0.034	0.121	0.054	**0.218**					
	(6) Winkelspruit	0.055	0.035	0.024	**0.185**	0.029				
O. harrisoni	(7) Yemen	**0.175**	**0.120**	**0.138**	**0.284**	**0.238**	**0.102**			
	(8) Ethiopia	**0.135**	**0.103**	**0.105**	**0.231**	**0.192**	**0.108**	**0.088**		
	(9) Kenya	**0.157**	**0.134**	**0.119**	**0.164**	**0.213**	0.084	**0.065**	**0.078**	
O. madagascariensis	(10) Toliara	**0.310**	**0.307**	**0.288**	**0.403**	**0.374**	**0.308**	**0.319**	**0.301**	**0.349**

Table 3.5. Mean relatedness values (*r*) calculated using TrioML for colonies of three Afro-Malagasy species of *Otomops*: *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*). Values are given for males only, females only and overall (males and females included). Where it was not possible to calculate relatedness (singular male/female or absence of males/females), lack of a value has been indicated (-).

	O. martiensseni							*O. harrisoni*				*O. madagascariensis*
	Overall	Umgeni	Gillitts	Glenashley	Greenwood	Pinetown	Winkelspruit	Overall	Yemen	Ethiopia	Kenya	Madagascar
Males	0.037	-	0.000	-	0.010	-	-	0.022	0.000	-	0.000	0.048
Females	0.060	0.037	0.000	0.048	0.053	0.049	0.000	0.055	0.024	0.009	0.000	0.038
Overall	0.067	0.049	0.078	0.058	0.074	0.075	0.011	0.069	0.035	0.086	0.032	0.056

Table 3.6. Kinship assignments generated using CERVUS and COLONY for 10 colonies of three Afro-Malagasy species of *Otomops*: *O. martiensseni, O. harrisoni and O. madagascariensis.* The number of kin groups and the percentage of related individuals (in parentheses) are given for each kinship association. Kinship was examined for within-colony, total within-colony, total extra-colony and total within-lineage groupings, where applicable. Total % = individuals in kinship associations; *n* = number of individuals; *r* = mean relatedness value; PO = parent/offspring kin groups; FS = full-sibling kin groups; HS = half-sibling kin groups.

Species (Lineage)	Colony	n	PO ($r \geq 0.50$)	FS ($r \geq 0.50$)	HS ($r \geq 0.25$)	Total %
O. martiensseni	Umgeni	8	0 (0)	0 (0)	1 (25)	25
	Gillitts	6	2 (50)	0 (0)	0 (0)	50
	Glenashley	9	0 (0)	1 (22.2)	2 (44.4)	55.6
	Greenwood	10	2 (40)	1 (20)	1 (20)	70
	Pinetown	5	0 (0)	1 (40)	0 (0)	40
	Winkelspruit	5	0 (0)	0 (0)	0 (0)	0
Within-colony total	All	43	4 (16.3)	3 (14)	4 (18.6)	44.2
Extra-colony total	All	43	0 (0)	3 (14)	12 (34.9)	46.5
Lineage total	All	43	4 (16.3)	6 (27.9)	16 (48.8)	74.4
O. harrisoni	Yemen	6	0 (0)	0 (0)	2 (50)	50
	Ethiopia	8	4 (100)	0 (0)	0 (0)	100
	Kenya	6	0 (0)	0 (0)	0 (0)	0
Within-colony total	All	20	4 (40)	0 (0)	2 (15)	55
Extra-colony total	All	20	0 (0)	0 (0)	1 (10)	10
Lineage total	All	20	4 (40)	0 (0)	3 (25)	60
O. madagascariensis	Toliara	8	0 (0)	1 (25)	3 (62.5)	75

The presence of full-sibling (FS) relationships is indicative of two individuals sharing the same mother and father; however, in these analyses potential within- and extra-colony mother/father candidates for each of the FS pairs were not identified, possibly due to absence from the roost at the time of sampling. FS pairs ($r \geq 0.50$) were assigned in four of the 10 colonies. Glenashley (22.2%: **DBN_21** and DBN_22), Greenwood (20%: DBN_35 and **DBN_37**) and Pinetown (40%: DM8421 and DP_4) contained within-colony, FS dyads (Table 3.6 and 3.7). Extra-colony FS relationships occurred between individuals of the Umgeni, Glenashley, Winkelspruit and Pinetown colonies, (14%) (Table 3.7). Overall, 27.9% of the *O. martiensseni* individuals were in full-sibling relationships (Table 3.6). In contrast, there were no FS dyads within or among the *O. harrisoni* colonies (Table 3.5 and 3.6). Only one FS relationship was found within Toliara, accounting for 25% of the colony (Table 3.5).

Half-sibling (HS) relationships ($r \geq 0.25$) were assigned in the Umgeni, Glenashley, Greenwood, Yemen and Toliara colonies (Table 3.6). For *O. martiensseni*, 48% of individuals were in HS relationships, 18.6% within colonies and 30.2% between colonies (Table 3.6). Within the *O. harrisoni* lineage, two HS dyads were found within the Yemen colony, with only one additional extra-colony HS pair between individuals from Ethiopia and Kenya (Table 3.6 and 3.7). Within the *O. madagascariensis* lineage, five individuals from the Toliara colony were in HS relationships, constituting three (62.5%) HS pairs (Tables 3.6 and 3.7).

For *O. martiensseni*, 74.4% of individuals were part of kin associations, the most common being HS pairs (48.8%) (Tables 3.6 and 3.7). Within the *O. harrisoni* lineage, 60% of individuals were in kinship associations; most of these were made up of the PO relationships observed in the Ethiopian colony. In the case of *O. madagascariensis*, 75% of individuals from the Toliara colony were in kinship associations, of which HS pairs constituted 62.5% (Table 3.6).

Assignment/exclusion analyses revealed the majority of individuals from each of the lineages to be correctly assigned to the colonies from which they were sampled (Table 3.8). There were four exceptions ($P < 0.01$ threshold) from the *O. martiensseni* lineage: three individuals from the Gillitts colony were assigned to the Winkelspruit (2) and Umgeni (1) colonies, and one Winkelspruit individual was assigned to the Glenashley colony. Analysis of first generation migrants also indicated that most individuals remained within their respective colonies, with the exception of five individuals ($P < 0.01$ threshold) (Table 3.8). Four of the 43 *O. martiensseni* individuals, from the Umgeni, Gillitts, Pinetown and Winkelspruit colonies, appeared as potential first generation migrants. The 5^{th} individual, from the Yemen colony, was detected as a potential migrant from the Kenya colony. All potential migrants were determined to be from other colonies within their respective lineages.

Table 3.7. Individuals associated in parent/offspring, full-sibling and half-sibling kinship assignments generated using CERVUS and COLONY within and among colonies of three Afro-Malagasy species of *Otomops*: *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. *n* = number of individuals; E = Ethiopia; GI = Gillitts; GL = Glenashley; GR = Greenwood; K = Kenya; P = Pinetown; U = Umgeni; W = Winkelspruit. Unknown sex is indicated (*) and males are indicated in boldface font.

Species (Lineage)	Colony	*n*	Parent/offspring		Full-sibling		Half-sibling	
			Parent	Offspring	Sibling 1	Sibling 2	Sibling 1	Sibling 2
O. martiensseni	Umgeni	8	-	-	-	-	DBN_1	**DBN_7**
	Gillitts	6	**DBN_10**	**DBN_12**	-	-	-	-
			DBN_10	DBN_14	-	-	-	-
	Glenashley	9	-	-	**DBN_21**	DBN_22	-	-
	Greenwood	10	DBN_28	DBN_34	DBN_35	**DBN_37**	**DBN_32**	DBN_34
			DBN_29	**DBN_31**	-	-	-	-
	Pinetown	5	-	-	DM 8421	DP_4	-	-
	All	43	-	-	DBN_23 (GL)	DBN_4 (U)	DBN_9 (GI)	**KZN_5** (W)
			-	-	DBN_25 (GL)	KZN_3 (W)	**DBN_10** (GI)	DBN_24 (GL)
			-	-	**DP_5** (P)	KZN_1 (W)	**DBN_10** (GI)	DP_2 (P)
			-	-	-	-	**DBN_12** (GI)	DP_2 (P)
			-	-	-	-	DBN_20 (GL)	DBN_4 (U)
			-	-	-	-	DBN_24 (GL)	DP_2 (P)
			-	-	-	-	DBN_27 (GL)	DBN_35 (GR)
			-	-	-	-	DBN_27 (GL)	**DBN_37** (GR)
			-	-	-	-	DP_3 (P)	KZN_2 (W)
			-	-	-	-	DBN_3 (U)	KZN_2 (W)

Table 3.7 continued.

Species (Lineage)	Colony	*n*	Parent/offspring		Full-sibling		Half-sibling	
			Parent	Offspring	Sibling 1	Sibling 2	Sibling 1	Sibling 2
O. martiensseni	All	43	-	-	-	-	DBN_5 (U)	KZN_2 (W)
			-	-	-	-	DBN_5 (U)	DP_3 (P)
O. harrisoni	Yemen	6	-	-	-	-	NMP 91812	NMP 91816
			-	-	-	-	NMP 91814	NMP 91816
	Ethiopia	8	pb2512	pb2512x*	-	-	-	-
			NMP 91201	NMP 91201*	-	-	-	-
			NMP 91202	NMP 91202*	-	-	-	-
			NMP 91203	NMP 91203*	-	-	-	-
	All	20	-	-	-	-	NMP 91201 (E)	**NMK 15465** (K)
O. madagascariensis	Toliara	8	-	-	UADBA 43204	FMNH 209264	**UADBA 43205**	FMNH 209264
			-	-	-	-	UADBA 43206	**FMNH 209262**
			-	-	-	-	UADBA 43206	**FMNH 209263**

Museum samples: DM – Durban Natural Science Museum; NMK – National Museum of Kenya; NMP – National Museum of the Czech Republic, Prague. Field collection samples: DBN – Durban; DP – Durban Pinetown; FMNH – Field Museum of Natural History; KZN – KwaZulu-Natal; pb – Petr Benda; UADBA – Université d'Antananarivo, Département de Biologie Animale.

Table 3.8. Exclusion of individuals and detection of first generation migrants generated in GENECLASS from colonies of three Afro-Malagasy species of *Otomops*: *O. martiensseni*, *O. harrisoni* and *O. madagascariensis* (probability of inclusion to reference population < 0.01 threshold). GI = Gillitts; P = Pinetown; U = Umgeni; W = Winkelspruit; Y = Yemen. Males are indicated in boldface font.

Species (Lineage)	Exclusion of individuals		Detection of first generation migrants	
	Excluded individual	Assigned colony	Potential migrant	Source colony
O. martiensseni	DBN_9 (GI)	Winkelspruit	DBN_2 (U)	Gillitts
	DBN_11 (GI)	Winkelspruit	DP_2 (P)	Glenashley
	DBN_13 (GI)	Umgeni	DBN_13 (GI)	Umgeni
	KZN_3 (W)	Glenashley	KZN_3 (W)	Glenashley
O. harrisoni	-	-	**NMP 91811** (Y)	Kenya

Museum samples: NMP – National Museum of the Czech Republic, Prague. Field collection samples: DBN – Durban; DP – Durban Pinetown; KZN – KwaZulu-Natal.

4. DISCUSSION

4.1 Lineage-level structure

One of the aims of this study was to provide a nuclear (microsatellite) perspective on genetic variation in *Otomops*, and to compare this with analyses of the same sample set based on mitochondrial DNA, as well as published studies based on mtDNA sequences.

With respect to our first objective, as hypothesised, the statistical parsimony analysis based on mtDNA divided the sample into three major lineages corresponding to *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. A variety of population genetic analyses based on nuclear microsatellites were congruent with the mitochondrial analysis based on the same dataset in supporting the currently circumscribed species boundaries within Afro-Malagasy *Otomops* spp. (Lamb *et al*., 2008; Ralph *et al*., 2015). Analysis of molecular variance yielded significant subdivision, with 15.07% of the variation occurring among species level lineages. There was no evidence of migration between lineages. Assignment/exclusion analyses, as well as parentage and kinship analyses, showed that no individuals were related to or assigned to individuals or colonies outside of their respective species lineages, further supporting the existence of three species within the Afrotropical region. Such lack of gene flow between species level lineages is to be expected and has also been found by Campbell *et al*. (2006), Racey *et al*. (2007) and Bodanowicz *et al*. (2012) among species groups of *Cynopterus*, *Pipistrellus* and *Myotis*, respectively. The morphometric data of Richards *et al*. (2012) further support the existence of these three Afro-Malagasy species level lineages of *Otomops* based on craniodental and landmark-based morphometric data.

Microsatellite analyses revealed lower levels of structure between the mainland Africa lineages (F_{ST} 0.087) than between either of these and the Malagasy lineage, from which they are substantially differentiated (F_{ST} 0.247 and 0.276, respectively). This supported the mtDNA sequence studies of Lamb *et al*. (2008), which showed a closer phylogenetic relationship between the mainland Africa sister species (*O. martiensseni* and *O. harrisoni*; divergence ~1.2 million years ago) than between either of these and *O. madagascariensis*, from which they diverged at an earlier time (~1.5 million years ago; Lamb *et al*., 2008, 2011).

4.2 Within-lineage (colony level) structure

Our second objective was to determine whether our data reveal subdivision within each of the species lineages, and if so, whether different colonies are genetically distinct from each other. Analyses of

mitochondrial markers revealed little structure within species level lineages. At a 95% connection limit, each species formed a single parsimony network (Fig. 3.3). AMOVA, based on mtDNA sequence data, showed that only 3.2% of variance occurred among species lineages, whereas 91% of the variation occurred within populations, indicating a lack of genetic structure within the species groupings (Lamb *et al*., 2008). Such low levels of structure might be expected in a strong-flying bat such as *Otomops*, which is capable of covering large distances, in the absence of behavioural/social patterns (such as philopatry) which might promote structure.

We found somewhat greater levels of nuclear structure within lineages than were observed in studies based on mitochondrial data (Lamb *et al*., 2008), with 22% of the variation occurring among colonies within lineages and 62% occurring among individuals. This suggests the presence of some structuring at colony level. In support of this, all *O. harrisoni* colonies were significantly distinct from each other (F_{ST} 0.065 – 0.088), and one third of comparisons among *O. martiensseni* colonies yielded significantly different F_{ST} values (0.159 – 0.218). However, mean relatedness values within colonies were relatively low and overall relatedness values among all individuals within each lineage were comparable to those within colonies, consistent with relatively low levels of structuring at colony level.

Moderate to high observed heterozygosity (0.33 – 0.70) and a lack of inbreeding (low positive non-significant inbreeding coefficients) suggests a relatively high level of genetic diversity within colonies, consistent with the results of the AMOVA, which showed the greatest source of variation (62.82%) to be among individuals (Table 3.2). Demonstrated migration between colonies suggests that heterozygosity in *Otomops* colonies is maintained by gene flow due to extra-colony matings, consistent with the lack of significant deviation from HWE (Table 3.3) (Bogdanowicz *et al*., 2012). This does not preclude the occurrence of intra-colony matings, although it should be noted that, besides mating, there are other benefits of colony membership; these include a reduced risk of predation due to temporal clustering upon exiting the roost, reduced thermoregulation cost by altering the local environment and increasing the ambient temperature, and the social benefits of cooperation and information transfer (Altringham and Senior, 2005).

4.3 Colony-level relatedness and migration

4.3.1 Among-colony gene flow

The third objective of this study was to determine levels of gene flow among and within colonies, and migration between colonies. Given the high genetic diversity within species lineages observed in

mtDNA studies, the use of day roosts, and the strong dispersal ability of *Otomops*, we hypothesized that we would observe migration and gene flow between colonies, although not between species level lineages.

Both assignment/exclusion analyses and parentage/kinship analyses showed that no individuals were assigned to or related to individuals from colonies outside of their respective lineages, in support of the above hypothesis. It should be noted that although efforts were made to sample all individuals from each colony, completeness of sampling can not be guaranteed due to the roosting habits of this bat, including use of alternative roosting sites and the nature of roost sites, e.g. aggregations in cave sites make determine precise colony numbers difficult,

The microsatellite-based analyses are largely consistent with the occurrence of gene flow among colonies. Moderate to high heterozygosities were observed within species-level *Otomops* lineages (Table 3.1), typical of individuals from shared habitats maintaining a level of random mating and avoiding inbreeding through extra-colony mating (Frankham *et al*., 2002; Atterby *et al*., 2010; Bogdanowicz *et al*., 2012); this has also been documented in studies on other bats, for example *Plecotus auritus* (Burland *et al*., 1999) and *Myotis bechsteinii* (Kerth *et al*., 2003). The *O. martiensseni* lineage appears to be most diverse, with the highest observed heterozygosity and alleleic richness (Table 3.1); this may be a consequence of the relatively greater number of individuals sampled from this area (Rivers *et al*., 2005). Two thirds of the pairwise F_{ST} values among colonies within the *O. martiensseni* lineage are not significant, consistent with the occurrence of gene flow between colonies and a lack of population subdivision (Atterby *et al*., 2010). Additionally, STRUCTURE analysis does not show a clear subdivision of samples into their assigned colonies (Fig. 2). Individuals from the *O. martiensseni* Gillitts and Winkelspruit colonies were reassigned to new colonies within this lineage, and the detection of first generation migrants indicates that bats from certain colonies had previously belonged to another colony (Table 3.8), consistent with gene flow among colonies. As only one colony was sampled from Madagascar, it was not possible to make conclusions on gene flow among colonies from this island.

In summary, our third hypothesis, that we would observe migration and gene flow between colonies, but not between species level lineages, was well supported by analyses of our micosatellite data.

4.3.2 Within-colony gene flow

Our fourth objective was to search for parent-offspring, full-sibling and half-sibling relationships among and within colonies. High genetic variability in analyses based on mtDNA sequences suggest the hypothesis that kinship between colonies will be observed.

Levels of relatedness within each of the colonies were relatively low (all relatedness (*r*) values were <0.1), indicating that not all individuals within a colony are related (Table 3.5). Overall mean relatedness values might be low in cases where colony members are not paternally related ($r = 0$) even if close matrilineal relatives exist within the colony (Rivers *et al*., 2005) or vice versa. Thus colonies containing maternally-related half-sibling pairs ($r = 0.25$) could show mean relatedness vaues below 0.1 in situations where females mate with different males from year to year, yielding offspring that are maternally, rather than paternally, related. Additionally, the presence of immigrant females within colonies may also contribute to low within-colony relatedness. Thus, there is support for the hypothesis that there is migration and gene flow among colonies within lineages, and consequent avoidance of inbreeding, although it is acknowledged that the reliability of the conclusions may have been affected by the presence of null alleles in that data.

4.4 Among- and within-colony kinship

Approximately 70% of sampled individuals shared some form of kinship, whether PO, FS or HS. The only PO relationships detected in the *O. harrisoni* lineage were within the Ethiopia colony, a result of direct sampling of mother and foetus pairs (Table 3.6). Approximately 16% of *O. martiensseni* individuals were involved in within colony PO relationships (two females: one with a male and the other with a female offspring, and a single male fathering a male and female), and none between colonies (Table 3.6). Assignment of a single Gillitts male as the parent of two individuals within the colony may imply that this is a dominant male, having fathered these individuals with two unidentified females. Individuals with unassigned parentage (*O. martiensseni*: ~91%; *O. harrisoni*: 80%; *O. madagascariensis*: 100% of individuals) are likely to have resulted from matings with extra-colony males/females, matings with unsampled members of the colonies concerned, or the absence/death of parents. Greater sampling numbers and the use of additional markers would be needed to better elucidate PO relationships between individuals (Putman and Carbone, 2014).

Full-sibling (FS) relationships were found mainly in the *O. martiensseni* lineage (~28% of individuals, Table 3.6). Although possible FS relationships have been identified, a minimum 15 to 20 microsatellite markers are required to distinguish full-siblings from unrelated pairs with 90% confidence (Blouin, 2003). The only other FS relationship was found in the Malagasy colony (25% of MAD individuals). The relatively larger number of FS relationships in the *O. martiensseni* lineage is likely to reflect greater sampling from this region (n=43). Since *Otomops* gives birth to only one offspring per year (Fenton *et al*., 2002; Taylor, 2005; Andriafidison *et al*., 2007), FS status among individuals may be a result of females mating with the same male (perhaps the dominant male) in sequential years, as was observed in the *Myotis mystacinus* complex (Bogdanowicz *et al*., 2012).

Alternatively, FS status could result from females following a set dispersal pattern and frequenting the same colonies during the mating period each year before returning to their original roost. Such set dispersal patterns have been observed in bats that annually frequent the same swarming sites during the mating period and subsequently return to their roosts, e.g. *M. nattereri* (Rivers *et al*., 2005) and *Plecotus auritus* (Furmankiewicz and Altringham, 2007).

As hypothesised, most kinship within lineages takes the form of half sibling (HS) relationships, reinforcing the suggestion that *Otomops* engages in extra-colony mating. Within the *O. martiensseni* lineage, HS relationships were the most common form of sibship (~49%), with almost twice as many HS pairs explained by extra-colony mating (~35%) than within-colony mating (~19%). Two of the extra-colony HS relationships were also supported by exclusion and detection of first migrant analyses (Table 3.8), where DBN_9 was excluded from Gillitts, assigned to the Winkelspruit colony and exhibited a HS relationship with KZN_5 from Winkelspruit. Similarly, DP_2 (P) shared a HS relationship with DBN_24 (GL) and was assigned as a first migrant to the Glenashley colony. Although an extra-colony HS pair was assigned in the *O. harrisoni* lineage, this association should be considered with some caution as these were museum samples collected during different years. Finally, HS relationships were most common within the *O. madagascariensis* colony. The predominance of HS relationships suggests that, as in *P. auritus* (Burland *et al*., 1999, 2001), *Otomops* maintains gene flow through extra-colony mating and does not exhibit strict colony faithfulness.

4.5 General

We found little genetic structure within species lineages based on maternally-inherited mitochondrial sequence variation. Although haplotype diversity was high, individuals from the same colony tended not to share the same mtDNA haplotype. Thus it is unlikely that the social system of the Afro-Malagasy *Otomops* spp. studied here is based on female philopatry, in common with *Plecotus auritus* (Burland *et al*., 2001), *Rhynchonycteris naso* (Nagy *et al*., 2013) and *Saccopteryx bilineata* (Nagy *et al*., 2007). There was somewhat more structure in bi-parentally-inherited nuclear DNA, with 22.11% of variation occurring among colonies within lineages. However, the greatest proportion of variation (62.83%) occurred among individuals, consistent with a high level of gene flow and interbreeding among members of different colonies.

There is little evidence of male philopatry, although the data are consistent with a single male from the Gillitts colony having fathered two of the juveniles within that colony. During sampling trips

within the greater Durban area L. Richards (*pers. comm.*[1]) has observed *Otomops* males roosting alone. This may be indicative of philopatry as a means of maintaining dominance over a roost locality, natal group and/or resources in the expectation that females will join this roost thereby allowing them to gain higher reproductive success than any immigrant males (Nagy *et al*., 2007, 2013; Clutton-Brock and Lukas, 2011). Although the presence of solitary males in a roost is not common in bats, single *Saccopteryx bilineata* males in Costa Rica have been observed defending territory within a building roof in the absence of females (Altringham, 2011).

Relationships within the Ethiopian *O. harrisoni* colony are consistent with female-biased dispersal; the foetuses of the mother/foetus pairs bore no sibling relation to each other, implying that none of them shared a father, and that the mothers may have mated with other males in the vicinity. Further, members of this lineage which were excluded as colony members and detected as migrants were female (Table 3.8) consistent with migration of females between colonies.

It should be noted that very few maternal candidates were collected during sampling. This could be attributed to the timing of sampling, which was carried out in the months before females gave birth, as we did not want to disturb the roost during this period. Collection may have taken place during the mating season, when females may have been using alternative day roosts and/or frequenting nearby roosts in order to mate with extra-colony males.

Most relationships identified were at the half-sibling kinship level, within and more commonly between colonies within a species level lineage (Tables 3.6 and 3.7). Extra-colony HS relationships within the *O. martiensseni* lineage were 4 times more common than FS relationships. The larger number of potential HS relationships is consistent with an extra-colony mating structure. Dispersal in both male and female bats for mating purposes is well-known (McCraken and Wilkinson, 2000), for example when bats meet at known swarming sites in order to mate with unrelated partners thereby promoting gene flow among colonies and avoiding inbreeding (Bogdanowicz *et al*., 2012). *Otomops* from the greater Durban area does not, to our knowledge frequent any swarming sites but does utilise day roosts and night roosts. These day roosts could serve as alternative locations where either males or females (or both) are able to meet with potential mating partners before returning to their natal colonies. The use of day/alternative roosts for mating has been documented in the common vampire bat, *Desmodus rotundus* (Wilkinson, 1987) and *Nycticeus humeralis* (Bain and Humphrey, 1986; McCracken and Wilkinson, 2000). *Otomops harrisoni* and *O. madagascariensis* are known cave-dwellers, which may facilitate finding of suitable and unrelated mates in other parts of the caves,

[1] Dr. L.R. Richards. Durban Natural Science Museum, 151 K E Masinga Road, Durban, South Africa.

karsts and lava tunnels they inhabit. This behaviour would have to be confirmed with more extensive sampling from within the cave site as well as observation studies, if possible.

Microsatellite analysis does not appear to give a definitive indication of male or female philopatry. The high heterozygosity and low inbreeding co-efficient values all point to the maintenance of a random mating structure among colonies within the various lineages sampled; however, the sex responsible for maintenance of gene flow among colonies cannot be clearly elucidated. A lack of strong genetic differentiation among the various colonies (and thus harems) might be attributed to increased movement of males or females, subsequent natal dispersal (Storz *et al*., 2001a, 2001b) as well as random mating associations, which result in the negative inbreeding coefficients observed within the various colonies which are at HWE (Table 3.3). Consistent sampling and observation from the same roosts over a number of years would allow us to better elucidate any recurring behaviour patterns and ascertain relationship dynamics among individuals within the colony and how these change from year to year. Additionally, it would also be useful to incorporate a greater number of microsatellite markers in the analysis, especially in the case of kinship analyses (Blouin, 2003).

5. CONCLUSION

Our nuclear microsatellite and mitochondrial sequence data support the circumscription of Afro-Malagasy *Otomops* into three species, *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*. There is some evidence of structuring at colony level within species lineages, as 22.11% of the nuclear variation occurs among colonies, and three quarters of pairwise F_{ST}s among all colonies are significant. However, analyses in STRUCTURE do not show clear clustering of individuals at colony level. Further, AMOVA shows that the highest proportion of variance occurs among indivduals (62.82%) and most pairwise F_{ST}s between colonies are not significant. In addition, mean relatedness among individuals within colonies is similar to that among individuals within species level lineages, suggestive of considerable gene flow among colonies.

We observed PO relationships within, but not between colonies. FS and HS pairs were observed both within and between colonies within their respective lineages. However, most kinship within lineages takes the form of HS relationships, reinforcing the suggestion that *Otomops* engages in extra-colony mating.

We find little evidence to support the presence of a social system based on either female or male philopatry in Afro-Malagasy *Otomops*. Our results suggest that individuals do not exhibit strict colony faithfulness, and that gene flow is maintained through extra-colony mating.

6. ACKNOWLEDGEMENTS

We gratefully acknowledge Prof. Steve Goodman, Dr. Peter Taylor, Dr. Corrie Schoeman and Dr. Leigh Richards for their assistance with fieldwork. We also wish to thank Petr Benda and the Bat Interest Group of KwaZulu-Natal for contributing additional specimen material. eZemvelo KZN Wildlife and the Department of Environmental Affairs are thanked for assistance in obtaining a Threatened or Protected Species (ToPS) permit for the collection of supplementary genetic material. TMCR and JML acknowledge financial assistance of the South African National Research Foundation (NRF). TMCR also acknowledges the financial assistance provided by the South African Biosystematics Initiative (SABI).

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8. APPENDICES

Appendix 1. Sample table

Table 8.1. Sampled individuals of *Otomops* used in nuclear microsatellite and mitochondrial sequence data analysis.

Species	Geographic origin	Co-ordinates	Museum / Field number	Sex	Haplotype number	Genbank accession number	
						Cytochrome *b*	D-loop
O. martiensseni	RSA: 27 Hunters Way; Umgeni Heights; KZN	29.808° S, 31.025° E	DBN_1	F	4	KJ433684	KJ433743
- " -	- " -	- " -	DBN_2	F	5	KJ433685	KJ433744
- " -	- " -	- " -	DBN_3	F	6	KJ433686	KJ433745
- " -	- " -	- " -	DBN_4	F	7	KJ433687	KJ433746
- " -	- " -	- " -	DBN_5	F	8	KJ433688	KJ433747
- " -	- " -	- " -	DBN_6	F		-	-
- " -	- " -	- " -	DBN_7	M	9	KJ433689	KJ433748
- " -	- " -	- " -	DBN_8	F	6	KJ433690	KJ433749
- " -	RSA: 6 Firwood Rd; Gillitts; KZN	29.796° S, 30.808° E	DBN_9	F	10	KJ433691	KJ433750
- " -	- " -	- " -	DBN_10	M	11	KJ433692	KJ433751
- " -	- " -	- " -	DBN_11	F	12	KJ433693	KJ433752

Table 8.1 continued.

Species	Geographic origin	Co-ordinates	Museum / Field number	Sex	Haplotype number	Genbank accession number	
						Cytochrome *b*	D-loop
O. martiensseni	RSA: 6 Firwood Rd; Gillitts; KZN	29.796° S, 30.808° E	DBN_12	M	13	KJ433694	KJ433753
- “ -	- “ -	- “ -	DBN_13	F	14	KJ433695	KJ433754
- “ -	- “ -	- “ -	DBN_14	F	6	KJ433696	KJ433755
- “ -	RSA: 29 Glen Anil St; Glenashley; KZN	29.752° S, 31.037° E	DBN_19	F	8	KJ433701	KJ433760
- “ -	- “ -	- “ -	DBN_20	F	15	KJ433702	KJ433761
- “ -	- “ -	- “ -	DBN_21	M	16	KJ433703	KJ433762
- “ -	- “ -	- “ -	DBN_22	F	8	KJ433704	KJ433763
- “ -	- “ -	- “ -	DBN_23	F	17	KJ433705	KJ433764
- “ -	- “ -	- “ -	DBN_24	F	10	KJ433706	KJ433765
- “ -	- “ -	- “ -	DBN_25	F	6	KJ433707	KJ433766
- “ -	- “ -	- “ -	DBN_26	F	3	KJ433708	KJ433767
- “ -	- “ -	- “ -	DBN_27	F	18	KJ433709	KJ433768
- “ -	RSA: 10 Rosary Rd; Greenwood Park; KZN	29.789° S, 31.017° E	DBN_28	F	18	KJ433710	KJ433769
- “ -	- “ -	- “ -	DBN_29	F		KJ433711	KJ433770
- “ -	- “ -	- “ -	DBN_30	F	6	KJ433712	KJ433771

Table 8.1 continued.

Species	Geographic origin	Co-ordinates	Museum / Field number	Sex	Haplotype number	Genbank accession number	
						Cytochrome *b*	D-loop
O. martiensseni	RSA: 10 Rosary Rd; Greenwood Park; KZN	29.789° S, 31.017° E	DBN_31	M		KJ433713	KJ433772
- " -	- " -	- " -	DBN_32	M	3	KJ433714	KJ433773
- " -	- " -	- " -	DBN_33	F	6	KJ433715	KJ433774
- " -	- " -	- " -	DBN_34	F	18	KJ433716	KJ433775
- " -	- " -	- " -	DBN_35	F		KJ433717	KJ433776
- " -	- " -	- " -	DBN_36	F	6	KJ433718	KJ433777
- " -	- " -	- " -	DBN_37	M		KJ433719	KJ433778
- " -	RSA: 8 Buys Rd; Pinetown; KZN	29.757° S, 30.639° E	DM8421	F	1	EF216413	EF216447
- " -	- " -	- " -	DP_2	F	2	EF216414	EF216448
- " -	- " -	- " -	DP_3	F	2	EF216415	EF216449
- " -	- " -	- " -	DP_4	F	3	EF216416	EF216450
- " -	- " -	- " -	DP_5	M	-	-	-
- " -	RSA: Eden Sands; Winkelspruit; KZN	30.097° S, 30.859° E	KZN_1	F	6	KJ433720	KJ433779
- " -	- " -	- " -	KZN_2	F	7	KJ433721	KJ433780
- " -	- " -	- " -	KZN_3	F	19	KJ433722	KJ433781

Table 8.1 continued.

Species	Geographic origin	Co-ordinates	Museum / Field number	Sex	Haplotype number	Genbank accession number	
						Cytochrome *b*	D-loop
O. martiensseni	RSA: Eden Sands; Winkelspruit; KZN	30.097° S, 30.859° E	KZN_4	F	20	KJ433723	KJ433782
- “ -	- “ -	- “ -	KZN_5	M	19	KJ433724	KJ433783
O. harrisoni	Ethiopia: S of Omar Caves; Bale Province	6.900° N, 40.859° E	NMP 91201	F	27	EF216435	EF216467
- “ -	- “ -	- “ -	NMP 91201	U	27	EF216436	EF216468
- “ -	- “ -	- “ -	NMP 91202	F	26	EF216433	EF216465
- “ -	- “ -	- “ -	NMP 91202	F	26	EF216434	EF216466
- “ -	- “ -	- “ -	NMP 91203	F	24	EF216429	EF216461
- “ -	- “ -	- “ -	NMP 91203	U	24	EF216430	EF216462
- “ -	- “ -	- “ -	pb 2512	F	25	EF216431	EF216463
- “ -	- “ -	- “ -	pb 2512x	U	25	EF216432	EF216464
- “ -	Yemen: Hud Sawa Cave; Al Mawhit	15.483° N, 43.533° E	NMP 91811	M	21	KJ433729	KJ433788
- “ -	- “ -	- “ -	NMP 91812	F	22	KJ433730	KJ433789
- “ -	- “ -	- “ -	NMP 91813	M	21	KJ433731	KJ433790
- “ -	- “ -	- “ -	NMP 91814	F	23	KJ433732	KJ433791
- “ -	- “ -	- “ -	NMP 91815	F	21	KJ433733	KJ433792

Table 8.1 continued.

Species	Geographic origin	Co-ordinates	Museum / Field number	Sex	Haplotype number	Genbank accession number	
						Cytochrome *b*	D-loop
O. harrisoni	Yemen: Hud Sawa Cave; Al Mawhit	15.483° N, 43.533° E	NMP 91816	F	21	KJ433734	KJ433793
- " -	Kenya: Ithundu Caves	2.358° S, 37.717° E	NMK 15459	M	30	EF216441	EF216458
- " -	- " -	- " -	NMK 15460	F	31	EF216442	EF216460
- " -	- " -	- " -	NMK 15461	F	-	-	-
- " -	- " -	- " -	NMK 15462	M	28	EF216428	EF216455
- " -	- " -	- " -	NMK 15463	M	29	EF216440	EF216457
- " -	- " -	- " -	NMK 15465	M	-		
O. madagascariensis	Madagascar: Toliara Province, Bishiko Cave	23.548° S, 43.767° E	UADBA 43203	F	33	KJ433735	KJ433794
- " -	- " -	- " -	UADBA 43204	F	34	KJ433736	KJ433795
- " -	- " -	- " -	UADBA 43205	M	35	KJ433737	KJ433796
- " -	- " -	- " -	UADBA 43206	F	36	KJ433738	KJ433797
- " -	- " -	- " -	FMNH 209262	M	32	KJ433739	KJ433798
- " -	- " -	- " -	FMNH 209263	M	37	KJ433740	KJ433799

Table 8.1 continued.

Species	Geographic origin	Co-ordinates	Museum / Field number	Sex	Haplotype number	Genbank accession number	
						Cytochrome *b*	D-loop
O. madagascariensis	Madagascar: Toliara Province, Bishiko Cave	23.548° S, 43.767° E	FMNH 209264	F	38	KJ433741	KJ433800
- " -	- " -	- " -	FMNH 209265	M	38	KJ433742	KJ433801

Museum samples: DM – Durban Natural Science Museum; NMK – National Museum of Kenya; NMP – National Museum of the Czech Republic, Prague. Field collection samples: DBN – Durban; DP – Durban Pinetown; FMNH – Field Museum of Natural History; KZN – KwaZulu-Natal; pb – Petr Benda; RSA – Republic of South Africa; UADBA – Université d'Antananarivo, Département de Biologie Animale.

CHAPTER SIX:

Revision of Afro-Malagasy *Otomops* (Chiroptera: Molossidae) with the Description of a New Afro-Arabian Species

Zootaxa 4057 (1): 001–049
www.mapress.com/zootaxa/
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Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

http://dx.doi.org/10.11646/zootaxa.4057.1.1
http://zoobank.org/urn:lsid:zoobank.org:pub:59548C19-0DE6-40BA-9956-45484E2EFB95

Revision of Afro-Malagasy *Otomops* (Chiroptera: Molossidae) with the description of a new Afro-Arabian species

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Abstract

The paucity of data for the molossid bat *Otomops* throughout its range has hindered our ability to resolve the number of *Otomops* species present within the Afro-Malagasy region (including the Arabian Peninsula). This paper employed an integrative approach by combining morphometric (cranial morphology) and molecular (mitochondrial cytochrome *b* and D-loop sequences, nuclear intron sequences and microsatellites) data to identify the number of *Otomops* taxa occurring in the Afro-Malagasy region. Three taxa were identified, two of which could be assigned to existing species, i.e. *O. martiensseni* and *O. madagascariensis*. The third taxon, previously recognised as *O. martiensseni* (Matschie 1897), is described herein as a new species, *Otomops harrisoni* **sp. nov.**, and can be differentiated from *O. martiensseni* s.s. based on both molecular and morphometric data. Locality data of specimens belonging to *O. harrisoni* suggest that its distribution range extends from the Arabian Peninsula through to Eritrea and south to Ethiopia and Kenya.

Key words: systematics, molecular genetics, morphometrics, ecological niche modelling, northeastern Africa

Introduction

The Palaeotropical genus *Otomops* Thomas, 1913 (Molossidae) currently includes seven recognised species (Simmons 2005; Hutcheon & Kirsch 2006). Five of these are distributed in the Oriental region, including southern India, Java, Papua New Guinea, Cambodia, the Philippines and Indonesia (Alor Island), which suggests that this genus may have an Oriental origin (Lamb *et al*. 2008). The other two species have a wide but somewhat sparse distribution throughout the Afro-Malagasy region, including the Arabian Peninsula (Peterson *et al*. 1995; Simmons 2005). *Otomops martiensseni* (Matschie 1897) is known from Yemen on the Arabian Peninsula (Al-Jumaily 1999) and the African mainland from South Africa in the south, to Ethiopia and Eritrea in the northeast (Kock & Zinner 2004) and Ivory Coast to the west (Lamb *et al*. 2008). *Otomops madagascariensis* Dorst, 1953 mostly occurs on the drier, western parts of Madagascar (Goodman & Raherilalao 2014).

Otomops martiensseni and *O. madagascariensis* are considered to be separate species (Simmons 2005; Lamb *et al*. 2008) although *O. madagascariensis* was formerly classified as a subspecies of *O. martiensseni* (Long 1995). Historically, there has also been debate regarding the existence of *O. icarus* Chubb 1917 (Meester *et al*. 1986; Simmons 2005). *Otomops martiensseni* is the only mainland African species, although *O. icarus* (Durban, South Africa) was once considered a species, subspecies or synonym of *O. martiensseni* (Chubb 1917; Long 1995; Mickleburgh *et al*. 2008). Lamb *et al*. (2006) showed that populations from east Africa and South Africa are distinct but have low divergences in mitochondrial cytochrome *b* (2.50%) and D-loop sequences. Nuclear data (PCR-RAPDs), however, revealed an opposing result with high genetic similarities between east African and South African individuals (Lamb *et al*. 2006). Fenton *et al*. (2002) reported morphological differences between

Accepted by P. Velazco: 22 Oct. 2015; published: 9 Dec. 2015

specimens from Madagascar and/or Durban versus those from east Africa, the latter appearing significantly larger. Peterson *et al*. (1995) used multivariate analyses of craniodental and external characters to support Dorst's (1953) view that *O. madagascariensis* is a distinct species. The above morphological and genetic analyses suggest that there are two or perhaps three species within this species group.

More recently Lamb *et al*. (2008) identified three reciprocally-monophyletic cytochrome *b* and D-loop clades of Afro-Malagasy *Otomops*: *O. madagascariensis*, *O. martiensseni* from eastern and northeast Africa (Ethiopia, Kenya and Yemen) and *O. martiensseni* from southern (Burundi, South Africa, Tanzania and Zimbabwe) and western (Ivory Coast) Africa. The morphometric data of Richards *et al*. (2012) also supports the recognition of the above three Afro-Malagasy *Otomops* lineages, and includes additional *O. martiensseni* samples from previously unsampled regions of eastern, western and southern Africa. The aim of this study was to resolve the taxonomic status of these three clades by analysing an expanded dataset of mitochondrial and nuclear DNA markers, as well as cranial morphology.

Material and methods

Sampling. Material for both molecular and morphological datasets was obtained from 15 museums, including associated acronyms, and sampling of individuals from extant roost colonies (Appendix 1). Samples for DNA analyses were stored in lysis buffer or 90% ethanol and comprised heart, lung, liver, kidney and/or thoracic muscle tissue. Wing punches of individuals from Durban, South Africa, were collected under Ezemvelo KZN Wildlife and 'ToPS' permit numbers OP 853/2009 and OP 360/2013. Figure 1 illustrates the areas of the Afro-Malagasy region that were sampled.

Molecular data. *Mitochondrial cytochrome* b *and D-loop*. The methods used for obtaining DNA sequence data from the mitochondrial cytochrome *b* and D-loop regions are presented in Lamb *et al*. (2008) and include DNA isolation, PCR amplification of both regions and DNA sequencing protocols. Due to the relatively smaller sample size (n = 60) used in Lamb *et al*. (2008), the molecular dataset was expanded (n = 106) to include additional samples from across the Afro-Malagasy region (Appendix 1). Mitochondrial cytochrome *b* and D-loop sequences were placed into a concatenated dataset comprising 1322 nucleotides and jModelTest v.0.1.1 (Guindon & Gascuel 2003; Posada 2008) was applied using the Akaike Information Criterion (AIC) to determine the most appropriate model of evolution (GTR+I+G) for use in Bayesian analyses. Relative genetic p-distances between and within the groups were calculated in PAUP* v.4.0b10 (Swofford 2002). The dataset was analysed using maximum parsimony in PAUP* v.4.0b10 and Bayesian Inference as implemented in MrBayes v.3.1.2 (Huelsenbeck & Ronquist 2001). Samples from Tanzania and Zimbabwe were analysed on the basis of cytochrome *b* data only (1012 nucleotides) as we were unable to amplify the D-loop region of these samples; their relative positions have however been included in the resulting tree. For maximum parsimony analysis starting trees were obtained by stepwise addition. The tree-bisection-reconnection branch-swapping algorithm was used with a random addition sequence in which 1 tree was held at each step, with 10 replicates. One thousand bootstrap replicates were carried out using a heuristic search. Bayesian analyses were run using four Markov chains for 5 million generations each, sampling every 100 generations, ensuring that the standard deviation of the split frequencies was less than 0.01. The chains were heated with the temperature scaling factor T = 0.02. The first 50,000 trees were discarded as burn-in, in each case having checked in a preliminary run that this was more than sufficient to achieve stationarity. A 50% majority-rule consensus tree was constructed from the remaining trees.

Nuclear intron sequence data. Genomic DNA was isolated from 7 molossid species (n = 9), *Otomops martiensseni* s.s., *O. harrisoni* **sp. nov.**, *O. madagascariensis*, *Mops leucostigma*, *M. condylurus*, *Mormopterus francoismoutoui* and *M. jugularis*, using the DNeasy® DNA isolation kit (QIAGEN). A total of 5 nuclear introns were PCR amplified: feline sarcoma proto-oncogene (FES), growth hormone receptor (GHR), rhodopsin (RHO1) (Venta *et al*. 1996), protein-kinase C1 (PRKC1) (Matthee *et al*. 2001) and pyridoxine 5'- phosphate oxidase intron 3 (PNPO-Intron 3) (Igea *et al*. 2010). The optimised PCR amplifications were performed in 25 µl reactions containing 30-60 ng template DNA, 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Fermentas), 0.2 µl 5 U/µl *Taq* polymerase (Super-Therm) and 4 µl of 6 µM primer dilution (forward and reverse) per reaction (primer sequences available from Venta *et al*. 1996, Matthee *et al*. 2001 and Igea *et al*. 2010). The thermal cycling parameters used were as follows: 95 °C for 5 min; followed by 39 cycles of (95 °C for 30 s, primer-specific

annealing temperature for 30 s and 72 °C for 2 min); followed by 72 °C for 10 min. Primer-specific annealing temperatures were as follows: FES: 58 °C; GHR: 60 °C; RHO1: 55 °C; PRKC1: 55 °C; PNPO-Intron 3: 55 °C. Sequencing was performed on an ABI 3500 Genetic Analyzer (Applied Biosystems) at Inqaba Biotec, Pretoria, South Africa. Nuclear intron data from all 5 markers were placed into a concatenated data set comprising 2216 nucleotides. The dataset was then analysed using TCS v.1.21 (Clement *et al*. 2000) to create a statistical parsimony network at a 95% confidence (20 step connection limit) to illustrate possible connections between samples.

Nuclear microsatellite repeats. Methods used for the amplification of nuclear microsatellite repeats are presented in Ralph & Lamb (2013), detailing the optimal annealing temperatures, amplification reagents, primers and thermal cycling parameters for each of the six loci used. Every individual (n = 71) was genotyped separately to avoid any complications incurred by multiplex reactions. Patterns of genetic clustering among individuals were assessed using STRUCTURE v.2.3.4 (Pritchard *et al*. 2000), where six runs for each *K* were implemented ranging from the minimum to maximum number of sites per lineage, i.e. 1 to 6. Clustering patterns were investigated to determine the number of populations, assign individuals to these groups and to determine whether the groupings observed corresponded to geographic locality. FSTAT v.2.9.3.2 (Goudet 2002) was used to calculate the expected (H_E) heterozygosities for loci within each population.

Morphology. *Topotypic material*. Data and multivariate analyses presented in the current study are derived from Richards *et al*. (2012) and further investigations that include topotypic material of Indo-Australasian *Otomops*. We extended the previous study to include six of the seven currently recognised *Otomops* taxa: *O*. cf. *formosus* (Indonesia), *O. papuensis* (southeast Papua New Guinea), *O. secundus* (northeast Papua New Guinea), *O. wroughtoni* (India and Cambodia), *O. madagascariensis* (Madagascar) and *O. martiensseni* (Africa and Arabian Peninsula). We were unable to examine individuals of the seventh species *O. johnstonei*; where possible, we have included published morphological data on this species for comparative purposes. Specimens of *O. martiensseni* were sampled from several African countries: Burundi, Central African Republic, Ivory Coast, Democratic Republic of Congo, Djibouti, Ethiopia, Kenya, Malawi, South Africa, Tanzania, Uganda, Zambia, Zimbabwe and Yemen. Holotypes and paratypes examined in this study (n = 11): *O. madagascariensis*, type locality south of Soalala, Namoroka, Réserve naturelle intégrale no. 8, Madagascar, (MNHN 1953-1590); *O. martiensseni*, type locality Magrotto plantation, southeast Usambara Mountains, Tanzania (MNHU 97523); *O. secundus*, type locality Tapu, Madang Province, Papua New Guinea (BMNH 50.979-50.982); *O. wroughtoni*, type locality Barapede Cave, near Talewadi, South India (BMNH 12.11.24.1, BMNH 13.4.9.1-13.4.9.2, BMNH 13.4.9.4-13.4.9.6).

Craniodental morphometrics. Specimens were aged based on the degree of cusp degradation of maxillary molars, as well as skull size and shape (see Richards *et al*. 2012). Six age-classes were identified. Only adults assigned to age classes 4–6 were included in the study. Because Afro-Malagasy *Otomops* display significant heterogeneity in sexual dimorphism in cranial size and shape (Richards *et al*. 2012; Richards unpublished data), the sexes were segregated in analyses. Three morphometric data sets (craniodental measurements, dorsal and ventral landmark data) were recorded from *Otomops* crania. Analyses of these data sets produced consistent results, thus only the results of analyses of craniodental measurements, dorsal landmark data of males and ventral landmark data of females are presented herein.

Nine cranial and three dental measurements, defined in Richards *et al*. (2012), were recorded from 182 *Otomops* spp. specimens (n = 89 males, n = 93 females) by LRR using Mitutoyo callipers to 0.01 mm accuracy. The cranial measurements included: greatest skull length (GSL), braincase height (BCH), braincase breadth (BCB), mastoid breadth (MB), zygomatic breadth (ZB), inter-orbital width (IOW), palatal length (PL), tympanic bulla length (TBL), and moment arm of temporalis (MAT). The dental measurements included: maxillary toothrow length (MTR), maxillary inter-canine width (C^1C^1), and mandibular toothrow length (LTR). Principal components analyses (PCA) were performed on variance-covariance matrices of $\log_{10}$-transformed craniodental measurements of males and females, using IBM SPSS Statistics v.21 (IBM Corp. 2012) to explore patterns of morphological variation in the six Afro-Malagasy and Indo-Australasian taxa examined in this study.

Digital images of the dorsal (n = 102 males, n = 92 females) and ventral (n = 87 males, n = 91 females) views of *Otomops* spp. skulls were captured using a Fujifilm FINEPIX S8100 digital camera (Fujifilm Corporation, Japan) mounted on a tripod (x18 optical zoom, 5 megapixel resolution, 25 mm focal length, macro function). Fourteen dorsal and 16 ventral landmarks (see Richards *et al*. 2012) were captured in two dimensions from cranial images using the software program tpsDig, v.2.15 (Rohlf 2010a). Landmarks were only recorded from the left half of dorsal views and right half of ventral views of crania to avoid the effects of bilateral asymmetry. Analyses of

dorsal and ventral data sets showed minimal error levels with respect to image capture and landmark placement (data not shown). The programme tpsRelw, v.1.45 (Rohlf 2010b) was used to conduct a Generalized Procrustes Analysis (GPA) of landmark data sets. Landmark configurations of each individual were translated, rotated, scaled and superimposed to derive a consensus configuration of all specimens analysed. The GPA residuals variation was then decomposed into affine (*U*1 and *U*2) and non-affine (partial warp scores) components of shape change. Patterns of interspecific shape variation amongst Afro-Malagasy and Indo-Australasian individuals were investigated by PCA of the total shape matrix (U + W) for dorsal and ventral data sets of males and females using MorphoJ software (Klingenberg 2011). Thin plate splines that depict morphological shape changes were generated using tpsRelw (Rohlf 2010b).

Ecological niche modelling. The MaxEnt algorithm v.2.3 (Phillips *et al.* 2006) was used to predict the potential geographic ranges of two groups of Afro-Arabian *Otomops* spp. throughout sub-Saharan, central and western Africa as well as the Arabian Peninsula. The predicted environmental limits allow for the inference and comparison of the potential distribution of each group. Input data included the georeferenced distribution records (recorded to 0.001 decimal degrees) of Afro-Arabian *Otomops* (n = 30 records south/east/central/west OTU; n = 18 records northeast OTU) obtained from museum and literature records (dataset available from TMCR). Nine continuous environmental variables (WORLDCLIM database v.1.4, Hijmans *et al.* 2005) were used as predictor variables in the model, including: ALT1 (altitude); BIO1 (annual mean temperature); BIO4 (temperature seasonality); BIO5 (maximum temperature of warmest month); BIO6 (minimum temperature of coldest month); BIO12 (annual precipitation); BIO13 (precipitation of wettest month); BIO14 (precipitation of driest month) and BIO15 (precipitation seasonality). Variables were uncorrelated with a maximum value of 0.76. Bioclimatic data (grid files) were sampled at a spatial grid resolution of 2.5 arc minutes (approximately 5 km). Spatial grids of the bioclimatic variables were converted to ASC files using ArcMap v.9.3. Models were run separately with all distribution records for each respective species, a regularisation multiplier of 1.0, a maximum number of 1000 iterations and 5 replicates. Other MaxEnt settings were set to the default and the relative and absolute contribution of each bioclimatic variable to the model was assessed using the jackknife procedure in MaxEnt.

Results

Molecular analyses. *Mitochondrial cytochrome* b *and D-loop*. A concatenated data set comprising 1322 nucleotides of cytochrome *b* and D-loop data was analysed under the phylogenetic species concept using Bayesian and maximum parsimony methods; 15.20% of the sites were variable and 12.60% were parsimony informative. As both analyses produced largely congruent trees, we present a single tree showing nodal support values derived from both methods (Fig. 2). All *Otomops* spp. samples formed a strongly supported monophyletic clade (bootstrap support: 100%; posterior probability: 1.00) relative to the outgroups. The SECW (southeast, central and west African) clade comprises samples from Burundi, Ivory Coast, South Africa, Tanzania and Zimbabwe; the NEA (northeast African) clade comprises individuals from Ethiopia, Kenya and Yemen, whereas the MAD (Malagasy) clade comprises specimens from Madagascar. The position of the outgroups relative to the ingroups was also congruent across both resulting trees. Major clades within the tree were well-resolved and well-supported (clades A to G) (Fig. 2). Afro-Malagasy *Otomops* samples (clade C) divide into 2 reciprocally monophyletic clades (clades D and G) where clade G, comprising 17 haplotypes (19 samples) from Madagascar, and clade D, containing 54 African and Arabian haplotypes (87 samples), are separated by an average genetic p-distance of 3.30%. Clade D further subdivides into 2 reciprocally monophyletic sister lineages (subclades E and F) and are separated by a genetic p-distance of 2.10%. There is stronger support for the existence of discrete groups from SECW (subclade E; bootstrap support: 100%; posterior probability: 1.00) and NEA (subclade F; bootstrap support: 100%; posterior probability: 1.00) than there is for the existence of their combination into the currently-circumscribed *O. martiensseni* from mainland Africa and Yemen (clade D; bootstrap support: 90%; posterior probability: 0.88). This suggests that the subclades E and F are stronger groups than is clade D and supports their status as separate species. The mean p-distances within groups were also low within subclades E (0.70%) and F (0.30%) and within the Madagascar clade (G; 0.60%). By comparison, the mean within group p-distance for clade D was much larger (1.60%) than the distances within subclades E and F.

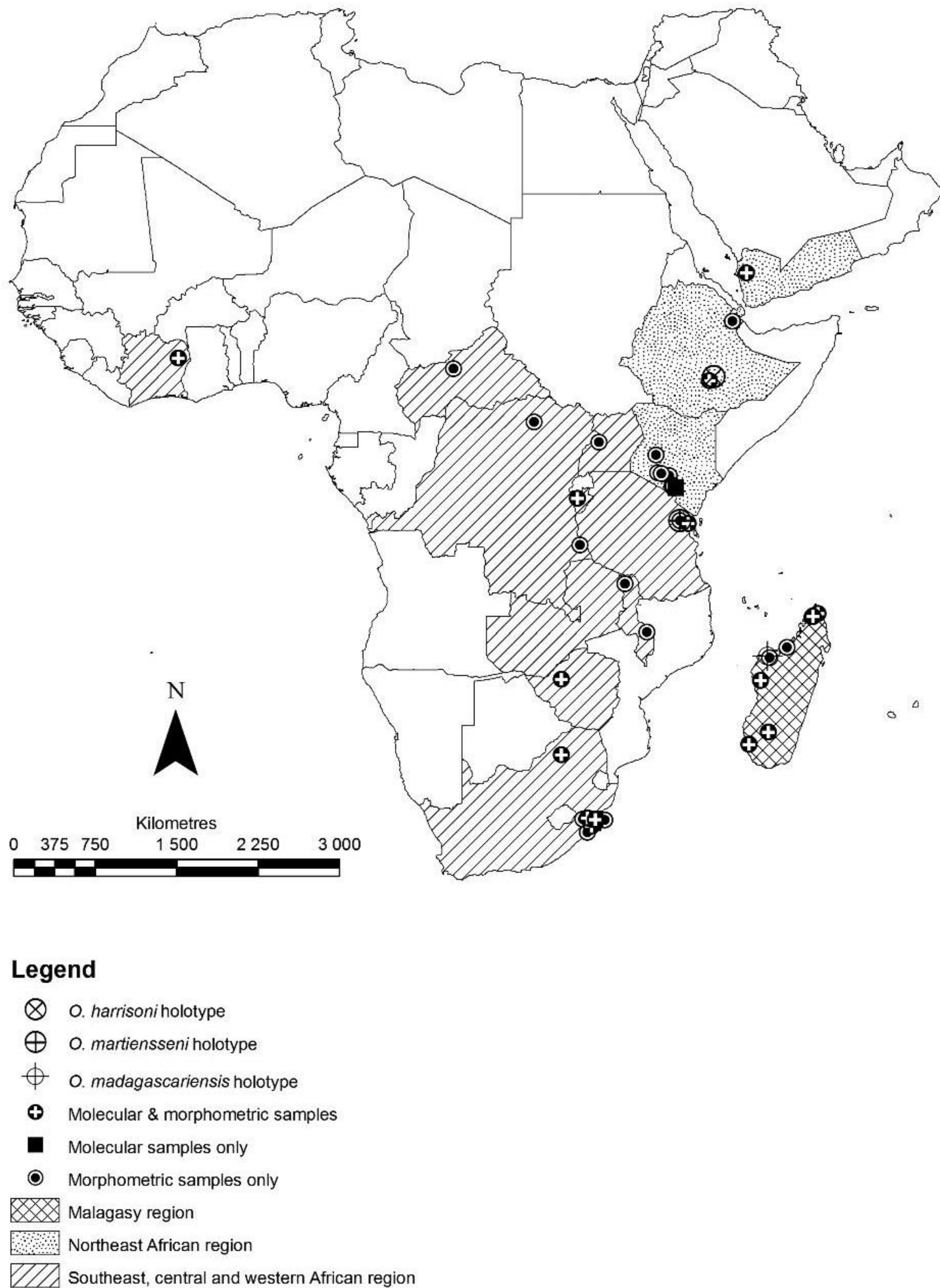

FIGURE 1. Map of the Afro-Malagasy region (including the Arabian Peninsula) depicting sampling regions and localities for specimens used for morphological and molecular analyses in this study and localities for holotype specimens.

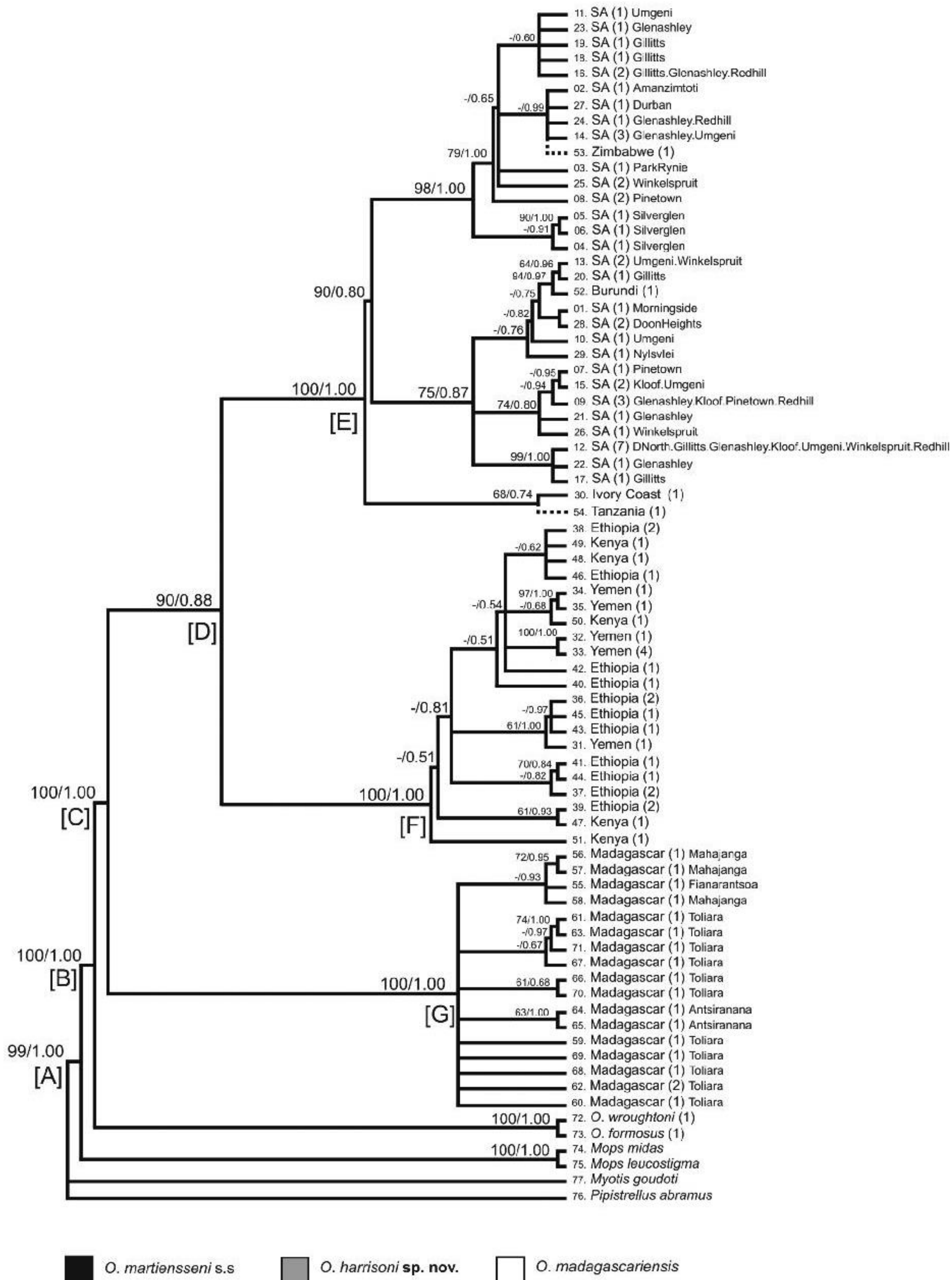

FIGURE 2. Tree based on maximum parsimony and Bayesian analyses of 1322 nucleotides of the concatenated mitochondrial cytochrome *b* gene and D-loop region sequences, depicting the relationship between 71 haplotypes of Afro-Malagasy *Otomops* species with respect to outgroups *O. wroughtoni*, *O. formosus*, *Mops midas*, *M. leucostigma*, *Pipistrellus abramus* and *Myotis goudoti* (haplotypes 72–77) (Appendix 1). Bootstrap and posterior probability support is given at the nodes according to parsimony (beginning) and Bayesian (end) analyses and dotted lines indicate relative positions of samples based on 1012 nucleotides of cytochrome *b* data only. SA = South Africa.

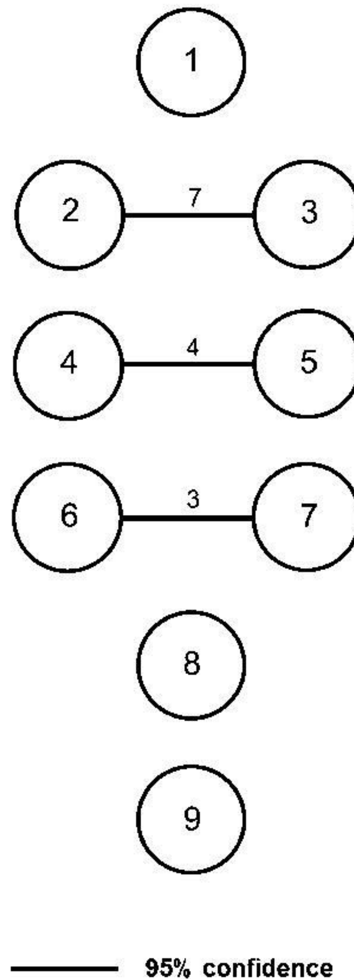

FIGURE 3. Six statistical parsimony networks formed at the 95% confidence level in an analysis of Afro-Malagasy region molossids based on 2216 nucleotides of 5 concatenated nuclear regions (FES, GHR, RHO1, PRKC1, PNPO-Intron 3). Numbers next to connection branches indicate the number of mutational steps between samples and solid lines indicate connection and confidence level. Numbers correspond to samples as follows: 1—*Otomops martiensseni* s.s. (South Africa), 2—*O. harrisoni* **sp. nov.** (Ethiopia), 3—*O. harrisoni* **sp. nov.** (Kenya), 4—*O. madagascariensis* (Madagascar), 5—*O. madagascariensis* (Madagascar), 6—*Mops leucostigma* (Madagascar), 7—*M. condylurus* (South Africa), 8—*Mormopterus francoismoutoui* (La Réunion) and 9—*M. jugularis* (Madagascar). Sample details are indicated in Appendix 1.

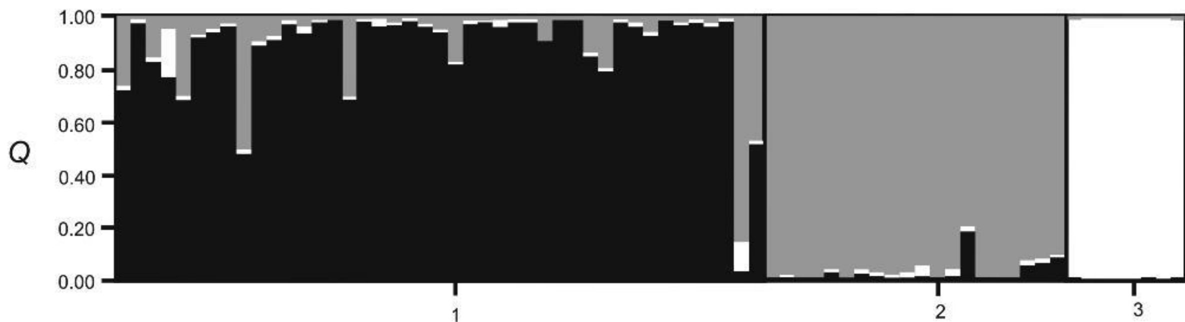

FIGURE 4. Graph illustrating probabilities of assignment of individuals to different genetic clusters (*Q*), where the number of clusters (*K*) = 3. Each column corresponds to an individual and each colour within the column corresponds to the relative likelihoods of that individual belonging to each of the 3 defined clusters. Numbers on the x-axis correspond to geographical areas: 1 = eastern region of South Africa (n = 43), 2 = northeast Africa (n = 20) and 3 = Madagascar (n = 8).

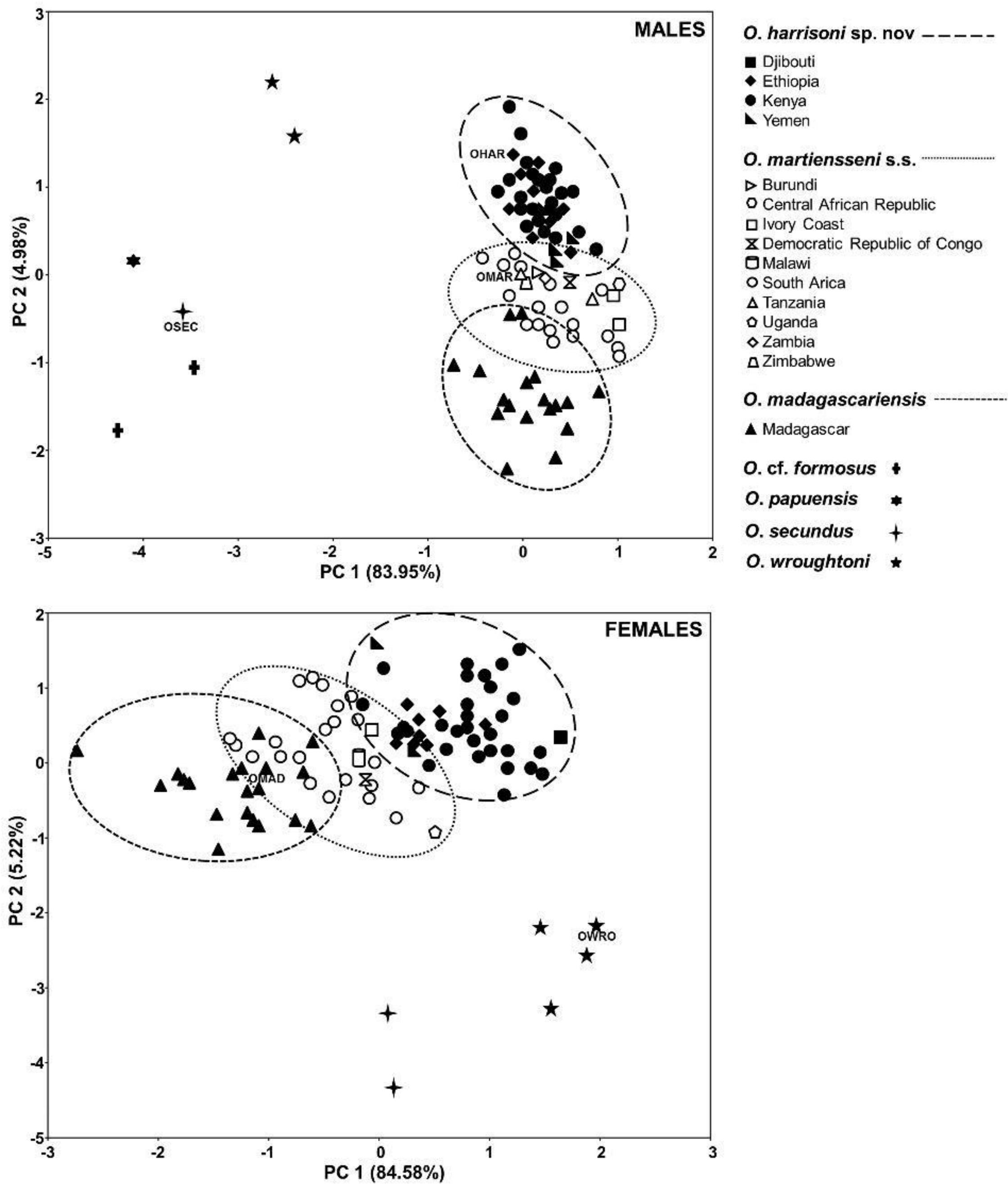

FIGURE 5. Biplot showing the first two components of principal component analyses of log–transformed craniodental variables of male and female *Otomops*. Holotypes: OHAR-*O. harrisoni* **sp. nov.**, OMAD-*O. madagascariensis*, OMAR-*O. martiensseni*, OSEC-*O. secundus*, OWRO-*O. wroughtoni*.

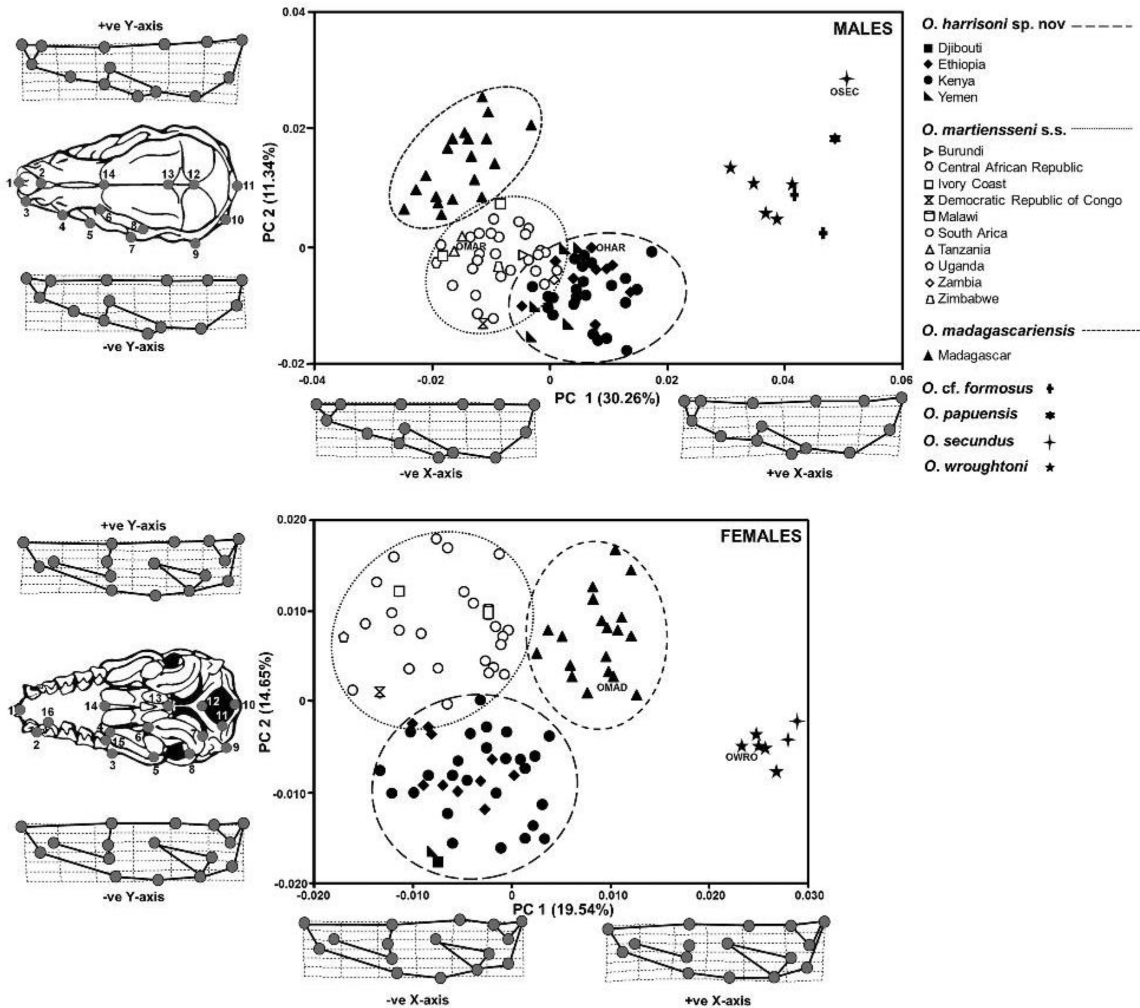

FIGURE 6. Biplot of the first two components of a principal component analysis of dorsal landmark data for males and ventral landmark data for females. Holotypes: OHAR-*O. harrisoni* **sp. nov.**, OMAD-*O. madagascariensis*, OMAR-*O. martiensseni*, OSEC-*O. secundus*, OWRO-*O. wroughtoni*. The thin plate splines depict deformations at the relevant skull landmarks (3x exaggerated).

Assuming the existence of two species, from SECW and NEA respectively, one would need to retain the name *O. martiensseni* and the other would receive a new name. The holotype of *O. martiensseni*, from Magrotto plantation in Tanzania, is included in the SECW clade based on morphometric analyses (Figs. 5–6). It was not possible to obtain DNA sequence data from the holotype, however the other experimental sample from Tanzania was also included in the SECW clade based on both morphometric and cytochrome *b* sequence (bootstrap support: 100%; posterior probability: 1.00) data (Figs. 2, 5–6). Additionally, genetic distances support this finding: p-distances between Tanzania and the remainder of the SECW clade and the NEA clade are 0.50% and 2.50%, respectively, indicating that the Tanzanian specimen used shows a closer association to the SECW clade. Based on this we propose that the members of the SECW clade retain the name *O. martiensseni*. Samples from the NEA subclade (F) would therefore constitute a new species: *O. harrisoni*, described herein. This currently comprises samples from Ethiopia, Kenya and Yemen, including the designated holotype HZM 60.36217 from Ethiopia (haplotype number 42). Studies based on more extensive samples would be needed to clarify whether there is any overlap in the distributional ranges of *O. martiensseni* s.s. and *O. harrisoni* especially in the area surrounding the

Kenya/ Tanzania border where the current ranges of the two clades abut. There is little to no structuring based on geographical locality within each of the major clades. Indo-Australasian *Otomops* representatives, *O. wroughtoni* and *O. formosus*, appear sister to the Afro-Malagasy species. *Otomops formosus* and *O. wroughtoni* were each separated by similar p-distances (10.20% and 9.60%, respectively) from each of the three Afro-Malagasy *Otomops* clades and *O. formosus* and *O. wroughtoni* were separated by a p-distance of 6.20%.

Nuclear intron sequence data. In the concatenated data set of 2216 nucleotides from 5 nuclear introns (FES, GHR, RHO1, PRKC1 and PNPO-Intron 3), 17.80% of the sites were variable and 10.00% were parsimony informative. Sequences were analysed using parsimony to create a statistical network (Fig. 3). When analysed at a 95% connection limit, 6 separate networks were formed, corresponding to: *O. harrisoni* (samples 2 and 3); *O. martiensseni* s.s. (sample 1); *O. madagascariensis* (samples 4 and 5); *Mops leucostigma*/*M. condylurus* (samples 6 and 7); *Mormopterus francoismoutoui* (sample 8); and *M. jugularis* (sample 9). The closely related *Mops condylurus* and *M. leucostigma* form a single network, but this is expected given that cytochrome *b* data analysis shows strong support for a monophyletic *Mops* clade, including *M. leucostigma*, *M. condylurus* and *M. midas* (Ratrimomanarivo *et al*. 2008).

Nuclear microsatellite repeats. STRUCTURE analysis clearly reveals genetic structure at a regional level (SECW, NEA, MAD), where the model $K = 3$ best fits the data (Fig. 4). Identical clustering patterns with similar cluster membership (Q) values were created for each $K = 3$ run. Local population structure appeared weak; clusters based on geographical location of collection sites could not be identified and individuals were inter-mixed across the various localities within each region. Dispersal movements across the three regions were not detected as all individuals were assigned to the region from which they were sampled. Nuclear microsatellite data analysed in STRUCTURE assignment tests showed high level gene flow among samples within the same regions (SECW, NEA, MAD), irrespective of distance between localities; maximum distances between colonies in SECW and NEA regions are ~37 km and 2250 km, respectively. Evidence of high gene flow, indicative of a random mating structure among individuals (Bogdanowicz *et al*. 2012), was also provided by the moderate to high expected heterozygosity (H_E) statistics within each of the Afro-Malagasy *Otomops* groupings: SECW = 0.7, NEA = 0.6 and MAD = 0.5.

Morphology. *Craniodental morphometrics*. In general, *Otomops harrisoni* is the largest taxon within the genus, both in overall body size (Table 1) and in cranial size (Table 2). The first three components from PCA of craniodental measurements explained 93.78% of morphological variation in males and 93.51% in females (Table 3). Males from the Afro-Malagasy region separated along PC2, as did those from the Indo-Australasian region, whereas females from the two biogeographic regions separated along PC1 (Fig. 5). Afro-Malagasy males separated from Indo-Australasian males along PC1, whereas the females from those regions separated along PC2. Palatal length (PL) and maxillary toothrow (MTR) loaded the highest on PC2 for females (Table 3), indicating that separation of African from Asian females was largely attributed to morphological changes localised within the palatal and rostral region. Afro-Malagasy males separated along PC2 into three distinguishable taxa representing *O. madagascariensis*, *O. martiensseni* s.s. and *O. harrisoni* (Fig. 5). Afro-Malagasy females separated according to size into three slightly overlapping groups, along PC1 (Fig. 5). Based on factor loadings presented in Table 3, male and female *O. harrisoni* were distinguished from other African individuals by greater inter-orbital width and proportionately wider braincases and greater braincase height (excl. tympanic bullae).

Principal component scatter plots based on landmark data for male and female *Otomops* show that Indo-Australasian *Otomops* were well separated from Afro-Malagasy individuals (Fig. 6). Indo-Australasian species were, in general, characterised by short nasals that were laterally flared, short yet broad rostra, broad occipital ridges, pointed supraoccipitals and long, wide braincases. As with craniodental measurements, dorsal landmark data indicated three morphologically identifiable Afro-Malagasy taxa (Fig. 6). Compared to Indo-Australasian *Otomops*, crania of African taxa were characterised by proportionately longer rostra, and round and shorter braincases. The crania of *O. harrisoni* were characterised by proportionately broader rostra, a less angular braincase (with reference to the mastoid region), larger nasals, and broader, longer bullae relative to *O. martiensseni* s.s. and *O. madagascariensis*.

TABLE 1. External measurements (mm) and mass (g) of Afro-Malagasy and Indo-Australasian *Otomops* species including *Otomops harrisoni* **sp. nov.** Mean, standard deviation, range and sample size (n) are provided. Measurements for all specimens, including those of the referred specimens are based on museum specimen records and published data. Hindfoot length is generally reported without the claw, unless otherwise stated.

Taxon	Total length	Tail length	Hindfoot length	Ear length	Forearm length	Body mass
O. harrisoni **sp. nov.** (males)						
Holotype HZM 60.36217[1]	151.2	47.3	14.5	38	72.8	–
Paratypes included in this study[1]	148.2 ± 4.55	48.4 ± 5.45	13.0 ± 0.26	35.9 ± 0.49	69.5 ± 1.42	–
	143.2–152.1, n=3	43.0–53.9, n=3	12.7–13.1, n=3	35.3–36.2, n=3	68.4–71.1, n=3	
Other referred specimens	151.1 ± 5.48	49.6 ± 2.92	13.2 ± 1.27	39.8 ± 2.37	70.7 ± 1.92	38. 8 ± 3.75
	139.5–163.0, n=33	42.6 –54.0, n=33	11.0–16.0, n=33	35.9–46.0, n=33	63.8–74.0, n=37	31.5–45.0, n=31
O. harrisoni **sp. nov.** (females)						
Paratypes included in this study[1]	139.5 ± 5.15	47.2 ± 3.48	13.4 ± 1.23	35.9 ± 1.44	69.1 ± 1.26	-
	130.0–145.0, n=10	40.0–53.1, n=10	12.0–16.1, n=10	33.6–36.8, n=10	67.2–70.8, n=10	
Other referred specimens[1]	151.1 ± 5.25	49.0 ± 4.48	13.0 ± 1.00	38.1 ± 1.94	69.5 ± 1.63	39.2 ± 4.77
	138.0–158.0, n=27	40.0–58.0, n=28	11.27–15, n=28	34.0–41.0, n=28	65.7–72.7, n=28	26.8–45.0, n=24
O. martiensseni (males)						
Holotype MNHU 97523[2]	–	43	–	37	66	–
Other specimens[1]	140.9 ± 8.50	44.4 ± 4.11	11.6 ± 1.12	37.6 ± 3.5	65.8 ± 1.60	31.3 ± 4.17
	130.0–155.0, n=15	39.0–52.0, n=17	10.0–14.0, n=16	32.0–42.0, n=14	60.5–68.0, n=23	25.0 –38.0, n=6
O. martiensseni (females)						
Adult specimens[1]	136.4 ± 6.41	43.2 ± 2.44	10.9 ± 1.25	34.6 ± 3.72	63.5 ± 1.53	28.2 ± 2.31
	127.0–148, n=18	40.0–49.0, n=18	9.0 -13.0, n=19	29.0–41.0, n=20	60.0–66.3, n=22	25.0–32.0, n=9
O. madagascariensis (males)						
Adult specimens[1]	139.3 ± 3.82	43.8 ± 3.27	9.8 ± 0.79	40.2 ± 1.32	63.2 ± 1.32	26.2 ± 1.94
	132–146, n=20	38–50, n=20	8.0–11.0, n=20	36.0–42.0, n=20	60.0–66.0, n=20	22.0–28.0, n=20
O. madagascariensis (females)						
Holotype MNHN 1985-1590[3]	128.3	44.5	–	31.3	62.6	–
Other specimens[1]	133.4 ± 4.47	41.3 ± 4.01	9.4 ± 0.63	37.3 ± 1.84	61.1 ± 1.31	24.2 ± 2.07
	126.0–142.0, n=16	34.0–49.0, n=16	9.0–13.0, n=16	33.0–41.0, n=16	60.0–64.0, n=16	19.5–25.5, n=16
O. wroughtoni (males)						
Other specimens including paratypes included in this study[1,4]	136.5 ± 4.88	44.2 ± 4.41	12.2 ± 1.38	32.7 ± 1.34	66.1 ± 0.64	–
	127.9–142.0, n=6	36.1–49.0, n=5	10.4–14.0, n=6	31.5–34.8, n=6	65.6–66.5	

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TABLE 1. (Continued)

Taxon	Total length	Tail length	Hindfoot length	Ear length	Forearm length	Body mass
O. wroughtoni (females)						
Holotype BM 12.11.24.1[4]	145	46	10.0	33	68	–
Other specimens including paratypes represented in this study[1,4]	131.0 ± 4.11	41.5 ± 4.16	11.7 ± 1.35	33.1 ± 1.35	66.1, ± 0.64	–
	127.9–137.0, n=4	36.1–46.0, n=4	10.4–13.5, n=4	31.5–34.8, n=4	65.5–66.5, n=2	
O. papuensis (male)						
Adult specimen (BMNH 73.136)[5]	–	–	–	–	49.6	–
O. papuensis (females)						
Holotype MCZ 45769[6]	97	30	10.6*	22.2	49.2	–
Adult specimen (BMNH 73.137)[5]	–	–	–	–	50.2	–
O. formosus (males)						
Holotype RMNH 15322[7, 8]	–	43	–	30	59.7	–
Other specimens[1]	120 ± 7.07	37.5 ± 2.12	10.5 ± 0.71	29.5 ± 0.71	59.0 ± 0	26.5 ± 0.71
	115–125, n=2	36.0–39.0, n=2	10.0–11.0, n=2	29.0–30.0, n=2	n=2	26.0–27.0, n=2
O. formosus (female)						
Paratype[8]	–	–	–	–	57.4	–
O. johnstonei (male only, no data available for females)						
Holotype M 37986[8]	123.1	43.7	11.9	31.1	60	19.5
O. secundus (male)						
Holotype BM 50.982[9]	108	37	10	24	58	–
O. secundus (females)						
Paratypes included in this study[9]	106.0 ± 0	37.0 ± 1.41	10.0 ± 0	24.6 ± 0.42	57.5 ± 0.71	–
	n=2	36.0–38.0, n=2	n=2	24.0–24.3, n=2	57.0–58.0, n=2	

[1] Taken from collectors notes and/or museum records; [2] Matschie (1897); [3] Dorst (1953); [4] Thomas (1913); [5] Hill (1983); [6] Lawrence (1948); [7] Chasen (1939); [8] Kitchener *et al.* (1992); [9] Hayman (1952). *With claw (c.u.)

TABLE 2. Craniodental measurements (mm) of Afro-Malagasy and Indo-Australasian *Otomops* species including *Otomops harrisoni* **sp. nov.** Mean, standard deviation, range and sample size (n) are provided. GSL = greatest skull length, BCH = braincase height, MB = mastoid breadth, ZB = zygomatic breadth, IOW = inter-orbital width, BCB = braincase breadth, PL = palatal length, MTR = maxillary toothrow length, UCW = maxillary inter-canine width, LTR = mandibular toothrow length, MAT = moment arm of temporalis and TBL = tympanic bulla length. Measurements taken by LRR, including those provided from published data in the literature, unless otherwise stated.

Taxon	GSL	BCH	MB	ZB	IOW	BCB
O. harrisoni **sp. nov.** (males)						
Holotype HZM 60.36217	28.6	9.5	14.3	14.9	6.7	11.7
Paratypes	27.9 ± 0.15	9.2 ± 0.28	13.6 ± 0.05	14.1 ± 0.12	6.2 ± 0.22	11.7 ± 0.18
	27.75–28.05, n=3	8.97–9.51, n=3	13.59–13.69, n=3	13.98–14.21, n=3	5.97–6.40, n=3	11.50–11.85, n=3
Other referred specimens	28.5 ± 0.33	9.4 ± 0.17	14.0 ± 0.16	14.5 ± 0.20	6.5 ± 0.18	11.6 ± 0.22
	28.03–29.26, n=34	9.08–9.82, n=34	13.52–14.34, n=34	14.10–14.89, n=34	6.13–6.74, n=34	11.17–12.05, n=34
O. harrisoni **sp. nov.** (females)						
Paratypes	26.6 ± 0.23	9.1 ± 0.14	13.3 ± 0.12	13.7 ± 0.15	6.1 ± 0.11	11.3 ± 0.12
	26.36–27.02, n=9	8.89–9.40, n=9	13.19–13.55, n=9	13.48–14.04, n=9	5.95–6.26, n=9	11.06–11.38, n=9
Other referred specimens	27.1 ± 0.35	9.1 ± 0.18	13.5 ± 0.16	13.9 ± 0.22	6.3 ± 0.13	11.4 ± 0.21
	26.23–27.92, n=32	8.70–9.46, n=32	13.05–13.83, n=32	13.38–14.30, n=32	5.93–6.58, n=32	10.96–11.98, n=32
O. martiensseni (males)						
Holotype MNHU 97523	27.2	8.4	13.6	14.2	6.4	11.5
Other specimens	27.5 ± 0.69	8.6 ± 0.24	13.3 ± 0.42	14.0 ± 0.33	6.2 ± 0.22	11.1 ± 043
	26.52–28.78, n=27	8.21–9.01, n=27	12.13–13.78, n=27	13.39–14.43, n=27	5.73–6.47, n=27	10.59–12.13, n=27
O. martiensseni (females)						
Adult specimens	25.5 ± 0.38	8.3 ± 0.23	12.8 ± 0.20	13.2 ± 0.24	5.9 ± 0.18	10.8 ± 0.22
	24.77–26.06, n=26	7.81–8.50, n=26	12.45–13.05, n=26	12.73–13.58, n=26	5.71–6.10, n=26	10.21–11.12, n=26
O. madagascariensis (males)[1]	25.7 ± 0.48	8.2 ± 0.20	12.7 ± 0.22	12.8 ± 0.26	5.4 ± 0.22	10.5 ± 0.25
	26.53 – 28.85, n=18	7.85–8.50, n=18	12.22–13.04, n=18	12.27–13.24, n=18	4.98–5.82, n=18	10.07–10.88, n=18

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TABLE 2. (Continued)

Taxon	GSL	BCH	MB	ZB	IOW	BCB
O. madagascariensis (females)						
Holotype MNHN 1985-1590	23.8	8.2	11.9	12.0	5.0	10.5
Other specimens	23.7 ± 0.50	7.9 ± 0.17	12.0 ± 0.24	12.1 ± 0.25	5.1 ± 0.15	10.1 ± 0.28
	22.83–24.51, n=19	7.59–8.22, n=19	11.50–12.36, n=19	11.57–12.58, n=19	4.87–5.38, n=19	9.65–10.60, n=19
O. wroughtoni (males)						
Other specimens including paratypes	24.0 ± 1.09	8.8 ± 0.21	12.6 ± 0.20	13.3 ± 0.22	6.3 ± 0.27	10.8 ± 0.18
	23.24–24.78, n=2	8.60–8.89, n=2	12.41–12.69, n=2	13.10–13.41, n=2	6.08–6.46, n=2	10.69–10.95, n=2
O. wroughtoni (females)						
Holotype BM 12.11.24.1	24.8	8.8	12.9	13.3	6.2	11.2
Other specimens	24.2 ± 0.30	8.6 ± 0.07	12.4 ± 0.22	12.7 ± 0.14	6.1 ± 0.03	11.0 ± 0.05
	23.99–24.51, n=3	8.47–8.60, n=3	12.23–12.66, n=3	12.59–12.86, n=3	6.07–6.13, n=3	10.90–10.96, n=3
O. formosus (male)						
Holotype RMNH 15322[2]	23.0	–	12.4	12.4	–	10.8
Other specimens	21.5 ± 1.12	7.7 ± 0.49	11.0 ± 0.84	11.1 ± 0.68	5.1 ± 0.33	9.6 ± 0.45
	20.68–22.27, n=2	7.39–8.08, n=2	10.39–11.58, n=2	10.66–11.62, n=2	4.89–5.36, n=2	9.27–9.91, n=2
O. formosus (female)						
Paratype[1]	23.3	–	12.5	12.5	–	10.4
O. papuensis (male)						
Adult specimen (BMNH 73.136)	19.5	7.5	10.6	10.4	5.2	9.5
O. papuensis (female)						
Holotype MCZ 45769[3]	20.2	–	10.4	10.6	–	9.5
O. johnstonei (male only, no data available for females)						
Holotype M 37986[2]	23.0	43.7	11.6	11.7	–	10.4
O. secundus (male)						
Holotype BMNH 50.982	21.2	7.4	11.5	11.1	5.1	9.8
O. secundus (females)	21.3 ± 0.13	7.6 ± 0.22	11.1 ± 0.08	11.2 ± 0.02	4.9 ± 0.01	10.2 ± 0.11
Paratypes represented in this study	21.21–21.40, n=2	7.45–7.76, n=2	11.08–11.20, n=2	11.15–11.18, n=2	4.91–4.93, n=2	10.16–10.31, n=2

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TABLE 2. (Continued)

Taxon	PL	MTR	UCW	LTR	MAT	TBL
O. harrisoni **sp. nov.** (males)						
Holotype HZM 60.36217	11.6	10.8	3.4	11.6	5.4	7.1
Paratypes	11.0 ± 0.26	10.4 ± 0.31	3.1 ± 0.02	11.2 ± 0.29	5.1 ± 0.04	7.2 ± 0.27
	10.75–11.26, n=3	10.23–10.78, n=3	3.05–3.08, n=3	10.96–11.52, n=3	5.10–5.18, n=3	6.89–7.37, n=3
Other referred specimens	11.6 ± 0.21	10.5 ± 0.21	3.2 ± 0.18	11.4 ± 0.19	5.4 ± 0.14	7.0 ± 0.16
	11.23–12.02, n=34	10.13–10.99, n=34	2.88–3.66, n=34	11.01–11.83, n=34	5.18–5.74, n=34	6.70–7.50, n=34
O. harrisoni **sp. nov.** (females)						
Paratypes	10.7 ± 0.18	10.0 ± 0.11	3.0 ± 0.19	10.6 ± 0.24	5.1 ± 0.07	6.8 ± 0.17
	10.47–11.04, n=9	9.83–10.21, n=9	2.70–3.23, n=9	10.14–10.97, n=9	5.0–5.24, n= 9	6.43–7.04, n=9
Other referred specimens	10.8 ± 0.30	10.0 ± 0.24	2.9 ± 0.18	10.7 ± 0.20	5.1 ± 0.14	6.8 ± 0.20
	10.21–11.27, n=32	9.53–10.50, n=32	2.43–3.25, n=32	10.28–11.05, n=32	4.88–5.39, n=32	6.42–7.13, n=32
O. martiensseni (males)						
Holotype MNHU 97523	11	10.2	3.4	11.1	5.3	6.7
Other specimens	11.2 ± 0.49	10.2 ± 0.36	3.2 ± 0.27	11.1 ± 0.33	5.4 ± 0.26	6.7 ± 0.20
	10.41–12.00, n=27	9.69–10.66, n=27	2.75–3.60, n=27	10.55–11.55, n=27	4.93–5.70, n=27	6.25–7.10, n=27
O. martiensseni (females)						
Adult specimens	10.3 ± 0.28	9.5 ± 0.19	2.9 ± 0.18	10.2 ± 0.24	5.0 ± 0.14	6.4 ± 0.20
	9.84–10.78, n=26	9.22–9.98, n=26	2.66–3.26, n=26	9.84–10.73, n=26	4.65–5.10, n=26	6.06–6.84, n=26
O. madagascariensis (males)[1]	10.3 ± 0.25	9.5 ± 0.25	2.8 ± 0.24	10.1 ± 0.21	4.8 ± 0.18	6.3 ± 0.26
	9.99–10.78, n=18	9.08–9.98, n=18	2.43–3.23, n=18	9.67–10.43, n=18	4.43–5.11, n=18	5.87–6.73, n=18
O. madagascariensis (females)						
Holotype MNHN 1985-1590	9.5	8.8	2.4	9.1	4.3	6.2
Other specimens	9.3 ± 0.28	8.8 ± 0.21	2.5 ± 0.14	9.2 ± 0.14	4.3 ± 0.14	6.0 ± 0.24
	8.67–9.71, n=19	8.40–9.11, n=19	2.30–2.78, n=19	8.71–9.57, n=19	4.02–4.50, n=19	5.56–6.52, n=19

……continued on the next page

TABLE 2. (Continued)

Taxon	PL	MTR	UCW	LTR	MAT	TBL
O. wroughtoni (males)						
Other specimens including paratypes	9.1 ± 0.04	8.7 ± 0.04	2.6 ± 0.05	9.3 ± 0.10	4.57 ± 0.01	6.4 ± 0.22
	9.04–9.10, n=2	8.70–8.75, n=2	2.60–2.67, n=2	9.18–9.32, n=2	4.56–4.57, n=2	6.23–6.54, n=2
O. wroughtoni (females)						
Holotype BM 12.11.24.1	9.3	8.9	2.7	9.7	5	6.4
Other specimens	9.0 ± 0.20	8.7 ± 0.18	2.8 ± 0.18	9.5 ± 0.12	4.4 ± 0.06	6.5 ± 0.06
	8.79–9.18, n=3	8.48–8.83, n=3	2.59–2.94, n=3	9.38–9.61, n=3	4.37–4.49, n=3	6.42–6.53, n=3
O. formosus (male)						
Holotype RMNH 15322[2]	8.0	9.1	–	–	–	6.0
Other specimens	8.1 ± 0.63	7.7 ± 0.43	3.4 ± 0.23	8.1 ± 0.42	3.9 ± 0.44	5.5 ± 0.28
	7.66–8.55, n=2	7.37–7.98, n=2	3.20–3.53, n=2	7.81–8.41, n=2	3.62–4.24, n=2	5.27–5.67, n=2
O. formosus (female)						
Paratype[1]	8.5	–	–	–	–	5.7
O. papuensis (male)						
Adult specimen (BMNH 73.136)	7.7	6.9	1.9	7.4	3.5	4.9
O. papuensis (female)						
Holotype MCZ 45769[3]	–	–	–	7.7	–	–
O. johnstonei (male only, no data available for females)						
Holotype M 37986[2]	8.2	8.9	–	–	–	6.6
O. secundus (male)						
Holotype BMNH 50.982	7.6	7.3	2.5	7.8	4	5.6
O. secundus (females)	8.2 ± 0.04	7.0 ± 0.52	2.3 ± 0.11	7.8 ± 0.03	4.0 ± 0.02	5.6 ± 0.02
Paratypes represented in this study	8.17–8.22, n=2	6.64–7.37, n=2	2.23–2.38, n=2	7.79–7.83, n=2	4.01–4.04, n=2	5.58–5.60, n=2

[1] Richards *et al.* (2012), [2]Kitchener *et al.* (1992); [3] Lawrence (1948)

TABLE 3. Factor loadings of 12 craniodental variables on the first three principal components for *Otomops*. Principal component analyses were conducted using the variance-covariance matrices of $\log_{10}$-transformed variables of males and females. Loading values in bold indicate craniodental variables with factor loading values > 0.750.

Craniodental measurement	Males (n = 89)			Females (n = 93)		
	PC1	PC2	PC3	PC1	PC2	PC3
Greatest skull length	**0.819**	0.489	0.255	0.612	0.712	0.300
Braincase height	0.411	**0.797**	0.278	**0.771**	0.496	0.188
Mastoid breadth	0.713	0.605	0.207	0.680	0.625	0.339
Zygomatic breadth	0.704	0.635	0.260	0.657	0.598	0.417
Inter-orbital width	0.384	**0.836**	0.279	**0.810**	0.384	0.383
Braincase breadth	0.524	**0.758**	0.178	**0.853**	0.368	0.194
Palatal length	0.869	0.378	0.254	0.415	**0.836**	0.304
Maxillary toothrow length	0.840	0.439	0.257	0.442	**0.829**	0.264
Maxillary inter-canine width	0.310	0.286	**0.906**	0.247	0.300	**0.918**
Mandibular toothrow length	**0.838**	0.456	0.252	0.534	0.741	0.362
Moment arm of the temporalis	**0.819**	0.387	0.315	0.552	0.624	0.459
Tympanic bulla length	0.654	0.640	0.222	0.693	0.525	0.242
Variance explained (%)	83.948	4.977	4.856	84.583	5.215	3.716

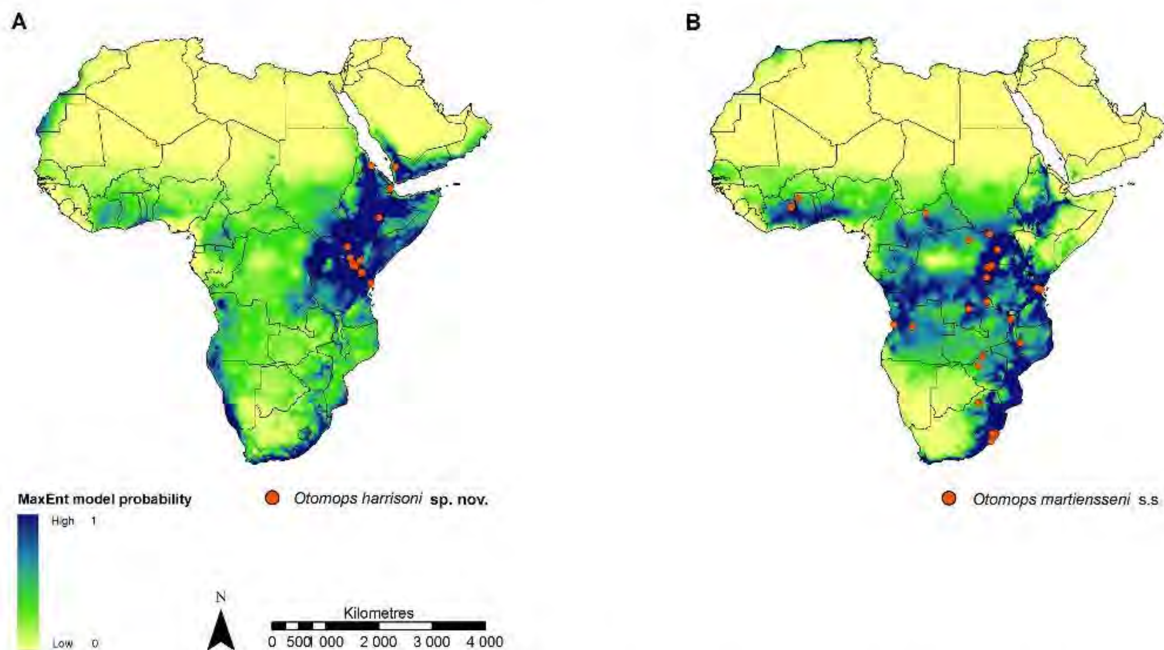

FIGURE 7. Occurrence probability for *Otomops harrisoni* **sp. nov.** (A) and *O. martiensseni* s.s. (B) in Africa and the Arabian Peninsula. Ecological niche models were generated based on current altitude and bioclimatic variables, and known occurrence records (indicated by the orange circles) for each species. Shading depicts the various grades of occurrence probability (based on habitat suitability), ranging from low (0) to high (1.00) suitability.

Ecological niche modelling. According to MaxEnt analysis, *O. harrisoni* is likely to occur mostly within the eastern regions of the sub-Saharan African mainland, including Kenya and Ethiopia, extending into Eritrea and the Arabian Peninsula (Fig. 7A). The distribution of *O. harrisoni* appears to be limited by the bioclimatic variables ALT1 (altitude), BIO4 (temperature seasonality), BIO12 (annual precipitation) and BIO14 (precipitation of driest month). Temperature seasonality limits potentially suitable habitat of *O. harrisoni* since values deviating from the

ideal result in steep declines in presence probability indicating that this species is better suited to a more stable bioclimate. This species also shows a preference for more than >20 mm of precipitation in the driest month, altitudes above 1500 m and annual precipitation below 500 mm.

Modelling results for *O. martiensseni* s.s. reveal a more extensive pattern of probable occurrence in the African region including: the coastal regions of South Africa, extending further north on the east coast through to Mozambique and eastern Tanzania, Uganda, Rwanda, Burundi and into Kenya and central Ethiopia (Fig. 7B). The potential distribution range also includes parts of the Democratic Republic of Congo in central Africa, and, further west, areas of Ivory Coast, Ghana, Togo, Benin and southwest Nigeria. Probabilities decrease as the range approaches the drier climes of the Saharan region and the Namib Desert. Distribution is mainly affected by variables BIO12 (annual precipitation), BIO14 (precipitation of the driest month) and BIO13 (precipitation of the wettest month). Areas with relatively lower annual precipitation levels (<1000 mm) present unsuitable habitats for *O. martiensseni* s.s. whereas optimal precipitation for the driest (0–40 mm) and wettest (~175 mm) months result in higher presence probabilities. Deviations from the optimum result in relatively steep declines in habitat suitability.

MaxEnt models show overlap in the potential distribution of both *Otomops martiensseni* s.s. and *O. harrisoni* in parts of Kenya, Ethiopia, Uganda and Tanzania. Annual precipitation influences the distribution of each species in contrasting ways. *Otomops harrisoni* appears more suited to drier climes, as the occurrence probability decreases as rainfall increases above 500 mm per year, whereas *O. martiensseni* s.s. appears better suited for wetter conditions, as presence probabilities decrease as rainfall decreases below 1000 mm per year. *Otomops harrisoni* appears to prefer higher altitudes, as habitat suitability decreases with altitudes below 1500 m. In contrast, *Otomops martiensseni* s.s. shows a slight drop in presence probability with increasing altitude (above sea level), however presence probability remains above 60% even at altitudes above 4000 m. Precipitation of the wettest month is a limiting factor for *O. martiensseni* s.s. and precipitation of the driest month are limiting factors for both *O. harrisoni* and *O. martiensseni* s.s.

Systematics

Family Molossidae Gervais, 1856

Genus *Otomops* Thomas, 1913

Otomops harrisoni sp. nov.

Harrison's large-eared giant mastiff bat
Figures 8–10

Synonymy

Otomops martiensseni martiensseni: Harrison, 1965:2 (part)
Otomops martiensseni martiensseni: Hill and Morris, 1971:46
Otomops martiensseni: Đulic and Mutere, 1973:62 (part)
Otomops martiensseni: Kinoti, 1973:129
Otomops martiensseni: Mutere, 1973:83
Otomops martiensseni: Epelu-Opio, 1974:229
Otomops martiensseni martiensseni: Largen *et al*., 1974:250
Otomops martiensseni: Kingdon, 1974:338 (part)
Otomops martiensseni: Warner *et al*., 1974:171 (part)
Otomops martiensseni: Kayanja and Mutere, 1975:166
Otomops martiensseni: Kayanja and Mutere, 1978:245
Otomops martiensseni: Valdivieso *et al*., 1979:6
Otomops martiensseni: Freeman, 1981:61 (part)
Otomops martiensseni: Norberg, 1981:365 (part)
Otomops martiensseni martiensseni: Aggundey and Schlitter, 1984:144
Otomops martiensseni: Fenton and Crerar, 1984:398
Otomops martiensseni: Freeman, 1984:400 (part)
Otomops martiensseni: Hickey and Fenton, 1987:381
Otomops martiensseni: Norberg, 1987:53 (part)
Otomops martiensseni: Norberg and Rayner, 1987:plate 1 (part)

Otomops martiensseni: Thollesson and Norberg, 1991:26 (part)
Otomops martiensseni: Long, 1995:1 (part)
Otomops martiensseni: Peterson *et al*., 1995:178 (part)
Otomops martiensseni: Yalden *et al*., 1996:91 (part)
Otomops martiensseni: Rydell and Yalden, 1997:72 (part)
Otomops martiensseni: Al-Jumaily, 1999:241
Otomops martiensseni: Monath, 1999:S130 (part)
Otomops martiensseni: Pearch *et al*., 2001:388
Otomops martiensseni: Debaeremaeker and Fenton, 2003:221 (part)
Otomops martiensseni: Jones and Rydell, 2003:303 (part)
Otomops martiensseni: Kock and Zinner, 2004:3
Otomops martiensseni: Kock *et al*., 2005:2
Otomops martiensseni: Taylor *et al*., 2005:26 (part)
Otomops martiensseni: Lamb *et al*., 2006:46 (part)
Otomops martiensseni: Lamb *et al*., 2008:25 (part)
Otomops martiensseni: Tong *et al*., 2009:483 (part)
Otomops martiensseni: Benda *et al*., 2011:25 (part)
Otomops martiensseni: Lamb *et al*., 2012:8 (part)
Otomops martiensseni: Patterson and Webala, 2012:5 (part)
Otomops martiensseni: Richards *et al*., 2012:913 (part)
Otomops martiensseni: Taylor *et al*., 2012:56 (part)
Otomops martiensseni: Kading *et al*., 2013:2394 (part)
Otomops martiensseni: Ralph and Lamb, 2013:4234 (part)
Otomops martiensseni: Tao *et al*., 2013:739 (part)
Otomops martiensseni: Yalden and Happold, 2013:480 (part)
Otomops martiensseni: Conrardy *et al*., 2014:259 (part)
Otomops martiensseni: Kassahun *et al*., 2015:168 (part)
Otomops martiensseni: Mortlock *et al*., 2015:1841 (part)

Holotype. HZM 60.36217 (field number A51) is part of a series of specimens collected by Paul J. J. Bates, M. J. Pearch and O. Nurhussein on 19 July 1998. This is an adult male presently preserved in 70% alcohol. The cranium and baculum have been extracted and prepared. External and craniodental measurements are presented in Tables 1 and 2, respectively. The cranium and mandible of the holotype are in good condition and are presented in figure 8. The baculum of the holotype was prepared following the methods of Hill & Harrison (1987) and Kearney *et al*. (2002), with slight modifications, and is illustrated in figure 9. This specimen was included in the morphological and mitochondrial DNA sequence-based analyses.

Type locality. Ethiopia, Bale District, Sof Omar Cave 06°54´N; 40°48´E; elevation 1340 m.

Paratypes. Thirteen adult specimens were collected from the same locality and on the same date as the holotype (n = 3 males, HZM 40.31315, HZM 44.31328, HZM 64.36221; n = 10 females, HZM 41.31316, HZM 42.31317, HZM 43.31318, HZM 56.36213, HZM 57.36214, HZM 58.36215, HZM 59.36216, HZM 61.36218, HZM 62.36219 (also DM 14750), HZM 63.36220). Measurements are provided in Tables 1–2.

Referred Specimens. Specimens originating from Djibouti, Eritrea, Ethiopia, Kenya and Yemen have been assigned to this taxon, as supported by molecular DNA and/or cranial morphological datasets, or based upon geographical location. Those specimens in bold have not been sequenced and their assignment to *O. harrisoni* is based on cranial morphological analyses alone. Djibouti: **BMNH 69.1256**, Day Forest National Park, Mount Day (11.767° N, 42.650° E, altitude 1400 m); Ethiopia: **HZM 45.31369**, **HZM 46.31370**, HZM 47.31371, **HZM 48.31372**, **HZM 49.33964**, **HZM 50.33965**, **SMF 41832**, **SMF 41833**, Bale District, Sof Omar Cave (6.900° N, 40.859° E, altitude 1300 m); Kenya: **ROM 48654–48657**, **ROM 48659–48664**, **ROM 48666–48667**, **ROM 63772**, **ROM 63779**, **ROM 63782**, **ROM 63808**, 19 km W of Makindu (2.300° S, 37.677° E, altitude 1000 m); **MRAC 38546–38549**, **SMNS 46077**, **SMNS 46079**, Chyulu Hills (2.583° S, 37.833° E, altitude 1930 m); **ROM 65875–65879**, Ithundu Caves, Kiboko (2.199° S, 37.717° E, altitude 920 m); **ROM 81198–81199**, Ithundu Caves, Makindu (2.333° S, 37.699° E, altitude 1100 m); **ROM 68360, ROM 68362, ROM 68364, ROM 68366**, Lake Baringo, Kampi Ya Moto (0.183° N, 35.867° E, altitude 1200 m); **MRAC 35264**, Machakos District (1.517° S, 37.267° E, altitude 1630 m); **ROM 78155–78158**, Makindu Cave, Makindu (2.300° S, 37.833° E, altitude 1000 m); **ROM 65873**, Makindu River (precise locality not defined); **ROM 36517**, **ROM 36519**, **ROM 41920**, **ROM 41924**, **ROM 41927–41928**, **ROM 41932**, **ROM 78147–78148**, **ROM 78151–78152**, **ROM 78154**, **ROM 91249–**

91250, Mount Suswa (1.150° S, 36.350° E, altitude 1895 m); **ROM 79677**, Nairobi (1.280° S, 36.817° E, altitude 1685 m); Yemen: **HZM 39.31195, HZM 51.33976,** HZM 52.33977, **HZM 53.33978, HZM 54.33979, HZM 55.33980,** NMP 91811–91816, **SMF 87648–87649,** SMF 87650, Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet (15.483° N, 43.533° E, altitude 2150 m).

Etymology. This species is named after the late renowned mammalogist, taxonomist and bat expert Dr. David L. Harrison (1926–2015). Harrison's numerous publications on Afro-Arabian Chiroptera, in particular the Molossidae, have significantly improved our knowledge of this poorly known family.

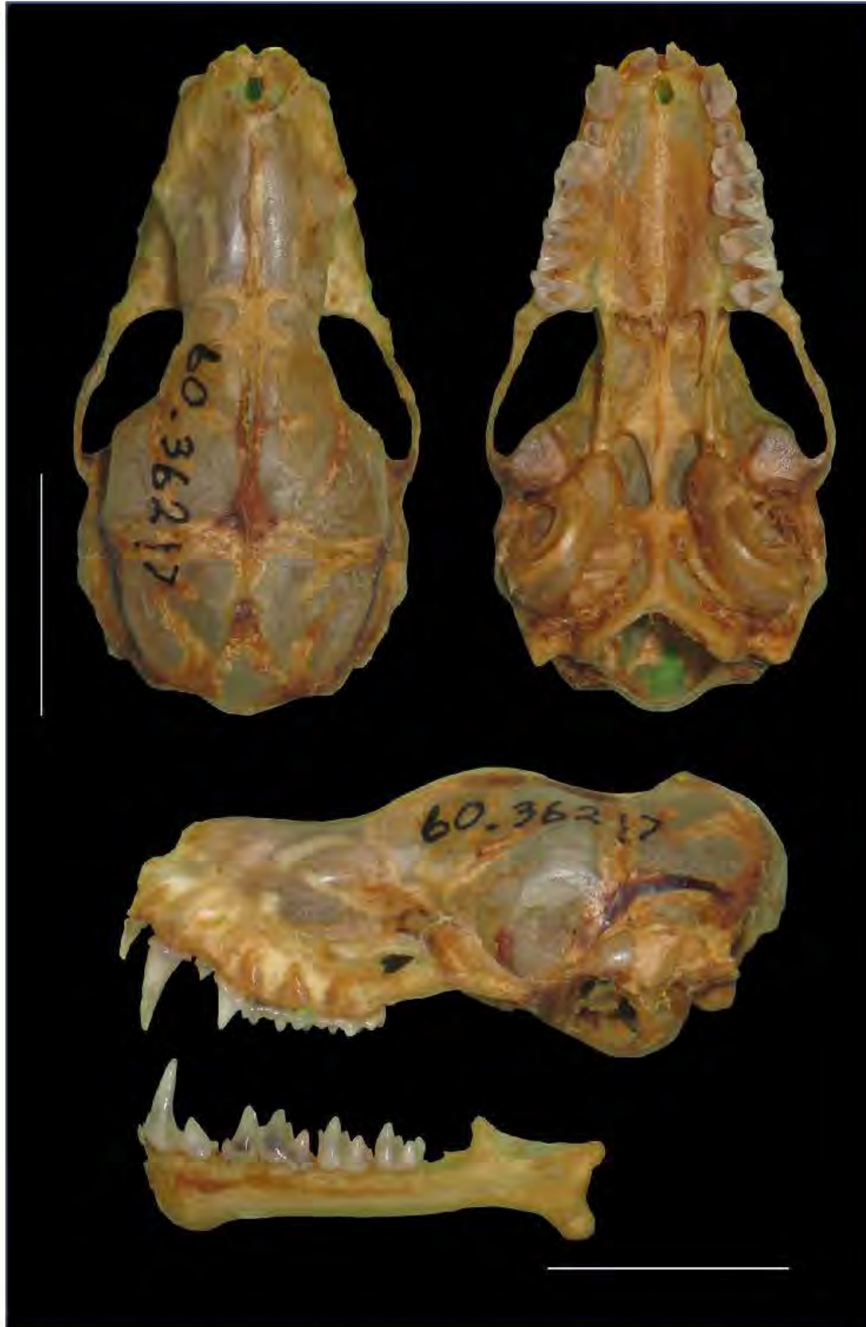

FIGURE 8. Cranium and mandible of the holotype of *Otomops harrisoni* **sp. nov.** (HZM 60.6217) in dorsal, ventral and lateral views. Scale bars = 10 mm. (Photographs taken by L.R. Richards).

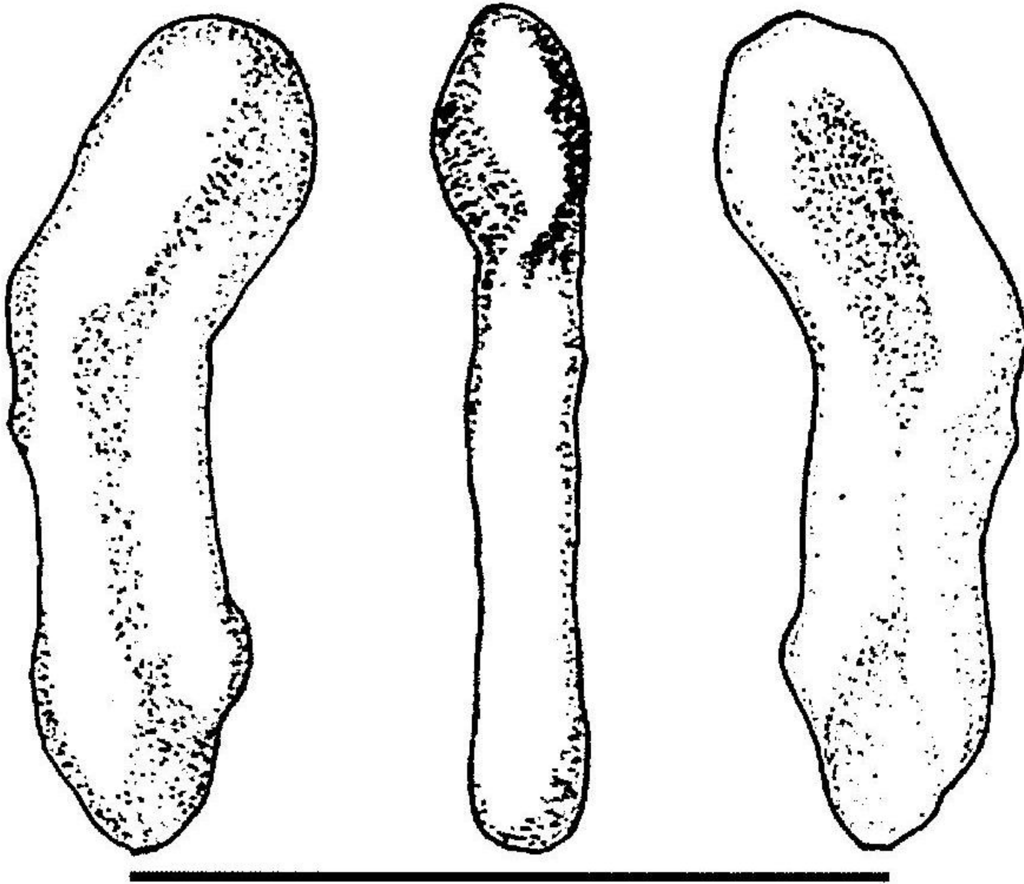

FIGURE 9. Dorsal, lateral and ventral views (from left to right) of the baculum of *Otomops harrisoni* **sp. nov.** holotype (HZM 60.36217). Scale bar = 1 mm.

Diagnosis. Diagnosis is based on statistically supported morphological differences and the occurrence of strongly supported reciprocally monophyletic lineages observed in analyses of concatenated mitochondrial cytochrome *b* and D-loop DNA sequences. Three lineages were strongly-supported within the Afro-Malagasy region; *O. madagascariensis* was sister to the reciprocally-monophyletic sister lineages, *O. martiensseni* s.s. and *O. harrisoni* (Fig. 2). *Otomops harrisoni* is separated from *O. martiensseni* s.s., its sister lineage, by a genetic p-distance of 2.10%, and from *O. madagascariensis* by 3.40%. *Otomops harrisoni* is further diagnosed by unique molecular characteristics, i.e. strict synapomorphies in both the cytochrome *b* gene and the D-loop region (Table 4).

In general, *O. harrisoni* is the largest and most robust member of the genus *Otomops* Thomas, 1913, overlapping in certain external and craniodental measurements with *O. martiensseni* s.s. The pelage is short and velvety, appearing dark chocolate brown on the upper parts, with a distinct cream-coloured collar that extends from the nape of the neck to the back. The ventral pelage consists of lighter brown-coloured hairs; it extends to the wings and covers a section of the plagiopatagium. The borders of the body and wing membranes of both the dorsal and ventral surfaces are marked by a distinct yet thin band of white fur, extending from the mid-humerus to the upper reaches of the thigh. Ears are long (35.2–46.0 mm for males, 33.6–41.0 mm for females), forward-directed and are united by a flap of skin across the nose. The species is sexually dimorphic, with males characterised by an average forearm length of 70.7 ±1.91 mm (64.0–74.0 mm, n = 41), whereas in females the average forearm length is 69.5 ±1.62 mm (65.7–72.7 mm, n = 38).

In general, *O. harrisoni* can be distinguished from other Afro-Malagasy taxa by its long cranium and notably high braincase (Fig. 8). *Otomops harrisoni* is characterised by an average greatest skull length of 28.5 ±0.36 mm

(27.8–29.3 mm, n = 38) and 27.0 ±0.36 mm (26.2–27.9 mm, n = 41), for males and females, respectively. The height of the braincase (excl. the tympanic bullae) of males averages 9.4 ±0.18 mm (9.0–9.8 mm, n = 38) and females average 9.1 ±0.17 mm (8.7–9.5 mm, n = 41). There is distinct and comparatively deep depression across the fronto-parietal area of the braincase. The braincase is markedly domed in the frontal region. The lambdoidal crests are moderately developed, join to form a "V-pattern", and extend to the sagittal crest at the highest point of the cranium. The sagittal crest remains slightly pronounced along the fronto-parietals, terminating at the posterior edge of a depression in the inter-orbital region. The rostrum is broad and robust, with fairly large, laterally flared nasals. The jugal process of the zygomatic arch is thickened and the zygomaxillary junction projects outwards. The external tympanic bullae are elongate and posteriorly broadened; they extend to the pterygoids and occupy a third of the braincase. The baculum of the holotype of *O. harrisoni* measures 1.16 mm (Fig. 9). The maximum width of the baculum shaft (SW) is 0.29 mm. The inflection of the baculum shaft (IB) in both the dorsal and ventral views is 54.53°.

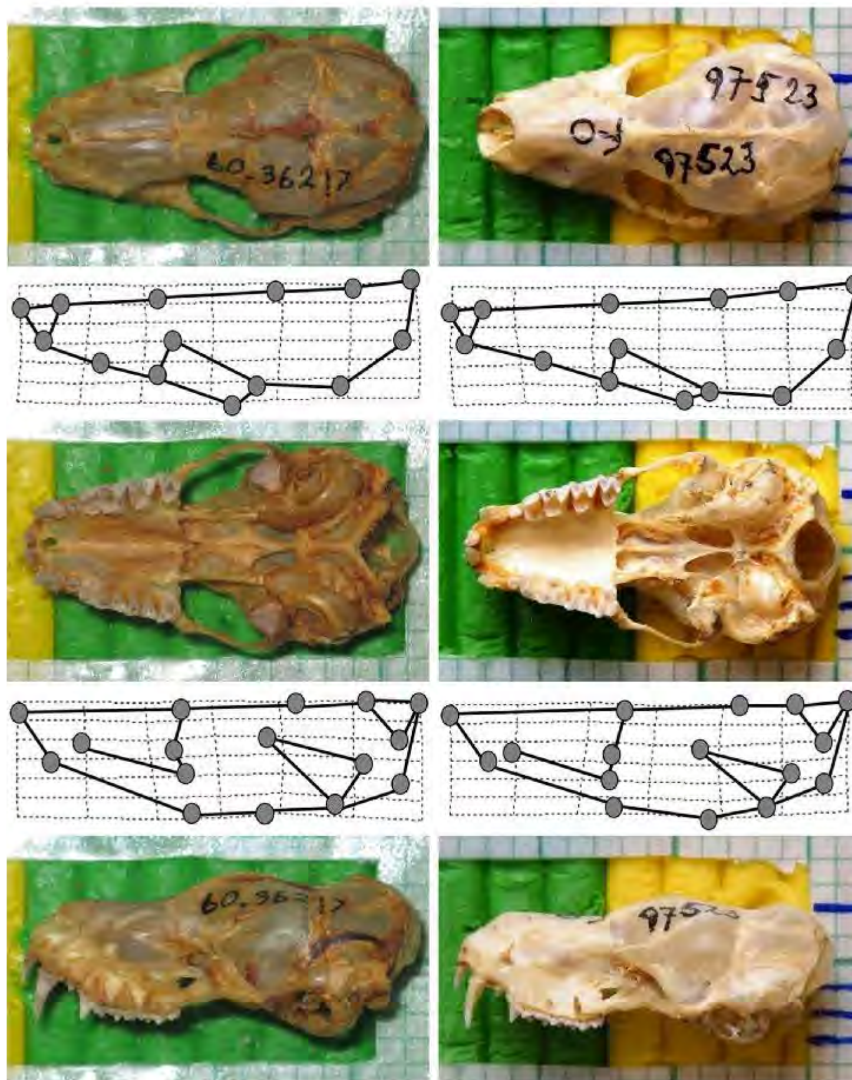

FIGURE 10. Comparative cranial views of the holotypes of *Otomops harrisoni* **sp. nov.** (HZM 60.36217), from Sof Omar Cave, Ethiopia on the left and *O. martiensseni* s.s. (MNHU 97523), from the foothills of the Usambara Mountains, near Tanga, Tanzania on the right. Thin plate splines illustrating the cranial morphology of *O. harrisoni* **sp. nov.** and *O. martiensseni* s.s., exaggerated 3x, are provided for the dorsal and ventral views.

TABLE 4. Unique synapomorphies of *O. harrisoni* **sp. nov.** in the mitochondrial cytochrome *b* gene and D-loop region. The first nucleotide gives the ancestral state, followed by the nucleotide position in each sequence, and the last nucleotide gives the derived state.

Haplotype number	Cytochrome *b*	D-loop
31	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	A(4)T; A(6)G; C(41)T; C(58)T; A(59)C; A(101)G; -(131)A; T(140)C; A(142)G; G(144)A; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; C(220)T; A(235)G; G(250)A; C(252)T; T(264)C
32	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; A(80)G; T(119)C; -(131)G; T(140)C; G(144)A; A(160)G; T(167)C; T(168)C; A(177)G; T(182)C; T(192)C; C(200)T; T(202)C; T(205)G; C(220)T; G(250)A; T(264)C
33	T (85) C; C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)G; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; A(80)G; T(119)C; -(131)G; T(140)C; G(144)A; A(160)G; T(167)C; T(168)C; A(177)G; T(182)C; T(192)C; C(200)T; T(202)C; T(205)G; C(220)T; G(250)A; T(264)C
34	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)G; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; -(131)G; T(140)C; A(142)G; G(144)A; A(160)G; T(167)C; A(177)G; T(182)C; T(190)C; A(191)G; C(200)T; T(202)C; T(205)G; C(220)T; C(252)T; G(250)A; T(264)C
35	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)G; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; -(131)G; T(140)C; A(142)G; G(144)A; A(160)G; T(167)C; A(177)G; T(182)C; T(190)C; C(200)T; T(202)C; T(205)G; C(220)T; G(250)A; C(252)T; T(264)C
36	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(798)C; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; A(6)G; T(25)C; C(41)T; C(58)T; A(59)C; G(81)A; A(101)G; -(131)A; T(140)C; A(142)G; G(144)A; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; C(220)T; A(221)G; A(235)G; G(250)A; C(252)T; T(264)C
37	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(798)C; T(810)C; T(831)C; C(837)T; T(933)C	A(4)T; T(5)C; A(15)G; C(41)T; A(59)T; -(131)G; T(140)C; A(142)G; G(144)A; T(166)C; G(176)A; A(177)G; T(182)C; A(195)G; C(200)T; T(202)C; T(205)A; A(206)G; C(220)T; G(250)A; T(264)C
38	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; -(131)G; T(140)C; A(142)G; G(144)A; A(160)G; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; C(220)T; A(221)G; G(250)A; C(252)T; T(264)C
39	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; G(304)A; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(798)C; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; A(101)G; -(131)G; T(140)C; G(144)A; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; G(250)A; C(252)T
40	C(231)T; G(232)A; C(243)A; A(258)C; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; A(101)G; -(131)G; T(140)C; A(142)G; G(144)A; A(160)G; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; C(220)T; G(250)A; C(252)T; T(264)C

......continued on the next page

TABLE 4. (Continued)

Haplotype number	Cytochrome *b*	D-loop
41	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; C(564)T; A(651)G; T(664)C; A(786)G; T(798)C; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; A(101)G; -(131)G; A(142)G; G(144)A; A(160)G; T(167)C; A(169)G; A(177)G; T(182)C; C(200)T; T(202)C; T(205)A; C(220)T; A(230)G; T(231)C; G(250)A; C(252)T; T(264)C
42	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(8)T; C(41)T; C(58)T; A(59)C; A(105)G; A(126)G; -(131)G; T(140)C; G(144)A; A(160)G; T(167)C; C(172)T; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; C(220)T; A(230)G; G(250)A; C(252)T; T(264)C
43	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; A(6)G; C(41)T; C(58)T; A(59)C; G(81)A; A(101)G; -(131)A; T(140)C; A(142)G; G(144)A; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; A(221)G; G(250)A; C(252)T; T(264)C
44	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(798)C; T(810)C; T(831)C; C(837)T; T(933)C	A(4)T; C(41)T; C(58)T; A(59)T; A(101)G; -(131)G; A(142)G; G(144)A; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)A; C(220)T; A(230)G; G(250)A; C(252)T; T(264)C
45	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; A(6)G; C(41)T; C(58)T; A(59)C; G(81)A; -(131)A; T(140)C; A(142)G; G(144)A; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; A(214)G; C(220)T; A(221)G; A(235)G; G(250)A; C(252)T; T(264)C
46	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; -(131)G; T(140)C; A(142)G; G(144)A; A(160)G; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; C(220)T; A(221)G; G(250)A; C(252)T; T(264)C
47	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; T(627)C; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)C; A(59)T; A(101)G; -(131)G; T(140)C; G(144)A; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; C(220)T; G(250)A; C(252)T
48	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; G(304)A; C(336)G; A(426)G; C(564)T; T(627)C; A(651)G; T(664)C; A(786)G; T(798)C; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; -(131)G; T(140)C; A(142)G; G(144)A; A(160)G; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)G; C(220)T; A(221)G; G(250)A; C(252)T; T(264)C
49	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; C(356)T; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; T(140)C; A(142)G; G(144)A; A(160)G; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; T(205)A; C(220)T; A(221)G; G(250)A; C(252)T; T(264)C
50	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; T(140)C; A(142)G; G(144)A; A(160)G; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; C(220)T; G(250)A; C(252)T; T(264)C
51	C(231)T; G(232)A; C(243)A; C(291)T; C(297)T; C(336)G; A(426)G; C(564)T; A(651)G; T(664)C; A(786)G; T(810)C; T(831)C; C(837)T; T(933)C	T(2)C; A(4)T; C(41)T; C(58)T; A(59)T; T(140)C; G(144)A; T(167)C; A(177)G; T(182)C; C(200)T; T(202)C; C(220)T; G(250)A; C(252)T

Description and comparisons. *Externals*. *Otomops harrisoni* bears all the external morphological diagnostic features of the genus as described by Thomas (1913) and subsequently refined by Freeman (1981). As with other *Otomops* spp. the pelage is conspicuous and is characterised by dark chocolate brown fur on the back and head, with slightly paler under parts. A cream-coloured collar extends from the dorsal surface of the neck to the throat, separating the head and back regions. This collar varies in size amongst the members of the genus. It is fairly pronounced in the Afro-Malagasy members (Monadjem *et al*. 2010; Goodman & Raherilalao 2014), and in some Indo-Australasian taxa (Thomas 1913; Kitchener *et al*. 1992), yet is reduced to a narrow pale band of hairs in *O. papuensis* (Lawrence 1948; Hill 1983). Characteristic of the genus, the large ears of *O. harrisoni* project forwards and join anteriorly above the nose. A series of brown spines is located along the anterior border of the ears (c. 15) and a flexible flange extends from the dorsal to the ventral surface of the pinnae; features shared by all *Otomops* spp. Similar to congenerics, the antitragus is absent and the tragus is minute. The two nostrils are stiffened by cutaneous ridges that run above and between them. These ridges are lined with small brown spines. The lips are characterised by loose folds and the anterior surface of the upper and lower lips are covered in short, light-brown bristles of hair. Common to other Afro-Malagasy and Indo-Australasian members, the upper lip projects beyond the lower lip. The feet of *O. harrisoni* are broad, with a comb of long white hairs. The ventral surfaces of the first and fifth toes are speckled with short light-coloured hairs. Adult males possess a distinct gular gland that is located on the lower portion of the throat. A similar gland has been reported from *O. martiensseni* s.s. (Chubb 1917), *O. madagascariensis* (Eger & Mitchell 2003), and *O. wroughtoni* (Thomas 1913). The first digit of *O. harrisoni* is noticeably thickened at its base and tapers to a point at the claw. Some individuals possess thumb pads that are slightly distended. A series of *O. martiensseni* s.s. examined from Durban displayed similar thumb pads; this feature appeared to be most pronounced in males. Based on external measurements, *O. harrisoni* is, in general, larger than other *Otomops* spp., including Afro-Malagasy congenerics. For instance, the average forearm length in *O. harrisoni* is 70.7 ±1.91 mm (63.8–74.0 mm, n = 41 males) and 69.5 ±1.62 mm (65.7–72.7 mm, n = 38 females), whereas in *O. martiensseni* s.s. this measurement is 65.8 ±1.60 mm (60.5–68.0 mm, n = 24 males) and 63.5 ±1.53 mm (60.0–66.3 mm, n = 22 females), and in *O. madagascariensis* average forearm length is 63.2 ±1.35 mm (60.0–66.0 mm, n = 20 males) and 61.2 ±1.32 mm (60.0–64.0 mm, n = 18 females).

Craniodental characters. The cranium of *O. harrisoni* is, in general, longer and broader relative to other Afro-Malagasy *Otomops* spp., yet there is some overlap in measurements with other Afro-Malagasy taxa, particularly *O. martiensseni* s.s. (Table 2). Given the morphological similarity between the two sister species, we have provided a side-by-side comparison of the crania of the holotypes of *O. harrisoni* and *O. martiensseni* s.s. to highlight the distinguishing features of the new species (Fig. 10). The average greatest skull length of *O. harrisoni* (males: 28.5 mm, range 27.8–29.3 mm; females: 27.1 mm, range 26.2–27.9 mm) is at least 1.04 times greater than the average greatest skull length of *O. martiensseni* s.s. (males: 27.5 mm, range 26.5–28.8 mm; females: 25.5 mm, range 24.7–26.1 mm), and 1.12 times that of *O. madagascariensis* (males: 25.7 mm, range 24.9–26.5 mm; females: 22.8 mm, range 22.8–24.5 mm). Multivariate analysis of craniodental data, demonstrated that *O. harrisoni* can be separated from *O. martiensseni* s.s. and *O. madagascariensis* based on greater inter-orbital width, braincase breadth and braincase height. The braincase of *O. harrisoni* is overall larger and higher than other *Otomops* spp. including *O. martiensseni* s.s.; this is most visible in the lateral view (Fig. 10). There is minimal overlap in braincase height (excl. tympanic bullae) between the corresponding sexes of *O. harrisoni* (males: 9.0–9.8 mm; females: 8.7–9.5 mm) and *O. martiensseni* s.s. (males: 8.2–9.0 mm; females: 7.8–8.8 mm), and there is no overlap of *O. harrisoni* with *O. madagascariensis* (males: 7.9–8.5 mm; females: 7.9–8.2 mm). Compared to *O. martiensseni* s.s., the frontal region of the cranium of *O. harrisoni* is markedly more inflated giving it a dome-like appearance (Fig. 10). Conversely, *O. martiensseni* s.s. is characterised by a more anteriorly slanted frontal region, giving the braincase a lower lateral profile than *O. harrisoni.* The depression that lies across the fronto-parietal suture of the braincase is less pronounced in *O. martiensseni* s.s. than in *O. harrisoni.* The sagittal crest and lambdoidal crests are slightly more developed in *O. harrisoni* than in *O. martiensseni* s.s. and *O. madagascariensis*. They join above the occiputs to produce a "V-pattern", giving the occipital region a rounded appearance; a characteristic feature of the genus. In *O. harrisoni* the sagittal crest remains slightly pronounced along the fronto-parietals, terminating at the inter-orbital depression by the junction of the nasals and frontals. In *O. martiensseni* s.s. and *O. madagascariensis* this feature is poorly developed. The anterior portion of the rostrum is more expansive in *O. harrisoni* than in *O. martiensseni* s.s. and *O. madagascariensis*. The mastoid projections are well-developed, elongate and contribute to the rounded appearance of the braincase when the cranium is viewed dorsally. In the lateral view, the foramen

magnum of *O. harrisoni* is situated higher than in *O. martiensseni* s.s. The lacrimal process is distinct in most *Otomops* spp., but is particularly prominent in the Afro-Malagasy members including *O. harrisoni*. The jugal process of the zygomatic arch is thickened and the zygomaxillary junction is more laterally displaced than in *O. martiensseni* s.s. A prominent vertical process is located on the dorsal surface of the zygoma; it is slightly broader and rounder in appearance than in *O. martiensseni* s.s. and *O. madagascariensis*. The basisphenoid pits are elongate, oval-shaped, and deep. They appear to be wider in the holotype of *O. martiensseni* s.s. than in that of *O. harrisoni*. The broadened occipital condyles of the northeastern African species project outwards. The posterior border of the hard palate extends to the posterior margin of M^3, similar to *O. martiensseni* s.s. *Otomops harrisoni* has external tympanic bullae that are longer and more posteriorly inflated than *O. martiensseni* s.s.

Typical of the genus, the mandible is thin and gracile, ending in an outwardly deflected angular process. The coronoid process is low-lying and the mandibular condyle is positioned in line with the lower toothrow. There is a distinct tubercle on the mandibular ramus between the third molar and the coronoid process. This tubercle has been observed in all three Afro-Malagasy species and has been reported from some Indo-Australasian species (Kitchener *et al*. 1992). The dental formula of *O. harrisoni* is I 1/2, C 1/1, P 2/2, M 3/3, characteristic of molossid bats. The upper incisors are moderately-developed and are separated from the canines by a slight diastema. This diastema is present in *O. martiensseni* s.s. and *O. madagascariensis*. The upper canines are long and fairly robust. The upper anterior premolar is small, reaching slightly past the cingulum of the posterior premolar, whereas the lower anterior premolar is half the size of the posterior premolar. The first two upper molars are similar in size, whilst the third molar is smaller in size and bears an unreduced commissure. The lower molars decrease in size from M_1 to M_3.

Molecular analyses. Analyses conducted using both mitochondrial and nuclear data are in agreement with regard to the number of extant clades/clusters within the Afro-Malagasy region. Genetic structure inferred by microsatellite analysis suggests strong genetic structure at regional (SECW, NEA), rather than population or colony level, and little gene-flow between the SECW and NEA regions, the habitats of *O. martiensseni* s.s. and *O. harrisoni*, respectively. The three groups formed, namely SECW, NEA and MAD (Fig. 4), correspond to the clades observed in the phylogram generated using concatenated cytochrome *b* and D-loop data (Fig. 2), the networks constructed using concatenated nuclear intron data (Fig. 3), as well as results reported by Lamb *et al*. (2008) and Richards *et al*. (2012). Clade G, comprising Malagasy *Otomops* specimens classified as *O. madagascariensis*, shows distinct and strongly supported (bootstrap support: 100%; posterior probability: 1.00) separation from the clades from mainland Africa (Fig. 2). Genetically, the sister lineages from mainland Africa and the Arabian Peninsula separate to form two independent reciprocally-monophyletic clades (subclades E and F), each having strong bootstrap and posterior probability support. These sister lineages are separated by a genetic p-distance of 2.10%. Nuclear sequence data supports this separation where, in figure 3, all *Otomops* spp. form independent networks at 95% confidence limit. The designation of *O. martiensseni* s.s. and *O. harrisoni* as separate species is supported based on the separation of individuals from the African mainland into 2 distinct groups derived from mitochondrial and nuclear sequence data and nuclear microsatellites.

Biology, distribution and conservation status. As mentioned above, specimens originating from Djibouti, Eritrea, Ethiopia, Kenya and Yemen have been assigned to this taxon. Therefore observations based on previously published literature ascribed to northeast African individuals now applies to *O. harrisoni*. While bioacoustic information is available from the literature for *O. martiensseni* s.s. (Fenton & Bell 1981; Fenton *et al*. 2002, 2004; Adams *et al*. 2015) and *O. madagascariensis* (Russ *et al*. 2003), information on individuals attributable to *O. harrisoni* is uncertain. Limited time expansion recordings of a single hand-released individual captured at Bungule, Taita Hills in Kenya showed a frequency of maximum energy of 12.00 kHz, minimum frequency of 10.50 kHz, maximum frequency of 16.50 kHz, and a call duration of 9.00 ms (Taylor *et al*. 2005); recorded echolocation parameters that lie within the previously described ranges of *O. martiensseni* s.s. (Fenton & Bell 1981; Fenton *et al*. 2002, 2004; Adams *et al*. 2015) and *O. madagascariensis* (Russ *et al*. 2003). However, as Bungule is equidistant from the nearest sampled localities included within this study for both *O. harrisoni* (Chyulu Hills) and *O. martiensseni* s.s. (Usambara foothills), the taxonomic assignment of the Taita Hills individual is uncertain. To the best of our knowledge, no information on animals that can be definitively assigned to *O. harrisoni* is presently available.

Scat analysis of Ethiopian *Otomops* inferred that this is an insectivorous bat feeding predominantly (97% by volume) on medium (size range: 1–5 cm) to large (wing span size range: 2.5–30 cm) Lepidoptera of the moth

families Noctuidae, Geometridae and Saturniidae (Rydell & Yalden 1997; Heppner 2008). *Otomops* has a diet that chiefly comprises a single prey category and is thus highly specialized for moth predation through its jaw and tooth morphology, flight style and echolocation system (Freeman 1981; Rydell & Yalden 1997; Jones & Rydell 2003). *Otomops martiensseni* s.s. bears similar morphological features as they relate to jaw architecture (Harrison 1965; Monadjem *et al*. 2010). As with *O. harrisoni*, the diet of *O. martiensseni* s.s. is comprised mostly of Lepidoptera (MC Schoeman, pers. comm.). This is in sharp contrast to the diet of the insular *O. madagascariensis* that consists of varying proportions of Lepidoptera, Diptera and Coleoptera (Andriafidison *et al*. 2007).

The reproductive cycle of two *Otomops* populations in Kenya, corresponding to the known distribution of *O. harrisoni*, was investigated by Mutere (1973) at the sites of Suswa and Ithundu Caves. Results indicated that males reach sexual maturity around one year of age, weighing approximately 25 g, at which time a gular gland had developed. Sexual maturity in females is indicated by lactation and/or the presence of a foetus, at approximately one year of age and at a similar mass to males. The breeding season occurs once a year and pregnant bats were found from October through January, birthing mainly in December. Females have a gestation period of around 3 months and give birth to a single, hairless young. For more on the reproductive biology of *Otomops* individuals from Kenya, see Long (1995) and references therein.

Members of *O. harrisoni* are known from a variety of habitats in northeast Africa and the Arabian Peninsula, including woodlands and shrublands of the Arabian Peninsula and Eritrea; montane grasslands, woodlands and forests of Ethiopia; xeric grassland and shrublands of Djibouti; and the bushlands and thickets of Kenya (Peel *et al*. 2007). According to the MaxEnt analysis conducted in this study (Fig. 7A), this range may include additional localities within Uganda, northern areas of Tanzania and southern areas of Somalia. These bats are found at high altitudes (>1000 m a.s.l) characterised by relatively drier climes (<500 mm annual precipitation), including warm semi-arid, tropical savanna, warm desert and, in the case of the Ethiopian highlands, temperate oceanic climates (Peel *et al*. 2007). Given these preferences, individuals roost predominantly in mountain-associated cave systems and lava caves. *Otomops* is known to congregate in the lava caves of the Rift Valley at Mount Suswa as well as the Ithundu caves of the Chyulu Hills in Kenya (Kingdon 1974; Kock *et al*. 2005) and is also found in the Sof Omar karst cave system of Ethiopia (Largen *et al*. 1974), the Hud Sawa caves at the Al-Rayadi Al-Gharbi Mountains in Yemen (Al-Jumaily 1999), the Day Forest National Park at the Goda Massif Mountains in Djibouti (Hill & Morris 1971) and within a disused railway line tunnel near Asmara in Eritrea (Kock & Zinner 2004). *Otomops harrisoni* roost sites are dark and poorly-ventilated, and consist primarily of natural structures with varying numbers of access points, e.g. whereas the tunnel housing the Asmara railway line has only one entry point (Kock & Zinner 2004), the Sof Omar and Mount Suswa caves have over 30 entrances each (Gunn 2004).

Otomops are usually found roosting in large colonies, comprising smaller groups of several hundred, tightly-packed individuals. Colonies observed at the Sof Omar, Ithundu and well-studied Mount Suswa roosts have been estimated to contain up to 15 thousand individuals per site; however, it has been suggested that Mount Suswa colonies may exhibit migratory behaviour, periodically leaving the breeding site caves in which they are normally found (Largen *et al*. 1974; Kock *et al*. 2005). At the time of publication, Al-Jumaily (1999) recorded the number of individuals in the Hud Sawa caves to be approximately 1500. Numbers in the Eritrean colony are comparatively lower than other roosts, i.e. approximately 500 individuals, since the railway tunnel would have only become suitable for habitation after 1974, making this a relatively young colony (Kock & Zinner 2004).

The IUCN classification of currently-circumscribed *O. martiensseni* is given as "Near Threatened" (Mickleburgh *et al*. 2008) and possibly close to qualifying as "Threatened" as population numbers have decreased over time. In light of the recircumscription of *O. martiensseni*, and the description of a new species, *O. harrisoni*, the conservation of both of these species will have to be assessed in future studies. Ethiopia is signatory to a number of conventions including the Conservation of Migratory Species, which lists *Otomops* as a species of interest. Although the Bale Mountains (Bale Mountains National Park and the Sof Omar National Monument included) are protected, protection measures are not clearly defined (Vreugdenhil *et al*. 2012). The large colony at Mt Suswa in Kenya is subject to human disturbance within the caves and does not appear to be protected (Kock *et al*. 2005). A protected area in the Day Forest National Park was assigned, although there may be a failure to implement prescribed conservation measures as a result of internal unrest in Djibouti (Hutson *et al*. 2001; Magin 2001). Conservation initiatives for the protection of *Otomops* within Yemen and Eritrea are unknown.

Discussion

This paper describes a new Afro-Arabian species of *Otomops*, *O. harrisoni*, based on material obtained from the Sof Omar caves in Ethiopia. This new species, which occurs in apparent parapatry with *O. martiensseni* s.s., has been characterized using differences in both genetic (mitochondrial and nuclear sequence and nuclear microsatellite) and morphological (craniodental measurements and dorsal and ventral landmarks) data. Samples obtained from localities in Djibouti, Eritrea, Kenya and Yemen have also been assigned to this taxon. Although phylogenetic (Lamb *et al*. 2006, 2008) and morphometric (Richards *et al*. 2012) studies of Afro-Malagasy *Otomops* have been published, it is hoped that this more inclusive paper will be able to resolve the number of species of *Otomops* from mainland Africa and the Arabian Peninsula. It has been suggested that the two Afro-Arabian *Otomops* groupings could represent evolutionary significant units (Lamb *et al*. 2008), however here we elevate their status to that of separate species.

Phylogenetic structure among Afro-Malagasy *Otomops* reveals the clear separation of samples into three strongly-supported clades which correspond to the species *O. martiensseni* s.s., *O. harrisoni* and *O. madagascariensis*. The mitochondrial DNA divergence between *O. martiensseni* s.s. and *O. harrisoni*, although relatively low (2.10% for concatenated cytochrome *b* and D-loop data), is not unusual since other bat species, although distinct at the morphological level, also possess low mitochondrial inter-specific divergences among sister taxa based on analysis of the same DNA regions. For example Goodman *et al*. (2010) recognises the separation of a *C*. '*pumilus*' clade from *C. leucogaster* by a cytochrome *b* genetic distance of 2.29%. Although 2.3% is considered relatively low for an inter-specific distance (Baker & Bradley 2006), mitochondrial mutation rates exhibit substantial variation between mammalian families (Nabholz *et al*. 2008) which raises the possibility that the molossid mitochondrial mutation rate may be lower than that in other bat families (Goodman *et al*. 2010).

Both nuclear intron sequence (95% confidence) and microsatellite analysis show congruence with phylogenetic analyses of mtDNA sequences, whereby Afro-Malagasy *Otomops* samples separate into three distinct networks/groups matching those found in the phylogenetic tree. The genetic separation of Afro-Arabian *Otomops* into two separate species indicates a lack of gene flow between the sister clades, even though members of *Otomops* may be capable of long-distance migrations. For example, *Otomops* individuals have been recorded from Molema Bush Camp (Tuli Block, Botswana), and the northern regions of South Africa including Kruger National Park and Mapungubwe National Park (Adams *et al*. 2015), as well as Modimolle (this study) and Mabelingwe near Bela-Bela (E. Balona, pers. comm.), where no records had previously existed within >600 km. Our results support the fact that both *O. martiensseni* and *O. harrisoni* are most likely characterised by homogenous gene pools across their range, although additional sampling would be required to collect individuals from the entire potential ranges of both species. Although isolating mechanisms have not been clearly identified, findings suggest that separation may be as a result of limiting bioclimatic factors: *O. harrisoni* occurs in regions of higher altitudes and lower annual precipitation whereas *O. martiensseni* s.s., although comparatively unaffected by altitude, is associated with areas of higher annual precipitation (see also Richards *et al*. 2012). Additionally, there may be as yet unknown ecological/biological factors at play, since geographical isolation may not be a factor for a species capable of long distance flight. Contrasting habitat preferences may have created barriers between *O. martiensseni* s.s. and *O. harrisoni*, allowing for both their physical and genetic separation over time.

Otomops harrisoni represents the largest species of the genus. In the Afro-Malagasy context, *O. harrisoni* can easily be distinguished from *O. madagascariensis* based on its comparatively larger and essentially non-overlapping size, particularly as it relates to the cranium. The species closely resembles *O. martiensseni* s.s. in its external appearance, yet the two can be distinguished from one another based on cranial size and shape. Multivariate comparisons of craniodental measurements and cranial landmark data for males and females showed separation of individuals assigned as *O. harrisoni* and *O. martiensseni* s.s. Our principal component analyses of craniodental characters revealed that this separation was largely based on the magnitude of the braincase breadth, braincase height, and inter-orbital width, with values for *O. harrisoni* greater than those reported for *O. martiensseni* s.s. The multivariate analyses of Richards *et al*. (2012) also showed braincase height as an important variable in distinguishing *O. martiensseni* s.s. from individuals currently identified as *O. harrisoni*. In contrast to this study, Richards *et al*. (2012), based on canonical variates analyses of craniodental measurements, described the northeastern species with a proportionately narrower inter-orbital region relative to *O. martiensseni* s.s. However, this morphological feature is most applicable to *O. madagascariensis* and not *O. harrisoni* as univariate analyses

and descriptive statistics of craniodental characters showed that *O. harrisoni*, in general, has a greater inter-orbital width than other Afro-Malagasy taxa (Tables 2–3 in Richards *et al*. 2012; this study).

To the best of our knowledge, we provided the first description of the baculum of *O. harrisoni*. The bacula of most *Otomops* species have not yet been described and intraspecific variation in bacula morphology within the Afro-Malagasy taxa is unknown. Further investigation is warranted to determine whether or not this morphological feature can be used in the taxonomic diagnoses of the various species.

Findings from this study illustrate the advantages of using a combined approach, based on morphological and molecular (nuclear and mitochondrial) analyses, with regards to the identification and description of new cryptic species. Although the taxonomic status of Afro-Malagasy *Otomops* has been assessed in this paper, it would be prudent to increase taxonomic sampling from additional localities on the African continent, specifically from central and western Africa.

Acknowledgements

We would like to extend our appreciation to Steve Goodman for his contribution of samples and additional assistance throughout the duration of this study. We are particularly grateful to Christiane Denys (MNHN), Paula Jenkins (BMNH), and Frieder Mayer (MNHU) for permitting access to type specimens. We also would like to thank the following curatorial staff for providing specimen material under their care (acronyms defined in Appendix 1): Paula Jenkins; the late William Stanley, Lawrence Heaney and John Phelps (FMNH); Paul Bates and Malcolm Pearch (HZM); Frieder Meyer; Christiane Denys; Wim Wendelen (MRAC); Bernard Agwanda (NMK); Petr Benda (NMP); Clare Mateke (NMZ); Judith Eger (ROM); Dieter Kock (SMF); and Teresa Kearney (TM). A special thanks to Paul Bates and Malcolm Pearch for rendering much needed assistance in accessing specimen information. We express our gratitude to Petr Benda, Paul van Daele and the Bat Interest Group of KwaZulu-Natal for contributing additional specimen material. eZemvelo KZN Wildlife and the South African Department of Environmental Affairs are thanked for assistance in obtaining a Threatened or Protected Species (ToPS) permit for the collection of supplementary genetic material. For assistance with fieldwork and analysis we thank M. Corrie Schoeman. TMCR, LRR, JML, MCN and PJT acknowledge financial assistance of the South African National Research Foundation (NRF). TMCR and LRR also acknowledge the financial assistance provided by the South African Biosystematics Initiative (SABI). We gratefully acknowledge two reviewers whose contributions significantly improved this work.

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APPENDIX 1. Sampled individuals of *Otomops* (n = 291) and outgroups (n = 8) used in molecular and morphometric analyses. Museum acronyms: BMNH—Natural History Museum; DM—Durban Natural Science Museum; FMNH—Field Museum of Natural History; HZM—Harrison Zoological Institute; MNHN—Muséum National d'Histoire Naturelle; MNHU—Museum für Naturkunde der Humboldt Universität; MRAC—Musée Royal Central Africa; NM—KwaZulu-Natal Museum; NMK—National Museum of Kenya; NMP—National Museum of the Czech Republic, Prague; NMZ—Livingstone Museum; ROM—Royal Ontario Museum; SMF—Senckenberg Museum; SMNS—Staatliche Museum für Naturkunde; TM—Ditsong Museum of Natural History; Field number acronyms: DBN—Durban genetic samples (wing punches); DP—Durban Pinetown genetic samples (wing punches); KZN—KwaZulu-Natal genetic samples (wing punches); PB—Pter Benda; SMG—Steven M. Goodman. Museum numbers in bold indicate samples used in nuclear microsatellite analysis, asterisks (*) indicate samples used for nuclear intron analysis and question mark (?) indicates that sex of the specimen is unknown.

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
Otomops harrisoni **sp. nov.**	Djibouti	Day Forest National Park, Mount Day	11.767° N, 42.650° E	BMNH 69.1256	F	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 41.31316	F	44	KJ509971	KJ509979	X	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 42.31317	F	45	KJ509972	KJ509980	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 43.31318	F	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 45.31369	F	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 49.33964	F	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 50.33965	F	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 56.36213	F	40	KJ509967	KJ509975	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 57.36214	F	41	KJ509968	KJ5009976	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 61.36218	F	43	KJ509970	KJ509978	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 63.36220	F	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	**NMP 91201**	F	39	EF216435	EF216467	-	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	**NMP 91201_B**	?	39	EF216436	EF216468	-	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	**NMP 91202**	F	38	EF216433	EF216465	-	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	**NMP 91202_B**	?	38	EF216434	EF216466	-	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	**NMP 91203**	F	36	EF216429	EF216461	-	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	**NMP 91203_B**	?	36	EF216430	EF216462	-	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	**PB 2512**	F	37	EF216431	EF216463	-	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	**PB 2512_B***	?	37	EF216432	EF216464	-	-	-
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 40.31315	M	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 44.31328	M	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 46.31370	M	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 47.31371	M	46	KJ509973	KJ509981	X	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 48.31372	M	-	-	-	X	X	X
Otomops harrisoni **sp. nov.** holotype	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 60.36217	M	42	KJ509969	KJ5009977	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	HZM 64.36220	M	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	SMF 41832	M	-	-	-	X	X	X
-''-	Ethiopia	Bale District, Sof Omar Cave	6.900° N, 40.859° E	SMF 41833	M	-	-	-	X	X	-
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48654	F	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 63772	F	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 63779	F	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 63782	F	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48655	M	-	-	-	X	-	-
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48656	M	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48660	M	-	-	-	-	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48661	M	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48663	M	-	-	-	-	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48664	M	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48666	M	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 48667	M	-	-	-	X	X	X
-''-	Kenya	19 km W of Makindu	2.300° S, 37.667° E	ROM 63808	M	-	-	-	X	X	X
-''-	Kenya	Chyulu Hills	2.350° S, 37.500° E	MRAC 38546	F	-	-	-	X	X	X
-''-	Kenya	Chyulu Hills	2.350° S, 37.500° E	MRAC 38547	F	-	-	-	X	X	X
-''-	Kenya	Chyulu Hills	2.350° S, 37.500° E	SMNS 46077	F	-	-	-	X	-	-
-''-	Kenya	Chyulu Hills	2.350° S, 37.500° E	SMNS 46079	F	-	-	-	X	-	-
-''-	Kenya	Chyulu Hills	2.350° S, 37.500° E	MRAC 38548	M	-	-	-	X	-	-
-''-	Kenya	Chyulu Hills	2.350° S, 37.500° E	MRAC 38549	M	-	-	-	X	X	X
-''-	Kenya	Ithundu Caves, Chyulu Hills, Makeuna District	2.358° S, 37.717° E	**NMK 15462** *	M	47	EF216428	EF216455	-	-	-
-''-	Kenya	Ithundu Caves, Chyulu Hills, Makeuna District	2.358° S, 37.717° E	NMK 15464	F	48	EF216439	EF216456	-	-	-
-''-	Kenya	Ithundu Caves, Chyulu Hills, Makeuna District	2.358° S, 37.717° E	**NMK 15460**	F	51	EF216442	EF216460	-	-	-

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-’’-	Kenya	Ithundu Caves, Chyulu Hills, Makeuna District	2.358° S, 37.717° E	**NMK 15463**	M	49	EF216440	EF216457	-	-	-
-’’-	Kenya	Ithundu Caves, Chyulu Hills, Makeuna District	2.358° S, 37.717° E	**NMK 15459**	M	50	EF216441	EF216458	-	-	-
-’’-	Kenya	Ithundu Caves, Kiboko	2.199° S, 37.717° E	ROM 65875	F	-	-	-	X	X	X
-’’-	Kenya	Ithundu Caves, Kiboko	2.199° S, 37.717° E	ROM 65878	F	-	-	-	-	X	X
-’’-	Kenya	Ithundu Caves, Kiboko	2.199° S, 37.717° E	ROM 65879	F	-	-	-	X	X	X
-’’-	Kenya	Ithundu Caves, Kiboko	2.199° S, 37.717° E	ROM 65876	M	-	-	-	X	X	X
-’’-	Kenya	Ithundu Caves, Kiboko	2.199° S, 37.717° E	ROM 65877	M	-	-	-	X	X	X
-’’-	Kenya	Ithundu Caves, Makindu	2.333° S, 37.699° E	ROM 81198	M	-	-	-	X	X	X
-’’-	Kenya	Ithundu Caves, Makindu	2.333° S, 37.699° E	ROM 81199	M	-	-	-	X	X	-
-’’-	Kenya	Lake Baringo, Kampi Ya Moto	0.183° N, 35.867° E	ROM 68360	F	-	-	-	X	X	X
-’’-	Kenya	Lake Baringo, Kampi Ya Moto	0.183° N, 35.867° E	ROM 68362	F	-	-	-	X	X	X
-’’-	Kenya	Lake Baringo, Kampi Ya Moto	0.183° N, 35.867° E	ROM 68364	F	-	-	-	X	X	X
-’’-	Kenya	Lake Baringo, Kampi Ya Moto	0.183° N, 35.867° E	ROM 68366	F	-	-	-	X	X	X
-’’-	Kenya	Machakos District	1.517° S, 37.267° E	MRAC 35264	F	-	-	-	X	X	X
-’’-	Kenya	Makindu Cave, Makindu	2.300° S, 37.833° E	ROM 78156	F	-	-	-	-	X	X
-’’-	Kenya	Makindu Cave, Makindu	2.300° S, 37.833° E	ROM 78155	M	-	-	-	X	X	-
-’’-	Kenya	Makindu Cave, Makindu	2.300° S, 37.833° E	ROM 78157	M	-	-	-	X	X	X
-’’-	Kenya	Makindu Cave, Makindu	2.300° S, 37.833° E	ROM 78158	M	-	-	-	X	X	X
-’’-	Kenya	Makindu River	-	ROM 65871	F	-	-	-	X	X	X
-’’-	Kenya	Makindu River	-	ROM 65872	F	-	-	-	X	X	X
-’’-	Kenya	Makindu River	-	ROM 65873	F	-	-	-	X	X	X
-’’-	Kenya	Mount Suswa	1.150° S, 36.350° E	MNHN 1966-186	F	-	-	-	X		-
-’’-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 41920	F	-	-	-	X	X	-
-’’-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 41924	F	-	-	-	X	X	X
-’’-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 41927	F	-	-	-	X	X	-
-’’-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 41928	F	-	-	-	X	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 41932	F	-	-	-	-	X	X
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 78147	F	-	-	-	X	X	X
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 78148	F	-	-	-	X	X	X
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 78154	F	-	-	-	X	X	-
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	MNHN 1966-185	M	-	-	-	X		-
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 36517	M	-	-	-	X	X	X
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 36519	M	-	-	-	-	-	X
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 78151	M	-	-	-	X	X	-
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 78152	M	-	-	-	X	X	X
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 91249	M	-	-	-	X	X	X
-”-	Kenya	Mount Suswa	1.150° S, 36.350° E	ROM 91250	M	-	-	-	X	X	X
-”-	Kenya	Nairobi	1.280° S, 36.817° E	ROM 79677	F	-	-	-	X	X	X
-”-	Kenya	Near Makindu, 192 km E of Nairobi	2.300° S, 37.833° E	ROM 48657	M	-	-	-	X	X	-
-”-	Kenya	Near Makindu, 192 km E of Nairobi	2.300° S, 37.833° E	ROM 48659	M	-	-	-	X	X	X
-”-	Kenya	Near Makindu, 192 km E of Nairobi	2.300° S, 37.833° E	ROM 48662	M	-	-	-	X	X	X
-”-	Kenya	Near Kibwezi	-	HZM 12.11899	F	-	-	-	X	-	-
-”-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	HZM 39.31195	F	-	-	-	X	X	X
-”-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	HZM 52.33977	F	32	KJ509966	KJ509974	-	-	-
-”-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	HZM 55.33980	F	-	-	-	X	X	X
-”-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	SMF 87650	F	31	EF216437	EF216469	-	-	-
-”-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	**NMP 91812**	F	34	KJ433730	KJ433789	-	-	-

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	**NMP 91814**	F	35	KJ433732	KJ433791	-	-	-
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	**NMP 91815**	F	33	KJ433733	KJ433792	-	-	-
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	**NMP 91816**	F	33	KJ433734	KJ433793	-	-	-
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	HZM 51.33976	M	-	-	-	X	X	X
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	HZM 53.33978	M	-	-	-	X	X	X
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	HZM 54.33979	M	-	-	-	X	X	X
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	**NMP 91811**	M	33	KJ433729	KJ433788	-	-	-
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	**NMP 91813**	M	33	KJ433731	KJ433790	-	-	-
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	SMF 87648	M	-	-	-	X	X	X
-"-	Yemen	Hud Sawa Cave, Ar-Rayadi Al-Gharbi Mountains, 3 km NW of Al-Mahweet	15.483° N, 43.533° E	SMF 87649	M	-	-	-	X	X	-
Otomops martiensseni s.s	Burundi	2.3 km N, 0.7 km W Teza, Kibira	3.200° S, 29.550° E	FMNH 137633	M	52	EF216423	EF216443	X	X	-
-"-	Central African Republic	Bamingui-Bangoran National Park	7.550° N, 19.290° E	BMNH 81.238	M	-	-	-	X	X	X
-"-	Ivory Coast	Comoé National Park	8.715° S, 3797° W	SMF 92048	M	-	-	-	X	X	X
-"-	Ivory Coast	Comoé National Park	8.715° S, 3797° W	SMF 92049	M	30	EF216420	EF216454	X	X	-
-"-	Ivory Coast	Comoé National Park	8.715° S, 3797° W	SMF 92050	F	-	-	-	X	X	X
-"-	Democratic Republic of Congo	Lufuko Stream, Marungu	7.400° S, 29.460° E	NZM 3395	M	-	-	-	X	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-"-	Democratic Republic of Congo	Welle River, Poko	3.080° N, 25.580° E	BMNH 19.3.92	F	-	-	-	X	X	X
-"-	Malawi	Mangoche Mountain	14.450° S, 35.483° E	NZM 3228	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Berea, 26 Waller Crescent	29.825° S, 31.002° E	DM4760	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Berea, Hime Road	29.800° S, 31°010' E	DM 4950	M	-	-	-	-	X	X
-"-	South Africa	KwaZulu-Natal, Bluff	-	TM 33867	F	-	-	-	X	-	-
-"-	South Africa	KwaZulu-Natal, Bluff	-	TM 38865	F	-	-	-	X	-	-
-"-	South Africa	KwaZulu-Natal, Bluff	-	TM 42514	F	-	-	-	X	-	-
-"-	South Africa	KwaZulu-Natal, Bluff, 296 Marine Drive	29.916° S, 31.024° E	DM 5425	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Bluff, 296 Marine Drive	29.916° S, 31.024° E	DM 5426	F	-	-	-	-	X	X
-"-	South Africa	KwaZulu-Natal, Bluff, 560 Marine Drive	29.917° S, 31.007° E	DM 5514	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Bluff, 560 Marine Drive	29.917° S, 31.007° E	DM 5516	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Bluff, 560 Marine Drive	29.917° S, 31.007° E	DM 5518	F	-	-	-	X	-	X
-"-	South Africa	KwaZulu-Natal, Bluff, 560 Marine Drive	29.917° S, 31.007° E	DM 5509	M	-	-	-	-	X	X
-"-	South Africa	KwaZulu-Natal, Bluff, 560 Marine Drive	29.917° S, 31.007° E	DM 5511	M	-	-	-	-	X	X
-"-	South Africa	KwaZulu-Natal, Bluff, 560 Marine Drive	29.917° S, 31.007° E	DM 5512	M	-	-	-	-	X	-
-"-	South Africa	KwaZulu-Natal, Brighton Beach, 137 Glenardle Road	29.934° S, 30.003° E	DM 6930	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Bluff, 296 Marine Drive	29.916° S, 31.024° E	DM 5427	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Carrington Heights, Marshall Grove	29.883° S, 30.967° E	DM 3518	F	-	-	-	-	X	X
-"-	South Africa	KwaZulu-Natal, Central Durban	29.850° S, 31.017° E	BMNH 16.10.9.1	M	-	-	-	-	X	X
-"-	South Africa	KwaZulu-Natal, Doonheights, 2 Mopani Road	30.065° S, 30.865° E	N/A	F	28	KJ433726	KJ433785	-	-	-
-"-	South Africa	KwaZulu-Natal, Doonheights, 2 Mopani Road	30.065° S, 30.865° E	N/A	M	28	KJ433727	KJ433786	-	-	-
-"-	South Africa	KwaZulu-Natal, Durban	-	DM 5936	F	-	-	-	-	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-”-	South Africa	KwaZulu-Natal, Durban	-	NM 379	F	-	-	-	-	X	X
-”-	South Africa	KwaZulu-Natal, Durban	-	DM 5392	M	-	-	-	X	-	-
-”-	South Africa	KwaZulu-Natal, Durban	-	DM 5936	M	-	-	-	-	X	-
-”-	South Africa	KwaZulu-Natal, Durban	-	DM 6904	M	-	-	-	X	X	X
-”-	South Africa	KwaZulu-Natal, Durban	-	N/A	M	27	KJ433725	KJ433784	-	-	-
-”-	South Africa	KwaZulu-Natal, Morningside, Brynderyn Flats	29.864° S, 31.040° E	DM 7909	M	1	EF216424	EF21644	X	X	X
-”-	South Africa	KwaZulu-Natal, Durban	-	NM 378	M	-	-	-	-	X	X
-”-	South Africa	KwaZulu-Natal, Durban North	-	DM 11731	M	-	-	-	X	X	X
-”-	South Africa	KwaZulu-Natal, Durban North, 229 Rinaldo Road	29.758° S, 31.044° E	DBN 18	M	12	KJ433700	KJ433759	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	DM 11434	F	-	-	-	X	X	X
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 19**	F	14	KJ433701	KJ433760	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 20**	F	21	KJ433702	KJ433761	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 21**	M	22	KJ433703	KJ433762	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 22**	F	14	KJ433704	KJ433763	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 23**	F	23	KJ433705	KJ433764	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 24**	F	16	KJ433706	KJ433765	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 25**	F	12	KJ433707	KJ433766	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 26**	F	9	KJ433708	KJ433767	-	-	-
-”-	South Africa	KwaZulu-Natal, Glen Anil, 29 Glen Anil Street	29.752° S, 31.037° E	**DBN 27**	F	24	KJ433709	KJ433768	-	-	-
-”-	South Africa	KwaZulu-Natal, Kloof, 99 Abelia Road	29.787° S, 30.839° E	DBN 15	F	12	KJ433697	KJ433756	-	-	-
-”-	South Africa	KwaZulu-Natal, Kloof, 99 Abelia Road	29.787° S, 30.839° E	DBN 17	F	9	KJ433699	KJ433758	-	-	-
-”-	South Africa	KwaZulu-Natal, Kloof, 99 Abelia Road	29.787° S, 30.839° E	DBN 16	M	15	KJ433698	KJ433757	-	-	-

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-"-	South Africa	KwaZulu-Natal, Gillits, 6 Firwood Road	29.796° S, 30.808° E	**DBN 09**	F	16	KJ433691	KJ433750	-	-	-
-"-	South Africa	KwaZulu-Natal, Gillits, 6 Firwood Road	29.796° S, 30.808° E	**DBN 11**	F	18	KJ433693	KJ433752	-	-	-
-"-	South Africa	KwaZulu-Natal, Gillits, 6 Firwood Road	29.796° S, 30.808° E	**DBN 13**	F	20	KJ433695	KJ433754	-	-	-
-"-	South Africa	KwaZulu-Natal, Gillits, 6 Firwood Road	29.796° S, 30.808° E	**DBN 14**	F	12	KJ433696	KJ433755	-	-	-
-"-	South Africa	KwaZulu-Natal, Gillits, 6 Firwood Road	29.796° S, 30.808° E	**DBN 10**	M	17	KJ433692	KJ433751	-	-	-
-"-	South Africa	KwaZulu-Natal, Gillits, 6 Firwood Road	29.796° S, 30.808° E	**DBN 12**	M	19	KJ433694	KJ433753	-	-	-
-"-	South Africa	KwaZulu-Natal, Hillary	-	DM 5935	M	-	-	-	-	X	-
-"-	South Africa	KwaZulu-Natal, Kingsway, Kingsway School	30.039° S, 30.894° E	DM 7914	M	2	EF216425	EF216445	X	X	X
-"-	South Africa	KwaZulu-Natal, La Lucia	-	DM 6936	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, La Lucia	-	DM 6937	F	-	-	-	X	-	X
-"-	South Africa	KwaZulu-Natal, Morningside, Percy Osbourne Road	29.817° S, 31.017° E	DM 11732	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Northdene, 20 Jan Smuts Avenue	-	DM 3886	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Northdene, 20 Jan Smuts Avenue	-	DM 3885	M	-	-	-	-	X	-
-"-	South Africa	KwaZulu-Natal, Pinetown, 8 Buys Road	29.757° S, 30.639° E	**DM 8421**	F	7	EF216413	EF216447	X	-	X
-"-	South Africa	KwaZulu-Natal, Pinetown, 8 Buys Road	29.757° S, 30.639° E	**DP 2**	F	8	EF216415	EF216448	-	-	-
-"-	South Africa	KwaZulu-Natal, Pinetown, 8 Buys Road	29.757° S, 30.639° E	**DP 3**	F	8	EF216415	EF216449	-	-	-
-"-	South Africa	KwaZulu-Natal, Pinetown, 8 Buys Road	29.757° S, 30.639° E	**DP 4**	F	9	EF216416	EF216450	-	-	-
-"-	South Africa	KwaZulu-Natal, Pinetown, 8 Buys Road	29.757° S, 30.639° E	**DP 5**	M	-	-	-	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 106 Bailey Road	29.771° S, 31.023° E	DM 6886	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Red Hill, 106 Bailey Road	29.771° S, 31.023° E	DM 6887	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Red Hill, 106 Bailey Road	29.771° S, 31.023° E	DM 6888	M	-	-	-	X	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 28**	F	24	KJ433710	KJ433769	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 29**	F	16	KJ433711	KJ433772	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 30**	F	12	KJ433712	KJ433771	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 33**	F	12	KJ433715	KJ433774	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 34**	F	24	KJ433716	KJ433775	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 35**	F	16	KJ433717	KJ433776	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 36**	F	12	KJ433718	KJ433777	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 31**	M	16	KJ433713	KJ33772	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 32**	M	9	KJ433714	KJ433773	-	-	-
-"-	South Africa	KwaZulu-Natal, Red Hill, 10 Rosary Road	29.789° S, 31.017° E	**DBN 37**	M	16	KJ433719	KJ433778	-	-	-
-"-	South Africa	KwaZulu-Natal, Silverglen, 473 Silverglen Drive	29.928° S, 30.903° E	N/A	?	4	EF216407	EF216451	-	-	-
-"-	South Africa	KwaZulu-Natal, Silverglen, 473 Silverglen Drive	29.928° S, 30.903° E	N/A	?	5	EF216409	EF216452	-	-	-
-"-	South Africa	KwaZulu-Natal, Silverglen, 473 Silverglen Drive	29.928° S, 30.903° E	N/A	?	6	EF216410	EF216453	-	-	-
-"-	South Africa	KwaZulu-Natal, St Winifred, 50 Winifred Drive	30.089° S, 30.851° E	DM 6220	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Umbilo, Fenniscowles Road	29.883° S, 30.967° E	DM 5344	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	**DM 10294**	M	15	KJ433689	KJ433748	X	X	X
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	DM 8419	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	DM 8420	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	**DM 10295**	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	**DBN 01**	F	10	KJ433684	KJ433743	-	-	-
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	**DBN 02**	F	11	KJ433685	KJ433744	-	-	-

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	**DBN 03**	F	12	KJ433686	KJ433745	-	-	-
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	**DBN 04**	F	13	KJ433687	KJ433746	-	-	-
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	**DBN 05**	F	14	KJ433688	KJ433747	-	-	-
-"-	South Africa	KwaZulu-Natal, Umgeni Heights, 27 Hunters Way	29.808° S, 31.025° E	**DBN 08**	F	12	KJ433690	KJ433749	-	-	-
-"-	South Africa	KwaZulu-Natal, Umhlanga, Westbrooke	-	DM 4490	F	-	-	-	-	-	X
-"-	South Africa	KwaZulu-Natal, Wentworth	-	HZM 1.2145	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Wentworth	-	HZM 1.2145	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Westville, 5 Springfield Drive	29.833° S, 30.933° E	DM 8571	M	-	-	-	-	X	X
-"-	South Africa	KwaZulu-Natal, Winkelspruit, Eden Sands	30.099° S, 30.859° E	**KZN 01**	F	12	KJ433720	KJ433779	-	-	-
-"-	South Africa	KwaZulu-Natal, Winkelspruit, Eden Sands	30.099° S, 30.859° E	**KZN 02**	F	13	KJ433721	KJ433780	-	-	-
-"-	South Africa	KwaZulu-Natal, Winkelspruit, Eden Sands	30.099° S, 30.859° E	**KZN 03**	F	25	KJ433722	KJ433781	-	-	-
-"-	South Africa	KwaZulu-Natal, Winkelspruit, Eden Sands	30.099° S, 30.859° E	**KZN 04**	F	26	KJ433723	KJ433782	-	-	-
-"-	South Africa	KwaZulu-Natal, Winkelspruit, Eden Sands	30.099° S, 30.859° E	**KZN 05**	M	25	KJ433724	KJ433783	-	-	-
-"-	South Africa	Ugu District, Park Rynie	30.317° S, 30.733° E	DM 5605	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Voortukker Strand, near Margate, Durban	30.850° S, 30.367° E	HZM 3.3077	F	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Voortukker Strand, near Margate, Durban	30.850° S, 30.367° E	HZM 4.3078	F	-	-	-	X	X	-
-"-	South Africa	KwaZulu-Natal, Park Rynie, Ocean View Farm	30.339° S, 30.731° E	DM 8031*	F	3	EF216426	EF216446	X	X	X
-"-	South Africa	KwaZulu-Natal, Park Rynie, Ocean View Farm	30.339° S, 30.731° E	DM 8032	M	-	-	-	X	X	X
-"-	South Africa	KwaZulu-Natal, Pietermaritzburg, Queen Elizabeth Park	29.573° S, 30.326° E	DM 10790	M	-	-	-	X	X	X
-"-	South Africa	Limpopo, 3 km of Modimolle (Nylstroom)	24.660° S, 28.130° E	DM 11526	F	29	KJ433728	KJ433787	X	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
Otomops martiensseni s.s. holotype	Tanzania	Magrotto Plantation, Magrotto Hill, near Tanga	5.070° S, 38.030° E	MNHU 97523	M	-	-	-	X	X	X
-''-	Tanzania	Tongwe F.R., Tanga, Muheza District	5.306° S, 38.728° E	SMF 79542	M	54	EF216422	-	X	X	X
-''-	Uganda	Budongo Forest, Bunyoro	1.450° S, 31.350° E	ROM 46695	F	-	-	-	X	X	X
-''-	Zambia	Mafinga Mountains	10.250° S, 33.500° E	Unaccessioned specimen	M	-	-	-	X	X	X
-''-	Zimbabwe	Hostes Nicolle Institute, Sengwa Wildlife Ranch	18.167° S, 28.217° E	ROM 83979	M	53	EF216421	-	X	X	X
Otomops madagascariensis	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Analamerana, Grotte de Barazibe	12.711° S, 49.473° E	FMNH 178849	F	-	-	-	X	X	X
-''-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Analamerana, Grotte de Barazibe	12.711° S, 49.473° E	FMNH 178850	F	-	-	-	X	X	X
-''-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Analamerana, Grotte de Barazibe	12.711° S, 49.473° E	FMNH 178851	F	-	-	-	X	X	X
-''-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Analamerana, Grotte de Barazibe	12.711° S, 49.473° E	FMNH 178852	M	-	-	-	X	X	X
-''-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, 3.5 KM se Andrafiabe	12.942° S, 49.055° E	FMNH 176355	M	-	-	-	X	X	X
-''-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, 3.5 KM se Andrafiabe	12.942° S, 49.055° E	FMNH 176356*	M	-	-	-	-	X	-
-''-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, 3.5 KM se Andrafiabe	12.942° S, 49.055° E	FMNH 176354	M	64	EF216381	EF216397	-	-	-
-''-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, 3.5 KM se Andrafiabe	12.942° S, 49.055° E	FMNH 176357*	M	65	EF216382	EF216400	X	X	-

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-"-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, 3.5 KM se Andrafiabe	12.942° S, 49.055° E	FMNH 176376	M				X	X	X
-"-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, Grotte Antsiroandoha	12.891° S, 49.098° E	FMNH 177398	F	-	-	-	X	X	X
-"-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, Grotte Boribe	13.000° S, 49.000° E	FMNH 183896	F	-	-	-	X	X	X
-"-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, Grotte Boribe	13.000° S, 49.000° E	FMNH 183897	F	-	-	-	X	X	X
-"-	Madagascar	Province d'Antsiranana, Réserve Spéciale d'Ankarana, Grotte Boribe	13.000° S, 49.000° E	FMNH 183927	F	-	-	-	X	X	X
-"-	Madagascar	Province de Fianarantsoa, 3.8 km NW, Ranohira, along Namaza River	22.540° S, 45.380° E	FMNH 166073	F	55	EF216372	EF216383	X	X	X
-"-	Madagascar	Province de Mahajanga, Grotte d'Anjohibe, 3.7 km NE Antanamarina	15.537° S, 46.886° E	FMNH 179316	F	-	-	-	X	X	X
-"-	Madagascar	Province de Mahajanga, Grotte d'Anjohibe, 3.7 km NE Antanamarina	15.537° S, 46.886° E	FMNH 179317	F	-	-	-	X	X	X
-"-	Madagascar	Province de Mahajanga, Grotte d'Anjohibe, 3.7 km NE Antanamarina	15.537° S, 46.886° E	FMNH 179318	F	-	-	-	X	X	X
-"-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18.245° S, 44.716° E	FMNH 169689	F	-	-	-	X	X	X
-"-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18.245° S, 44.716° E	FMNH 169693	F	-	-	-	X	X	X
-"-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18.245° S, 44.716° E	FMNH 169667	M	56	EF216373	EF216384	-	X	X
-"-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18.245° S, 44.716° E	FMNH 169692	M	-	-	-	X	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-''-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18.245° S, 44.716° E	FMNH 169694	M	57	EF216374	EF216385	-	-	-
-''-	Madagascar	Province de Mahajanga, Parc National de Bemahara, Grotte d'Anjohimbabazimba	18.245° S, 44.716° E	FMNH 169695	M	58	EF216375	EF216386	-	-	-
Otomops madagascariensis holotype	Madagascar	Province de Mahajanga, Réserve south of Province de Mahanja, Namoroka, Réserve naturelle intégrale no. 8	16.230° S, 45.280° E	MNHN.CG 1953-1	F	-	-	-	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23.540° S, 43.767° E	FMNH 172397	M	-	-	-	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23.540° S, 43.767° E	FMNH 172934	M	61	EF216378	EF216389	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23.540° S, 43.767° E	FMNH 172936	M	-	-	-	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23.540° S, 43.767° E	FMNH 172938	M	59	EF216376	EF216387	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23.540° S, 43.767° E	FMNH 172939	M	-	-	-	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23.540° S, 43.767° E	FMNH 172940	M	60	EF216377	EF216388	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23.540° S, 43.767° E	FMNH 172942	M	-	-	-	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172949	F	-	-	-	X	X	X
-''-	Madagascar	Province de Toliara, Grotte d'Ambanilia, 3.7 km SSE Sarodrano	23.540° S, 43.767° E	FMNH 172941	M	-	-	-	X	X	X
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172943	F	-	-	-	X	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172944	F	62	EF216379	EF216390	X	X	X
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172945	F	-	-	-	X	X	X
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172949	F	-	-	-	X	X	X
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172952	F	-	-	-	X	X	X
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172953	F	-			X	X	X
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	**SMG 16453**	F	66	KJ433735	KJ433794	-	-	-
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	**SMG 16454**	F	67	KJ433736	KJ433795	-	-	-
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	**SMG 16456**	F	69	KJ433738	KJ433797	-	-	-
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	**SMG 16459**	F	71	KJ433741	KJ433800	-	-	-
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172947	M	-	-	-	X	X	X
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172948	M	63	EF216380	EF216391	X	X	X
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172950	M	-	-	-	X	X	X
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	FMNH 172951	M	-	-	-	X	-	-
-"-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	**SMG 16455**	M	68	KJ433737	KJ433796	-	-	-

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	**SMG 16457**	M	62	KJ433739	KJ433798	-	-	-
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	**SMG 16458**	M	70	KJ433740	KJ433799	-	-	-
-''-	Madagascar	Province de Toliara, Grotte de Bishiko, 0.75 km E of St Augustin	23.548° S, 43.716° E	**SMG 16460**	M	71	KJ433742	KJ433801	-	-	-
Otomops wroughtoni	Cambodia	Preah Vihear Province, Chhep District	13.893° N, 105.267° E	HZM 3.33440	M	72	EF504251	EF504253	-	X	X
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	BMNH 13.4.9.1	M	-	-	-	X	X	X
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	BMNH 13.4.9.2	M	-	-	-	-	X	X
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	BMNH 13.4.9.3	M	-	-	-	X	-	-
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	BMNH 13.4.9.4	M	-	-	-	-	X	X
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	HZM 2.5005	M	-	-	-	-	X	X
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	BMNH 12.11.24.1	F	-	-	-	X	X	X
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	BMNH 13.4.9.5	F	-	-	-	X	X	X
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	BMNH 13.4.9.6	F	-	-	-	X	X	X
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	HZM 1.25004	F	-	-	-	-	X	-
-''-	India	Kamataka, 0.5 km from Talewadi Village, Barapede Caves	15.417° N, 74.367° E	MNHN 1985-1590	F	-	-	-	X	X	X

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APPENDIX 1. (Continued)

Species	Country	Locality	Coordinates	Museum No.	Sex	Haplotype No.	Cyt *b*	D-loop	Traditional Morphometrics	Geometric Morphometrics (dorsal)	Geometric Morphometrics (ventral)
Otomops secundus	New Guinea	Tapu, Upper Ramu River Plateau	-	BMNH 50.982	M	-	-	-	X	X	X
-''-	New Guinea	Tapu, Upper Ramu River Plateau	-	BMNH 50.979	F	-	-	-	X	X	X
-''-	New Guinea	Tapu, Upper Ramu River Plateau	-	BMNH 50.980	F	-	-	-	X	X	X
Otomops cf. *formosus*	Philippines	Luzon Island, Kalinga Province, Balbalan Municipality, Balbalasang Brgy, Magdallao	17.458° S, 121.068° E	FMNH 167240	M	73	EF504252	EF504254	X	X	X
-''-	Philippines	Mindanoa Island, Bukidnon Province, Sumilao Municipality, 10.6 km S, 2.8 km W Sumilao	8.189° S, 124.92°2 E	FMNH 167382	M	-	-	-	X	X	X
Otomops papuensis	Papua New Guinea	Milne Bay Province, Mount Suckling, Maul	-	BMNH 73.136	M	-	-	-	X	X	X
Mops midas	Madagascar	Antanimbary	-	FMNH 185120	M	74	EF474039	EF474062	-	-	-
Mops leucostigma	Madagascar	Ambondramamy	16.436° S, 47.156° E	FMNH 184701	M	75	EF474029	EF474076	-	-	-
-''-	Madagascar	Mahajanga	15.708° S, 46.312° E	FMNH 184698*	?	-	-	-	-	-	-
Mops condylurus	South Africa	Phinda Private Game Reserve	27.871° S, 32.344° E	DM 6332*	M	-	-	-	-	-	-
Mormopterus francoismoutoui	Mascarene Islands, Reunion	Sainte Clotilde	20.918° S, 55.483° E	FMNH 194015*	M	-	-	-	-	-	-
Mormopterus jugularis	Madagascar	Ankazobe	18.314° S, 47.114° E	FMNH 184835*	M	-	-	-	-	-	-
Myotis goudoti	Madagascar	Befotaka	23.830° S, 46.970° E	FMNH178603	?	77	GU116768	GU116752	-	-	-
Pipistrellus abramus	Japan	not given in original paper	-	not given in original paper	?	76	NC_005436	NC_005436	-	-	-

CHAPTER SEVEN:

Summary and General Conclusions

SUMMARY AND GENERAL CONCLUSIONS

Molossidae is the fourth largest family within the Chiroptera but remains one of the most understudied due to the relative lack of available specimens for analysis (Ammerman *et al*., 2012). This study focused on aspects of the genetics and molecular ecology of the Afro-Malagasy members of the Old World genus *Otomops* found in mainland Africa, Madagascar and the Arabian Peninsula. Prior to this study, only one paper had been published on the genetics of Afro-Malagasy *Otomops*, focussing on *O. martiensseni* from populations in South Africa and Kenya (Lamb *et al*., 2006). This study used a multi-level (genus, species, population, colony and individual) approach, employing a variety of data types, techniques and analyses to address issues related to the systematics, population genetics, distribution and social structure of Afro-Malagasy *Otomops*.

Genus-level relationships in the Molossidae and the status of Otomops

Higher-level associations among molossid genera were historically investigated via phenetic analysis of morphological data (Freeman, 1981; Ammerman *et al*., 2012). This project was designed to complement these analyses and test established phylogenetic hypotheses at family, genus and species level with a molecular genetic approach based on sequencing of mitochondrial and nuclear DNA regions (Chapter 3; and see Lamb *et al*., 2011).

Nuclear RAG2 and mitochondrial cytochrome *b* sequence analysis showed that the molossid genera formed a single monophyletic clade, with *Mormopterus* sister to the other genera (divergence date: 36.56 MYA; 95% confidence interval: 35.56 – 37.50 MYA) and *Otomops* forming a well-supported and discrete clade, with no apparent association with other molossid genera (divergence date: 21.31 MYA; 95% confidence interval: 11.02 – 30.57 MYA). The genus *Tadarida* was not monophyletic, showing phylogenetic associations with *Chaerephon jobimena* and *Sauromys*; this raises questions relating to its validity as a genus and the manner in which taxa are assigned to this genus, and points to the need for further studies based on greater taxonomic representation to resolve its generic status. Species of *Chaerephon* and *Mops* formed a strongly-supported monophyletic group (divergence date: 19.07 MYA; 95% confidence interval: 10.69 – 27.86 MYA), within which *Mops* taxa were basal to *Chaerephon*. *Mops* taxa did not form a monophyletic clade, and *Chaerephon* taxa appear as a derived subclade of *Mops*, raising questions about these genera as currently circumscribed, and indicating the need for further studies based on greater genetic and taxonomic representation. Freeman (1981) and Legendre (1984) define *Sauromys* as a subgenus of *Mormopterus*; however, we find *Mormopterus* to be a monophyletic clade whereas *Sauromys* occupies a position sister to the *Mops*/*Chaerephon* clade.

Further, Legendre (1984) classified *Chaerephon* and *Mops* as subgenera of *Tadarida* whereas we find *Chaerephon* and *Mops* to be distinct from *Tadarida*.

Otomops remains both morphologically and genetically discrete. At the population level, the roosting behaviour of *Otomops* is different from that of other Molossidae. For example, *Chaerephon* and *Mops*, which form a single clade and whose status as distinct genera has been questioned by the results of this study, have been shown to share roost spaces (Goodman and Cardiff, 2004), whereas *Otomops* has never been observed sharing a roost with members of other molossid genera.

Phylogenetic data, such as that of this study, are an important complement to taxonomies based on morphological measurements. Many bat species appear morphologically similar and morphological measurements used in systematics can be subject to homoplasy and convergence, which may confound phylogenies (Mayer *et al*., 2007; Evin *et al*., 2008). For example, within the family Vespertilionidae, a complex of morphologically similar bats, *Myotis myotis* (Borkhausen, 1797) and *M*. *blythii* (Tomes, 1857) were initially defined based on traditional morphometric analyses. However molecular studies by Castella *et al*. (2000) revealed an additional grouping within these taxa; this was elevated to full species rank by Simmons (2005), and named *M. punicus* (Elvin *et al*., 2008). Molossid species have also been known to exhibit morphological similarity, e.g. *Chaerephon jobimena* shares similar size range with *C. ansorgei*, *C. bemmeleni*, *C. bivittatus*, *C. jobensis*, *C. johorensis*, *C. nigeriae*, *C. plicatus*, *C. pumilus* and *C. russatus* (Goodman and Cardiff, 2004), and according to Freeman (1981), some species can only be differentiated on the basis of somewhat subtle anatomical differences, e.g. the degree of ear joining, shape of the antitragus and wrinkles on the lips. In light of these morphological similarities, molecular-based phylogenies contribute greatly to the circumscription of taxonomic units within the Molossidae.

Species-level analyses of Afro-Malagasy Otomops

Results from Chapter 2 (subsequently published in a peer-reviewed journal under Lamb *et al*., 2008) and Chapter 6 (Ralph *et al*., 2015) reveal the primary division of Afro-Malagasy *Otomops* into two strongy-supported reciprocally-monophyletic clades, from Madagascar and mainland Africa. Analyses based on the mitochondrial cytochrome *b* gene and D-loop confirmed the Malagasy clade as a species in its own right, *O. madagascariensis*. The mainland Africa clade was subdivided into two strongly supported reciprocally monophyletic sister lineages containing samples from southern and western Africa (Burundi, Ivory Coast, South Africa, Tanzania and Zimbabwe) and northeast Africa and the Arabian Peninsula (Ethiopia, Kenya and Yemen). In Lamb *et al*. (2008) we referred both mainland Africa clades to *O. martiensseni*. Statistical parsimony haplotype network analysis

supported this pattern and AMOVA confirmed that 80% of variance occurred between the three geographically-defined clades (Chapter 2, Tables 3.17 and 3.15).

Based on more extensive taxonomic sampling, a molecular dataset which included both mitochondrial and nuclear sequence data, and morphometric and ecological niche modelling analyses, we subsequently described the northeast African clade as a species in its own right, *O. harrisoni* (Ralph *et al*., 2015). This increased the number of species found in the region from two to three, including *O. martiensseni* and *O. madagascariensis*. Use of mitochondrial markers (cytochrome *b* gene and D-loop region) for phylogenetic and phylogeographic analysis showed that *Otomops* may have an Oriental origin since outgroups *O. wroughtoni* and *O. formosus* are basal to their Afro-Malagasy counterparts.

Otomops madagascariensis was separated from the mainland Africa clades by a mean concatenated cytochrome *b* and D-loop p-distance of 3.30%, whereas *O. martiensseni* and *O. harrisoni* were separated by 2.10% (Chapter 6), consistent with a more recent divergence. This is also supported by lower pairwise F_{ST} values between *O. martiensseni* and *O. harrisoni* (0.087) than between *O. madagascariensis* and *O. martiensseni*/*O. harrisoni* (average 0.262) (Chapter 5, Table 4). Nuclear sequence and microsatellite data presented in Chapters 5 and 6, including a haplotype network (Chapter 5, Fig. 3.3), STRUCTURE plot (Chapter 6, Fig. 4) and a Delta K vs. K plot (Chapter 5, Fig. 3.1), each showed division of samples into the 3 species groups, congruent with results obtained using mtDNA data.

Isolation by distance did not appear to influence patterns of genetic structure in this strong flying molossid genus (Chapter 5). However, it is likely that the 482 – 950 km Mozambique Channel separating Madagascar from the mainland forms a vicariant barrier contributing to the separation of *O. madagascariensis* from its generic counterparts in mainland Africa. Ecological niche modelling suggests that each species group has become adapted to their respective habitats. *Otomops harrisoni* from northeastern Africa and the Arabian Peninsula is unable to tolerate variability in conditions, and is affected most by changes in altitude, precipitation and seasonality of temperature. MaxEnt analysis suggests that the potential range of *O. harrisoni* extends from the Arabian Peninsula in the north through to Kenya and Ethiopia in the sub-Saharan mainland, with preference for localities at high altitudes (>1500 m a.s.l) with relatively drier climates (<500 mm annual precipitation) (Chapter 6, Fig. 7A). These areas are typically characterised by warm semi-arid, tropical savanna and warm desert climates. *Otomops martiensseni* has a comparatively broader distribution, from the coastal regions of South Africa extending further north on the east coast through to Mozambique and eastern Tanzania, Uganda, Rwanda, Burundi and into Kenya and central Ethiopia (Chapter 6, Fig. 7B). Parts of the Democratic Republic of Congo in central Africa, and areas of Ivory Coast, Ghana, Togo, Benin and southwest Nigeria are also included within the potential range. The distribution of *O. martiensseni* is

mainly restricted by precipitation levels, and it shows a preference for areas with high annual precipitation (>1000 mm) (Chapter 6, Fig. 7B). *Otomops madagascariensis* is primarily distributed in the drier, western part of Madagascar (Goodman and Ramasindrazana, 2014), although diversity and neutrality analyses based on the mitochondrial D-loop indicate population size expansion in this species (Chapter 2; Table 3.17). Evidence of population expansion provides support for the suggestion made by Racey *et al*. (2010) that further field surveys need to be undertaken to confirm the distribution of bat species on the island. In addition to ecological niche modelling, haplotype network structure was also seen to be predominantly influenced by differences in altitude (Chapter 2, Figs. 3.15 and 3.25). It is also possible that separation of the two mainland African *Otomops* species, *O. martenisseni* and *O. harrisoni*, may be maintained by the use of alternate migration routes (boreal and austral) when following opposing insect migration patterns around the Inter Tropical Convergence Zone (ITCZ). The existence of three species of Afro-Malagasy *Otomops* is supported not only by genetic and ecological analyses but also by morphological analyses. Traditional and geomorphometric analyses of cranio-dental morphology also supported the existence of three taxa within the region, namely *O. madagascariensis* from Madagascar, and *O. martiensseni* and the newly described *O. harrisoni* from mainland Africa and the Arabian Peninsula (Chapter 6, Figs. 5 and 6).

Otomops *population- and colony-level findings*

Population genetic analyses support the division of Afro-Malagasy *Otomops* into three species, as they are consistent with the presence of three major groups, as defined by the DNA sequence-based, morphometric and niche-modelling analyses, and further indicate no migration or gene flow between these species-level lineages. There is little subdivision within these lineages according to colony or geographical locality and evidence of migration and gene flow among colonies. This is consistent with relatively low and predominantly non-significant pairwise F_{ST} values (< 0.10) among colonies within species lineages, low mean within-colony relatedness values and a lack of inbreeding within colonies. These findings suggest that gene flow is maintained by a random mating structure based on extra-colony mating as well as instances of local migration or dispersal of individuals into new colonies. A radio-tracking study shows that South African *Otomops* move in and out of areas, utilising a number of day and night roosts and various foraging areas (Fenton *et al*., 2002). AMOVA based on microsatellite data showed the highest percentage of variance (62.82%) to be among individuals within colonies and observed and expected heterozygosity values within colonies were moderate to high (H_O range: 0.33 – 0.70; H_E range: 0.51 – 0.75), which suggests a relatively high level of genetic diversity and high gene flow within and among the colonies, indicative of random mating structure among individuals (Bogdanowicz *et al*., 2012). Phylogenetic and haplotype analyses based on mitochondrial sequence data (Chapter 5, Fig. 3.3 and Chapter 6, Fig. 2) support this as they show little

subdivision by locality but rather show an admixture of individuals from their respective larger geographical regions.

Half-sibling relationships, in which individuals share either a mother or a father, were the most common form of kinship observed. These, combined with the lack of mitochondrial and/or nuclear genetic structure within species lineages, are consistent with male and female-biased dispersal and extra-colony mating in Afro-Malagasy *Otomops*. Fenton *et al*. (2002) observed that both male and female South African *Otomops* change day roosts, and are able to travel great distances (at least 10 km) from the original day roost.

A harem structure implies that dominant males may have increased reproductive success with colony females. However, mature males have been observed in association with females and young outside of the mating season, which suggests that harem associations are not exclusively related to mating. Other benefits from maintaining such a structure may include reduced predation risk, reduced thermoregulation cost, information transfer and protection from invading males by the dominant male (Kerth and Van Schaik, 2012). If roost structure is maintained for reasons other than mating, it is plausible that females may mate outside the colony and return to their original roost thereafter. Such a situation is not uncommon in bats and has been observed in *Cynopterus sphinx*, where males are significantly more faithful to the colony than females (Garg *et al*., 2015). In the case of *Cynopterus*, the authors suggest that the reason for this loyalty lies in the increased chance of reproductive success since females, able to move between harems and colonies, will be able to access males in a stable space. McCracken and Wilkinson (2000) acknowledge that it is more common for one or both sexes to mate outside of the harem, as a result of which the preferential reproductive ability of resident males has not been documented for many bat species.

Otomops martiensseni colonies in South Africa, where roosts tend to be in the roof-spaces of houses, tend to be considerably smaller than those of *O. harrisoni* or *O. madagascariensis*, which roost in caves. This may reflect the relative abundance of suitable roof spaces, compared with caves (Fenton *et al*., 2002). Observations at the various roost sites suggest the existence of a harem structure, which is most noticeable in the house-dwelling *O. martiensseni* of South Africa. Although this roost structure suggests that dominant males may exhibit philopatry, males are also known to disperse and utilise day and night roosts. Solitary males in roosts, such as those we had observed during collection of our samples, may have dispersed or be unable to attract suitable mates (Fenton *et al*., 2002). However, use of numerous day/night roosts makes it possible that solitary individuals may have been using an alternative roost site at the time of capture or may have remained in the roost to defend their territory. A harem structure may exist in the northeast African and Malagasy colonies, but may be

more difficult to observe due to the nature of the roost sites (caves) as well as the comparatively larger numbers comprising each colony (Fenton *et al*., 2002).

Otomops *conservation implications*

Phylogenetic and population genetic studies will have implications for the the conservation status and future protection of the various Afro-Malagasy *Otomops* species. In particular, the conservation status of the newly described *O. harrisoni*, from northeast African and the Arabian Peninsula, will need to be assessed and appropriate conservation strategies put in place. This will also have implications for the conservation status of *O. martiensseni*, whose range has been redefined. *Otomops martiensseni*, as previously circumscribed, was classified in the IUCN (The World Conservation Union) Red List of Threatened Species as “Vulnerable” from 1996 to 2004 (Mickleburgh *et al*., 2004) due to the paucity of data, suggesting that the number of individuals for this species was particularly low and thus warranted active protection. This classification was changed to “Near Threatened” in 2008 (Mickleburgh *et al*., 2008) based on a slower rate of global decline in population numbers (Mickleburgh *et al*., 2008). Population numbers of *O. martiensseni* from the southern and western regions of Africa have shown an increase in recent years and could retain their “Near Threatened” IUCN status, however it is suspected that population numbers of *O. harrisoni* from northeastern Africa and the Arabian Peninsula are still declining due to habitat disturbance and thus may require a “Threatened” or “Vulnerable” IUCN status after further threat assessment (Mickleburgh *et al*., 2008). With additional studies on the diversity, biology and behavioural ecology of Afro-Malagasy *Otomops*, the conservation status of these three species can be re-evaluated and both global and local legislation for its conservation appropriately updated and implemented.

Future Research

Future investigations should be based on more extensive taxonomic sampling; sample numbers should be increased, and the sampling range should be widened to include as-yet-unsampled Afrotropical localities. This will help to refine the distributions of *O. martiensseni*, *O. harrisoni* and *O. madagascariensis*, and has the potential to uncover further *Otomops* species, particularly from west Africa, from which only one Ivory Coast sample was available. This sample was strongly supported as part of the *O. martiensseni* clade, which has a wide range including south, central, west and east Africa. However it was separated from the rest of its clade by a relatively large cytochrome *b* genetic distance of 2.1% (compared to 3.1% between *O. martiensseni* and *O. harrisoni*); the haplotype network showed it to be an outlier among the *O. martiensseni* samples, and of these it was clearly the

most similar to *O. harrisoni* (Lamb *et al*., 2008). More extensive sampling in central and west Africa would clarify the position of this sample.

Fine scale analyses based on nuclear microsatellites would benefit from more extensive sampling at colony level. Additional colonies should be identified and sampling of colonies should be carried out over a number of years. This will allow us to gain a better understanding of social structure and behaviour at colony level. More extensive genetic sampling, by the identification and use of more microsatellite loci in the analyses will allow determination of kinship within colonies to be made with greater reliability. Combination of microsatellite-based analyses at population level with ecological data, including radio tracking and observation studies, will allow for a more comprehensive understanding of the biology and social systems of *Otomops* spp.

Markers and techniques used in this study could potentially be applied to the study of Asian *Otomops* species, given the availability of suitable samples, and possibly other molossid species. More complete phylogenetic, population genetic, morphometric and ecological analyses will enable more accurate assessements of the conservation status of *Otomops* species and potentially, taxonomic units below the species level will need to be translated into updated conservation legislation in the countries in which *Otomops* is found. While this study has advanced our knowledge of the biology of *Otomops*, there is clearly potential for further work, which will enhance our understanding of this genus within not only Africa, Madagascar and the Arabian Peninsula, but also Asia.

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APPENDICES

Appendix 1

Lamb, J.M., Ralph, T.M.C., Goodman, S.M., Bogdanowicz, W., Fahr, J., Gajewska, M., Bates, P.J.J., Eger, J., Benda, P. and Taylor, P. (2008). Phylogeography and predicted distribution of African-Arabian and Malagasy populations of giant mastiff bats, *Otomops* spp. (Chiroptera: Molossidae). *Acta Chiropterologica* **10**: 21-40.

Appendix 2

Lamb, J.M., Ralph, T.M., Naidoo, T., Taylor, P.J., Ratrimomanarivo, F., Stanley, W.T. and Goodman, S.M. (2011). Toward a molecular phylogeny for the Molossidae (Chiroptera) of the Afro-Malagasy region. *Acta Chiropterologica* **13**: 1-16.

Appendix 3

Lamb, J.M., Abdel-Rahman, E.H., Ralph, T.M.C., Fenton, M.B., Naidoo, A., Richardson, E.J., Jacobs, D.S., Denys, C. and Taylor, P.J. (2006). Phylogeography of southern and northeastern African populations of *Otomops martiensseni* (Chiroptera: Molossidae). *Durban Museum Novitates* **31**: 42-53.

Acta Chiropterologica, 10(1): 21–40, 2008
PL ISSN 1508-1109 © Museum and Institute of Zoology PAS
doi: 10.3161/150811008X331063

Phylogeography and predicted distribution of African-Arabian and Malagasy populations of giant mastiff bats, *Otomops* spp. (Chiroptera: Molossidae)

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Otomops martiensseni is sparsely distributed throughout sub-Saharan Africa and southwestern Arabia (Yemen). *Otomops madagascariensis* from the dry portions of Madagascar is widely recognised to be a distinct species. Based on mitochondrial DNA sequences of the cytochrome *b* gene (1,004 base pairs; $n = 50$) and the control region (D-loop, 290 base pairs; $n = 52$), two Oriental outgroup species (*O. wroughtoni* and *O.* cf. *formosus*) formed a monophyletic clade that was the sister group to the Afro-Malagasy taxa, composed of *O. martiensseni* and *O. madagascariensis*. Within the Afro-Malagasy clade, we discovered three well-supported but genetically similar clades (inter-clade genetic distances of 3.4–4.4%) from 1) north-eastern Africa and Arabia, 2) African mainland except northeast Africa, and 3) Madagascar. Taken together, haplotype networks, estimated divergence times, regional species richness and historical demographic data tentatively suggested dispersal from Asia to Africa and Madagascar. To understand ecological determinants of phylogeographic, biogeographic and genetic structure, we assessed the potential distribution of *O. martiensseni* throughout sub-Saharan Africa with ecological niche modelling (MaxEnt) based on known point localities ($n = 60$). The species is predicted to occur mainly in woodlands and forests and in areas of rough topography. Continuity of suitable habitats supported our inferred high levels of continental gene flow (relatively low genetic distances), and suggested that factors other than habitat suitability have resulted in the observed phylogeographic structure (e.g., seasonal mass migrations of insects that might be tracked by these bats). Based on a Bayesian relaxed clock approach and two fossil calibration dates, we estimated that African and Oriental clades diverged at 4.2 Mya, Malagasy and African clades at 1.5 Mya, and African clades 1 and 2 at 1.2 Mya. Integrating phylogenetic, phylogeographic, population genetic and ecological approaches holds promise for a better understanding of biodiversity patterns and evolutionary processes.

Key words: *Otomops*, mitochondrial DNA, phylogeography, Africa, Madagascar, ecological niche modelling

INTRODUCTION

The Palaeotropical genus *Otomops* Thomas, 1913 currently comprises seven species of large-bodied (25–50 g — Mutere, 1973) molossid bats. Five species of Oriental origin have very restricted distributions in southern India, Cambodia, Java, Alor Island (Indonesia), the Philippines, and Papua New Guinea, suggesting an Oriental centre of origin for the genus. The two Afro-Malagasy species are widely but sparsely distributed in Africa and Madagascar (Peterson *et al.*, 1995; Simmons, 2005). *Otomops martiensseni* (Matschie, 1897) occurs on the Arabian Peninsula (Yemen) and the African mainland from Ethiopia and Eritrea in the north, to Ivory Coast in the west and Durban, South Africa in the south. *Otomops madagascariensis* Dorst, 1953 is only known from the drier regions of Madagascar

(Goodman *et al.*, 2005). These high-flying bats have seldom been captured in mist nets and the vast majority of distributional information derives from day-roost sites. In Madagascar, these sites are typically associated with caves in areas of exposed sedimentary rock. In northeast and east-central Africa, *O. martiensseni* colonies frequently occupy caves in volcanic hills and mountains (such as the lava tunnels on Mt. Suswa in Kenya), largely coinciding with the entire length of the Great African Rift Valley from Eritrea and Djibouti in the north to the Zambezi Escarpment of northern Zimbabwe in the south. The genus is conspicuously absent from the central Congo Basin (with scattered records in Angola, Central African Republic, and the Democratic Republic of the Congo along the periphery of the basin), but is present in low-lying areas of west Africa (Ivory Coast) and the Durban metropolitan region and surroundings on the east coast of South Africa.

Chubb (1917) described *O. icarus* Chubb, 1917 from Durban, South Africa, as a species distinct from *O. martiensseni*. While this view is not currently upheld by most authors (Harrison, 1957; Meester *et al.*, 1986; Koopman, 1993; Bronner *et al.*, 2003; Simmons, 2005), populations from east Africa (*martiensseni*) and South Africa (*icarus*) have been shown to be distinct in cytochrome *b* and D-loop mitochondrial sequences, albeit at a low sequence divergence (Kimura 2-parameter-corrected) of 2.5% for cytochrome *b* (Lamb *et al.*, 2006). However, nuclear markers (PCR-RAPDs) showed very high genetic similarities between individuals from Kenya and South Africa (Lamb *et al.*, 2006).

In east Africa, *O. martiensseni* has usually been found in colonies of hundreds to tens of thousands of individuals roosting in caves (Mutere, 1973; Kock *et al.*, 2005), where apparent substantial declines in populations have been used to justify the IUCN listing as 'Near Threatened' (Mickleburgh *et al.*, 2004; but see Kock *et al.*, 2005, for a contrasting view). Around Durban, colonies are smaller and individuals roost in buildings. Elsewhere, for example in central, east, and west Africa, they are known to roost in hollow trees (Decher *et al.*, 1997; Fenton *et al.*, 2002). In Madagascar, except for one case discussed below, *O. madagascariensis* is unknown from synanthropic settings and most cave colonies are less than 70 individuals (Andriafidison *et al.*, 2007; SMG, unpublished data). Roosts in Durban typically comprise up to 30 individuals, one adult male and one to 10+ adult females with young, suggesting a harem social structure (Richardson and Taylor, 1995; Taylor, 1998, 2000; Fenton *et al.*, 2002). In South Africa, due to its restricted distribution, *O. martiensseni* has recently been assigned a 'Vulnerable' IUCN rating (Friedmann and Daly, 2004).

Our study has five objectives. The first was to delimit Afro-Malagasy species of *Otomops* based on evidence from the mitochondrial cytochrome *b* and the D-loop regions of samples from 20 localities. Specifically, our first aim is to test the validity and distributional limits of the described forms, *martiensseni*, *icarus*, and *madagascariensis*. Mitochondrial DNA sequences have contributed significantly towards delimiting species and revealing cryptic species diversity in bats. A recent study of the mitochondrial ND1 gene increased the known diversity of western Palaearctic bats from 46 to 54 in an apparently well-studied fauna (Mayer *et al.*, 2007). This includes the description of cryptic lineages within five European vespertilionid species found on the Iberian Peninsula (Ibáñez *et al.*, 2006) and new species recognized within *Myotis mystacinus* (Kuhl, 1817), *Plecotus austriacus* (Fischer 1829) (Mayer and von Helversen, 2001) and the *Pipistrellus pipistrellus-pygmaeus* complex within the Mediterranean Region (Hulva *et al.*, 2004). Similarly, DNA sequences have led to increases in recognized species diversity in numerous New World bats including, for example in the genus *Carollia* Gray, 1838 (Baker and Bradley, 2006).

Our second aim was to compare and contrast genetic structure and demographic history of Afro-Malagasy *Otomops* populations from regions known to have diverse roosting habitats and possibly different breeding systems. To this end we estimated population genetic and demographic population history parameters separately for three genetically defined clades from mainland Africa and Madagascar. Understanding patterns and causes of underlying genetic structure is critical to predicting modes of speciation, past historical events (e.g., bottlenecks which result in reduced heterozygosity), possible evolutionary trajectories of diverging species, and the presence of 'evolutionary significant units' (ESU) or unique gene pools, which merit formal conservation protection (Moritz, 1994; Brown and Houlden, 2000).

Thirdly, we attempted to infer historical patterns of colonization of *Otomops* from Asia, Africa and Madagascar. Since population genetic data can illuminate alternative dispersal hypotheses (Russell *et al.*, 2007), we combined phylogenetic, phylogeographic, and population genetic data to investigate

patterns of colonization of *Otomops* from Madagascar and Africa in the light of available evidence, which suggests a predominant pattern of dispersal from the African mainland to Madagascar but not vice versa (Eger and Mitchell, 2003; Ratrimomanarivo *et al*., 2007; Russell *et al*., 2007).

Fourthly, we used a Bayesian approach, with relaxed clock model, to date nodes using fossil calibrations of earliest *Mops* divergence in the mid-Miocene (11.2–16.4 Mya — McKenna and Bell, 1997; Jones *et al*., 2005) and earliest molossid divergence in Africa (17.5–18.0 Mya — Arroyo-Cabrales *et al*., 2002).

Fifthly and finally, we examined phylogeographic and population genetic patterns in the light of current ecological and biogeographical data. On the one hand, morphological capability of *Otomops* for long distance dispersal (Rydell and Yalden, 1997), and the likelihood that this species is capable of long-distance migration (Kock *et al*., 2005), would lead us to predict the absence of deep phylogeographic structure at continental and local scales. On the other hand, scattered distribution and paucity of collecting records indicates a seemingly rare species complex for which we might hypothesize some degree of geographical isolation and phylogeographic differentiation, yet such a situation may be an artefact of the difficulty of catching these animals or access to roosting sites. Using the MaxEnt program, we modelled the potential distribution of *Otomops* throughout sub-Saharan Africa under current environmental conditions to assess if patterns of phylogeographic structure and potential distribution are concordant. Such integration of ecological and phylogeographic approaches has rarely been attempted previously (but see Rissler *et al*., 2006; Peterson and Nyári, 2007; Raxworthy *et al*., 2007), but should be further explored since current biogeographic patterns are the result of both ecological and historical processes. Our study models only current ecology but it is possible with advanced climatic models to predict past climatic effects such as Pleistocene refugia (Peterson and Nyári, 2007).

Materials and Methods

Samples

Otomops samples were obtained from Yemen, sub-Saharan Africa (Burundi, Ethiopia, Ivory Coast, Kenya, Tanzania, Zimbabwe and South Africa), the drier portions of Madagascar, Cambodia (*O. wroughtoni*) and the Philippines (*O*. cf. *formosus* — Appendix). South African samples included those from residential areas within the Durban metropolitan region and on the adjacent north and south coastlines of eastern South Africa (Ballito, Durban, Pinetown, Silverglen, Scottburgh). For the cytochrome *b* study, the following molossid outgroups were sequenced in our Durban laboratory: *Tadarida fulminans* (Thomas, 1903), *Mops midas* (Sundevall, 1843), *M. condylurus* (A. Smith, 1833) and *M. leucostigma* G. M. Allen, 1918. Sequences for *Pipistrellus abramus* (Temminck, 1838) were taken from the NCBI GenBank (No. NC005436). As D-loop sequences were used for haplotype and population genetic analysis and not analysed phylogenetically, we did not use the specific outgroups.

DNA Sequencing and Analysis

Genetic variation of *Otomops* was investigated using mitochondrial cytochrome *b* (n = 50) and D-loop sequencing (n = 52). *Otomops* DNA was isolated, using a DNeasy® DNA isolation kit (Qiagen), from liver, heart, kidney or muscle tissues previously stored in 80% ethanol or EDTA. Cytochrome *b* trees were further rooted on both molossid (*Tadarida fulminans*, *Mops midas*, *M. condylurus*, *M. leucostigma*) and non-molossid (*Pipistrellus abramus*) outgroups.

PCR amplifications for all samples except *O. wroughtoni* and *O*. cf. *formosus* were performed in the laboratory of JL in South Africa, whilst DNA from the two Asian samples was amplified and sequenced in the laboratory of WB. For the majority of the samples, the cytochrome *b* gene was PCR-amplified (Saiki *et al*., 1988) as two overlapping double-stranded fragments using the following primer pairs: L 14723 (5'-ACC AATGCAATGAAAAATCATCGTT-3') and H 15553 (5'-TAGGCAAATAGGAAATATCATTCTGGT-3'); L15146 (5'-CATGAGGACAAATATCATTCTGAG-3') and H15915 (5'-TCTCCATTTCTGGTTTACAAGAC-3') (Irwin *et al*., 1991). In order obtain a complete sequence, primers L14723 and L46RC (5'-CTCAG AAAG ATATTTG TCCTCATG-3'), as well as H53RC (5'-AC CAGAATGATATTTCCTATTTGCC-TA-3') and H15915, were used to obtain additional data on the first and last ≈400 bp of sequence, respectively. Amplifications with these primers were performed in 25 µl volumes. Each reaction contained 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Roche Diagnostics), 0.2 µl Taq polymerase (5 U/µl) (Super-Therm) and 4 µl of each primer (6 µM) (forward and reverse) per reaction. The thermal cycling parameters used were as follows: 94°C for 4 min; 36 cycles of (94°C for 40 s, 50°C for 45 s and 72°C for 40 s); 72°C for 10 min. Target fragments were purified from excised gel bands using a QIAquick® Gel Extraction Kit (QIAGEN Inc.) and sequenced at Inqababiotec, Pretoria, South Africa.

For the Asian taxa, amplification and sequencing of the entire cytochrome *b* gene was carried out using primers: rhin1L (5'-ATGACATGAAAAATCACCGTTGTA-3') and Nyc2H (5'-GTTTACAAGACCRGKGTAATKDAT-3'). Additional primers Oto420L (5'-CTGAGGAGCAACAGTCATCA-3'), Oto751L (5'-GGAGACCCCGACAATTACAC-3'), Oto1029H (5'-TAC TGGTTGGCCTCCRATTC-3') and Nyc4H (5'-ACDGARAA DCCVCCTCARATTCA-3') were used for sequencing, which was carried out with a CEQ8000 sequencer (Beckman-Coulter).

Amplification of sequences from the Asian taxa was performed in 50µl total volume containing 3–5 µl template, 0.2 µM of each primer, 5× amplification buffer (250 mM KCl, 7.5 mM

$MgCl_2$, 50 mM Tris, pH 8.4), 1 mM of each dNTP [Sigma-Aldrich, Germany]) and 2U REDTaq Polymerase (Sigma-Aldrich, Germany). The thermal cycling parameters used were as follows: 95°C for 2 min; 35 cycles of (94°C for 15 s, 50°C for 20 s and 72°C for 1 min); 72°C for 3 min.

The D-loop regions of all samples were amplified as a single fragment using primers P (5'-TCCTACCATCAGCACCCA AAGC-3') and E (5'-CCTGAAGTAGGAACCAGATG-3') (Wilkinson and Chapman, 1991). Amplifications were performed as described above for cytochrome *b*. The thermal cycling parameters used were as follows: 95°C for 2 min; 40 cycles of (95°C for 60 s, 55°C for 90 s and 72°C for 120 s); 72°C for 7 min. Target fragments were purified from excised gel bands using a QIAquick® Gel Extraction Kit (QIAGEN Inc.) and sequenced at Inqababiotec, Pretoria, South Africa. For the two Asian samples, sequencing, was carried out in a CEQ8000 sequencer (Beckman-Coulter).

All fragments were sequenced in both directions. Sequences were deposited in GenBank (Appendix). Sequences were aligned using the CLUSTAL W option (Thompson *et al.*, 1994) of the BioEdit program (Version 5.0.9 for Windows 95/98/NT) and by visual inspection. Sequences were trimmed to a common length of 1,004 nucleotides (nt) for the cytochrome *b* gene and 290 nt for the D-loop. In all analyses, indels were treated as a fifth character, rather than as missing data.

We used MODELTEST 3.7 (Posada and Crandall, 1998) to determine that the cytochrome *b* sequences best fit a model of (GTR+I+G). This model specifies unequal base frequencies and a gamma-distributed mutation rate with shape parameter α = 1.1064. The proportion of invariant sites was 0.470. Subsequent analyses were carried out using the assumptions of this model and parameter values specified.

Phylogenetic and Phylogeographic Analysis

We used a Bayesian likelihood analysis to look for phylogenetic signal within our *Otomops* cytochrome *b* sample (MrBayes version 3.0b4; Huelsenbeck and Ronquist, 2001). For each analysis, we ran four Markov chains for 5 million generations each, sampling every 100 chains. The chains were heated with the temperature scaling factor T = 0.02. We discarded the first 25,000 trees as burn-in, and constructed a 50% majority rule consensus tree from the remaining trees. We identified three *Otomops* clades based on the architecture of these trees (see below), and calculated GTR genetic distances between these clades and outgroups in PAUP for the cytochrome *b* data (Swofford, 1998).

In some intraspecific analyses, a hierarchical tree may be inappropriate for representing relationships among haplotypes because the period of time over which the samples have evolved is so short that ancestral and descendant haplotypes exist (Posada and Crandall, 2001; Kratysberg *et al*., 2004). In such instances, a haplotype network is more appropriate to illustrate relationships among the sampled haplotypes by using multiple pathways to reflect possible homoplasy or reverse mutations. Since our D-loop data showed greater variability than for cytochrome *b*, we used this dataset to construct a haplotype network using the TCS computer programme (Clement *et al.*, 2000).

Analysis of Molecular Variance

In order to test for significant molecular variance structure between the three Afro-Malagasy clades defined by phylogenetic analyses, the sampled populations were subdivided into groups ('south-east-central-west', n = 9 populations; 'north-east', n = 3 populations; Madagascar, n = 4 populations), and the cytochrome *b* dataset (n = 1,004 nt) was analysed by hierarchical Analysis of Molecular Variance (AMOVA) using the program Arlequin 3.01 (Excoffier *et al.*, 2005). Fixation indices were calculated (for individuals, populations and groups) in a conventional fashion and their significance tested using a non-parametric permutation approach described in Excoffier *et al.* (1992), consisting of permuting haplotypes, individuals or populations, among individuals, populations or groups of populations. After each permutation round, all statistics were recomputed to obtain their null distributions.

Population Genetic Analysis

Because of its higher variability, the D-loop dataset was also used for population genetic analyses; these were performed separately for each of the three genetically-defined groups. Following Rogers and Harpending (1992) and Russell *et al.* (2005), we used haplotype (h) and nucleotide (π) diversity values, neutrality tests (Fu's 1997 F_s and Fu and Li's 1993 D* and F*), and mismatch distribution analysis (distribution of observed pairwise nucleotide differences) to estimate whether each population group was stationary or had undergone an historical population expansion. High h with low π, a unimodal pairwise difference distribution, significant F_s but non-significant D^* and F^*, and a high ratio of number of variable sites (S) to average number of pairwise differences (d) (S/d), are indicators of an historical population expansion event (Russell *et al.*, 2005 and references therein). These analyses were carried out with DnaSP version 4.0 (Rozas *et al.*, 2003). Based on the distribution of pairwise nucleotide differences, the time since expansion, tau (τ), could be calculated in mutational units. Although males and females may reach sexual maturity in one year (Mutere, 1973), data from captive bats (E. J. Richardson and W. White, personal communication) indicate an estimated average generation time of approximately two years for the species. Given this generation time and two independent estimates of D-loop mutation rates based on divergence rates per million years of 17.3% ($\mu = 1.73 \times 10^{-7}$ per generation) and 33% ($\mu = 3.3 \times 10^{-7}$ per generation) (Rogers and Harpending, 1992), this approach allowed approximation of the absolute time of expansion, using the formula $\tau = 2u$ t, where u was calculated as the product of the mutation rate (μ: mutations per site per generation) and sequence length (290 bp), and t was the time (in generations) since expansion.

Dating

Our cytochrome *b* data were used to estimate nodal dates. Recent studies have attempted to calibrate molecular dates of deeper (family-level) nodes of the chiropteran phylogeny based on a robust higher phylogeny and compilation of data from earliest fossil occurrences of bats (Jones *et al*., 2005; Teeling *et al*., 2005). Fossil calibrations of bat molecular phylogenies have also been attempted at the intrageneric level, e.g., in *Myotis* where a cytochrome *b* divergence rate of 4% per Myr (mutation rate of 0.02 substitutions/site/Myr) was applied based on the divergence of two species (Ruedi and Mayer, 2001; Hulva *et al*., 2004). In our study, the 'mid-Miocene' fossil date (11.2–16.4 Mya — Böhme, 2003; given as 13.8 Mya by Jones *et al.*, 2005) for the first fossil occurrence of *Mops* (McKenna and Bell,

1997) was available to calibrate the earliest cytochrome *b* sequence divergence (13.8%; see Table 1) for the three *Mops* species included in this study (*M. midas*, *M. condylurus* and *M. leucostigma*), giving a divergence rate of 1%. Additionally, using the date of the oldest known African molossid (17.5–18.0 Mya; *Tadarida rusingae* — Arroyo-Cabrales *et al.*, 2002) and the deepest divergence between the three African molossid genera sampled in this study (Table 1: 19.0–20.0% obtained between *M. leucostigma* and *O. wroughtoni*), we again obtain a divergence rate of just over 1% per million years (mutation rate of 0.005 subsitutions/site/Myr). If however, one adopts the deepest estimated crown divergence date for all molossids (35–38 Mya; Jones *et al.*, 2005), the divergence rate decreases to just over 0.5% (mutation rate 0.0025 substitutions/site/Myr). These two rates (0.0025 and 0.005) are an order of magnitude lower than the 0.036 third codon substitutions/site/Myr for Chiroptera (n = 222 species) estimated by Nabholz *et al.* (2008), probably due to faster-evolving third codon position (neutral) rates, possible saturation in our data and non-correspondence of available fossil dates. The median third codon chiropteran rate of 0.036 obtained by Nabolz *et al.* (2008) is closer to the value of 0.02 obtained for *Myotis*. The latter estimate was based on all substitutions (as in our study); thus, we chose the value of 0.02 as a fixed mean rate for Bayesian dating analyses as described below.

Before using simple models to date nodes based on the molecular clock (as was common until recently), one should test the assumption of a global clock for a given dataset. Using the program PAUP version 4.0b10, and the cytochrome *b* dataset, likelihood scores were calculated for a pruned 13-taxon ML tree (all molossid species and two or three haplotypes for each Afro-Malagasy lineage), in which the strict molecular clock was enforced (-ln L = 3719.00) and not enforced (-ln L = 3704.90). Applying the Likelihood Ratio Test, with 11 degrees of freedom (number of taxa minus two), we obtained a chi-square test statistic of 28.20, which significantly ($P < 0.01$) rejected the molecular clock assumption. To overcome this problem, we used a Bayesian approach with an uncorrelated relaxed clock model assuming that branch-specific rates followed a log-normal distribution, which gives more biologically reasonable results compared to the exponential model (A. Rambaut, personal communication). Bayesian analysis (Markov Chain Monte Carlo [mcmc] algorithm, with length 5,000,000, sampled every 1,000 iterations with burn-in of 100,000) was used to estimate mean and 95% confidence limits of nodal dates assuming the relaxed clock model described above, the GTR+I+G substitution model (based on the results of MODELTEST 3.7) and a mean mutation rate of 0.02 substitutions/site/Myr. The analysis was achieved using the program BEAST v. 1.4 (Drummond and Rambaut, 2006a) in conjunction with the programs BEAUti v.1.4 (Drummond and Rambaut, 2006*b*) and Tracer v. 1.3 (Rambaut and Drummond, 2005).

Ecological Niche Modelling

Georeferenced distribution records (precision of 0.001 decimal degrees) of *O. martiensseni* (n = 60, data set available from JF — Fig. 3) were used to predict its potential distribution across sub-Saharan Africa south of 20°N. To avoid confounding influences due to phylogeny and geographical outliers, both the Arabian Peninsula (Yemen: 1 record) and Madagascar (14 records) were excluded from modelling. We employed the recently developed MaxEnt algorithm (version 2.3; Phillips *et al.*, 2006) that has been shown to perform particularly well with presence-only data (Elith *et al.*, 2006). Sixteen continuous environmental variables were used as predictors in the final model, including topography (SRTM30: log contrast [mean of maximum-minimum values], calculated with a rectangular 9 * 9 km moving window analysis), climate (WORLDCLIM version 1.4 [Hijmans *et al.*, 2005]: mean maximum temperature in April [T_{max}4], August [T_{max}8], October [T_{max}10], December [T_{max}12], mean minimum temperature in April [T_{min}4], August [T_{min}8], mean precipitation in April [Prec4], August [Prec8], December [Prec12], mean annual precipitation [Bio12], precipitation seasonality [Bio15]) and land cover (MODIS Vegetation Continuous Fields [=VCF], collection 3 [Hansen *et al.*, 2003]: VCF% tree cover and VCF% bare ground; Africa mosaic SPOT VGT data 2000 [Mayaux *et al.*, 2004]: unclassified Near Infra-Red-spectral channel, and unclassified Red-spectral channel). The environmental data were set to a spatial grid resolution of 30 arc seconds (≈ 1 km) and aligned with the digital elevation model (SRMT30) using GIS (ArcView 3.2a with Spatial Analyst 2.0a extension). The MaxEnt model was run with all distribution records (100% training), the regularization multiplier was set to 2.5 to account for statistical over fitting given the relatively large number of predictor variables, and maximum number of iterations was set to 1,000; other MaxEnt settings were kept unchanged. Model performance was assessed with proportion of presences correctly classified (sensitivity), proportion of absences correctly classified (specificity), and discrimination ability (area under the curve [AUC] of a receiver operating characteristic [ROC] plot). Since MaxEnt produces a continuous probability (ranging from 0 to 100% predicted area), we transformed the continuous model output to a map representing probabilities above three pre-defined thresholds: a) 10 percentile training presence, b) equal training sensitivity and specificity, and c) minimum training sensitivity plus specificity. The contribution of each explanatory variable to model performance was evaluated with a jackknife procedure implemented in MaxEnt, where variables are successively omitted and then used in isolation to measure their relative as well as absolute contribution to the model.

RESULTS

Phylogenetic Inference

Bayesian analysis of 1,004 nucleotides of the mitochondrial cytochrome *b* gene yielded a tree in which *Otomops* taxa studied formed a monophyletic clade (posterior probability [pp] 0.75 — Fig. 1). This clade was separated from the molossid outgroups (*Tadarida fulminans*, *Mops midas*, *M. leucostigma* and *M. condylurus*) by an average GTR-corrected genetic distance of 17.8%, and from the vespertilionid outgroup (*Pipistrellus abramus*) by 24.3% (Table 1). Neighbour-joining and maximum parsimony analysis yielded trees with congruent topology (data not shown).

Within the *Otomops* clade, the Oriental species, *O.* cf. *formosus* and *O. wroughtoni*, showed relatively deeper divergence (GTR-corrected cytochrome *b*

distance, 6.6%) than that between lineages within the Afro-Malagasy clade (GTR-corrected cytochrome *b* distance, 3.1 to 4.9%). The average genetic distances between the Afro-Malagasy clade and *O. wroughtoni* and *O.* cf. *formosus* were 11.2% and 10.6%, respectively (see Table 1 for between-groups genetic distances). The Afro-Malagasy *Otomops* clade was subdivided into two reciprocally-monophyletic lineages comprising all samples from Africa plus Yemen (pp 0.96) and Madagascar (pp 1.00), respectively. The mean genetic distance separating African from Malagasy lineages was 4.4%. The African lineage was further split into two well-supported (pp 1.00), reciprocally monophyletic sister-lineages comprising samples from 1) north-east Africa (Ethiopia, Kenya) and Yemen (hereafter, Clade 1) and 2) mainland sub-Saharan Africa (South Africa, Zimbabwe, Tanzania, Burundi, Ivory Coast), excluding north-east Africa and Yemen (Clade 2). A mean genetic distance of 3.4% separated these lineages from each other.

Haplotype Network

The Afro-Malagasy *Otomops* sample of 50 individuals comprised 32 D-loop haplotypes, with a haplotype diversity (h) of 0.986 and a nucleotide diversity (π) per site of 0.106. Most haplotypes (21) comprised only one individual, whilst the rest of the haplotypes comprised between two and five individuals. Statistical parsimony analysis of D-loop data yielded partial networks from the three previously mentioned geographical regions when set at the 95% connection limit. A single network including all *Otomops* samples was formed when the connection limit was fixed at 100 mutational steps (Fig. 2). Overall, haplotypes separated into the three groups defined previously by cytochrome *b* data. Thirty-two mutational steps separated South African samples (Pinetown1 and 4) from Madagascar (Toliara14). Clade 2 (Ivory Coast) was connected to Clade 1 (Kenya7) by 30 mutational steps. At this limit, no connection was evident between the Malagasy network and the Clade 1 network. The Oriental taxa appear closer to the Malagasy (51 mutational steps) group than the south-east-central-west Africa (Clade 2) group (57 mutational steps).

Population Genetics

Using D-loop data, genetic structure and demographic histories were examined separately for the three defined groups. Populations from Madagascar

TABLE 1. Net between-groups genetic distances for *Otomops* samples and outgroups based on cytochrome *b* sequences. GTR-corrected distances are represented below the diagonal, and p-distances above the diagonal

	Taxon	1	2	3	4	5	6	7	8	9	10	11
[1]	*Otomops* S-E-C-W Africa		0.016	0.020	0.030	0.100	0.096	0.140	0.142	0.152	0.148	0.199
[2]	*Otomops* Ivory Coast	0.021		0.033	0.047	0.103	0.097	0.144	0.147	0.153	0.149	0.204
[3]	*Otomops* N-E Africa	0.031	0.037		0.034	0.102	0.095	0.148	0.144	0.154	0.153	0.203
[4]	*Otomops madagascariensis*	0.038	0.049	0.044		0.100	0.096	0.149	0.149	0.157	0.151	0.203
[5]	*Otomops wroughtoni*	0.110	0.116	0.111	0.113		0.062	0.156	0.170	0.168	0.169	0.210
[6]	*Otomops* cf. *formosus*	0.105	0.108	0.106	0.105	0.066		0.148	0.164	0.164	0.160	0.210
[7]	*Mops midas*	0.158	0.162	0.169	0.171	0.178	0.168		0.144	0.123	0.118	0.228
[8]	*Tadarida fulminans*	0.161	0.166	0.164	0.170	0.197	0.190	0.163		0.156	0.155	0.222
[9]	*Mops condylurus*	0.173	0.173	0.175	0.180	0.194	0.189	0.138	0.177		0.029	0.241
[10]	*Mops leucostigma*	0.169	0.169	0.175	0.172	0.196	0.183	0.132	0.176	0.030		0.244
[11]	*Pipistrellus abramus*	0.235	0.240	0.241	0.240	0.250	0.250	0.285	0.274	0.295	0.300	

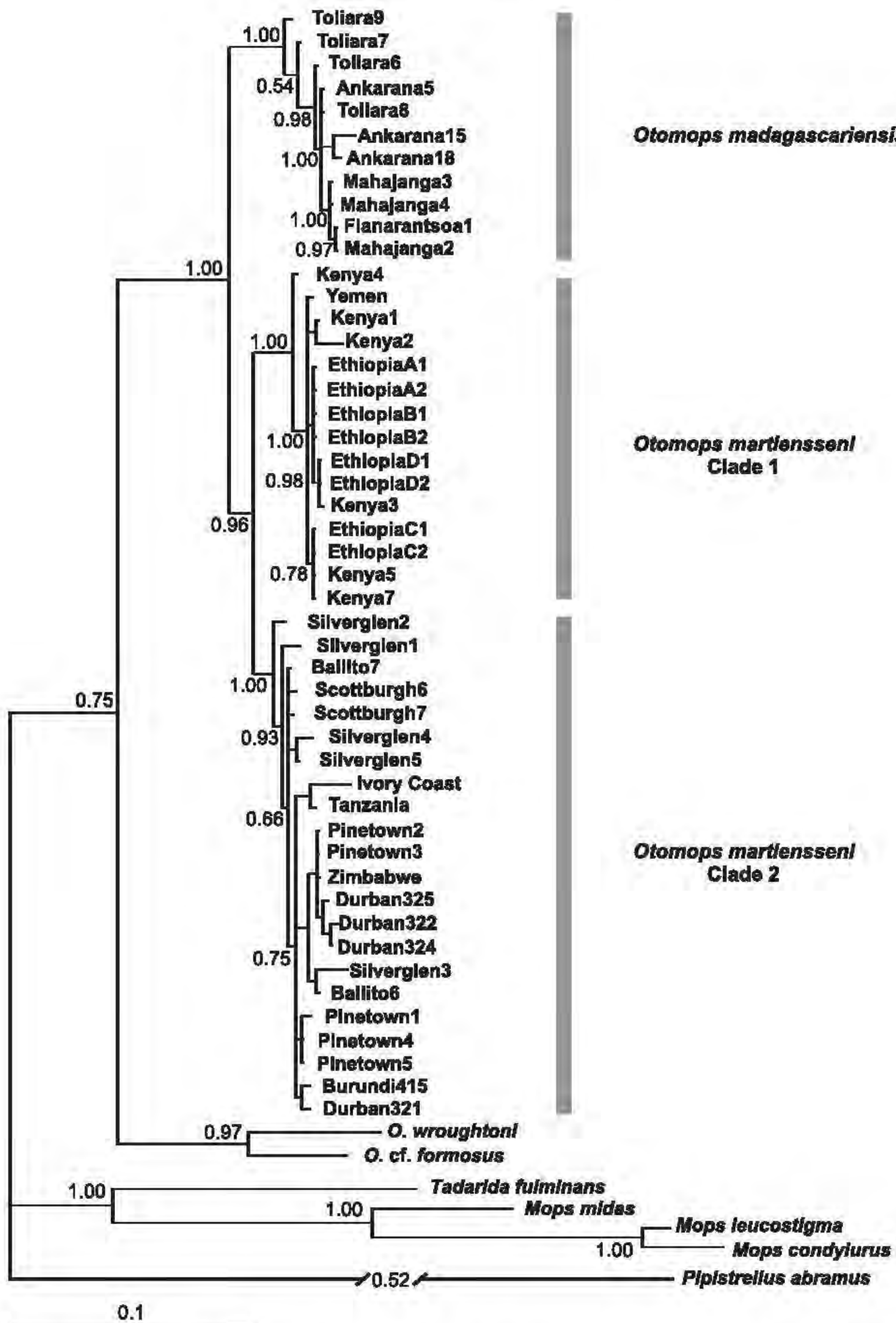

FIG. 1. Bayesian phylogram of cytochrome *b* data (1,004 nucleotides). See Appendix for explanation of locality information

closely fitted the expectations of a model of exponential demographic expansion, e.g., a unimodal mismatch distribution (Fig. 4), low nucleotide diversity but high haplotype diversity, and significant F_S (Table 2). On the other hand, the two mainland groups from Africa and Arabia, whilst meeting certain expectations, showed clearly multimodal mismatch distributions. Using D-loop divergence rates of 17.3% and 33%, we obtained a date for the Madagascar population expansion of between 27,400 and 52,200 yr BP (Table 2).

Analysis of Molecular Variance

AMOVA of cytochrome *b* data revealed significant variance among the three Afro-Malagasy clades (F_{CT} = 0.032; P < 0.01), whilst variance due to populations within groups was not significant at the 1% level, although it was at the 5% level (F_{SC} = 0.06, P = 0.04 — Table 3). Molecular variance was largely dominated by within-population (individual) variance, which explained 91.0% of the total (Table 3). The significant (P < 0.05) variance between populations within groups was a result of the variation in the south-east-central-west clade (Clade 2). In addition, AMOVAs were calculated for each of the groups separately to test for significance of molecular population variance (F_{ST} values). In the case of Madagascar and north-east Africa (Clade 1), F_{ST} values were non-significant while the south-east-central-west clade (Clade 2) was statistically significant at the 5% level (F_{ST} = 0.060, P = 0.03) due to divergence of the Ivory Coast population.

Dating

Based on cytochrome *b*, in spite of the reasonably high confidence limits for the relaxed clock

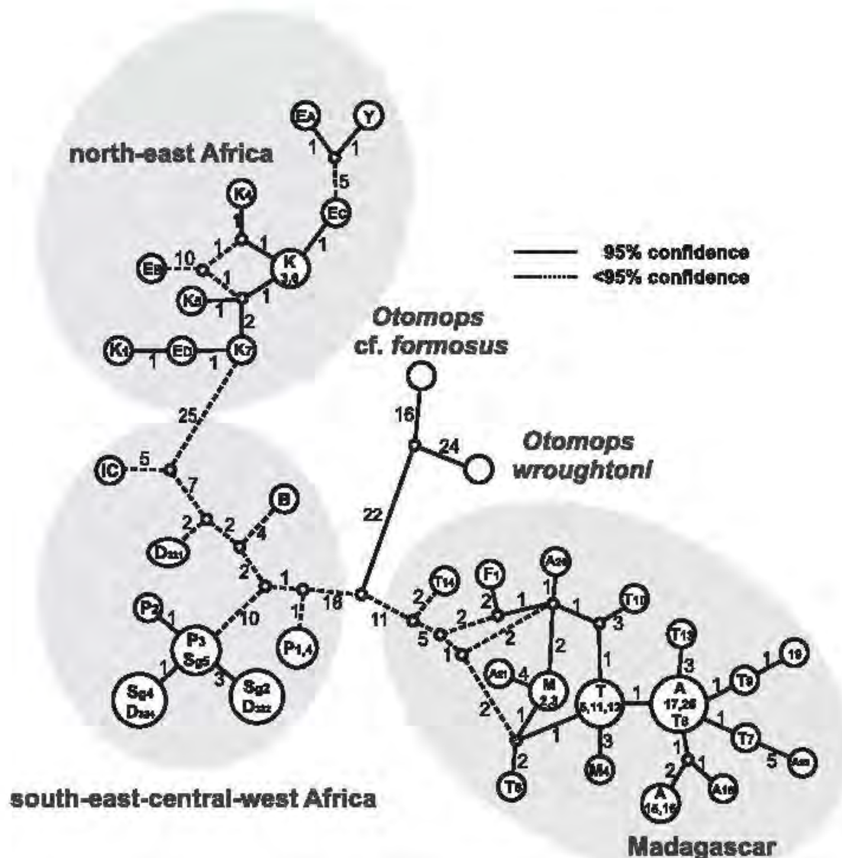

FIG. 2. Statistical parsimony network of D-loop (290 nucleotides) data. Figures next to connections indicate numbers of mutational steps between haplotypes. Locality codes as follows: Y = Yemen, E = Ethiopia, K = Kenya, IC = Ivory Coast, B = Burundi, P = Pinetown, D = Durban, S = Silverglen, A(15–18) = Ankarana, A(20–25) = Antsiranana, F = Fianarantsoa, M = Mahajanga, T = Toliara. See Appendix for further explanation of locality information

TABLE 2. Diversity and neutrality statistics based on 290 nucleotides of the mitochondrial D-loop

Parameter	South-east-central-west Africa	North-east Africa	Madagascar	Expectation#
Nucleotide diversity (π)	0.03856	0.03015	0.01959	Low
Haplotype diversity (h)	0.939	0.952	0.968	High
Expansion coefficient (S/d)	2.90	3.34	5.87	High
Fu and Li's (1993) F^*	0.43886	0.72155	-1.55917	NS
Fu and Li's (1993) D^*	0.45708	0.91022	-1.29140	NS
Fu's (1997) F_s	0.005	-0.509	-7.523*	Significant
Raggedness (rg)	0.0611	0.0434	0.0144	
Mismatch distribution	Multimodal	Multimodal	Unimodal	Unimodal
Tau (τ)	4.767	4.382	2.621	
Time since expansion (yr BP)			27,390–52,240 yr†	

— Expected trends for a model of demographic population expansion (Hull and Girman, 2005)
* — $P < 0.001$
† — Value obtained from formula $\tau = 2ut$, following Rogers and Harpending (1992), where u was the product of mutation rate (μ) per generation (two rates for the D-loop were used from Rogers and Harpending, 1992: 17.3% divergence per million years, or $\mu = 1.73 \times 10^{-7}$ mutations per site per generation, and 33.0% divergence or 3.3 mutations $\times$ 10^{-7}) multiplied by sequence length (290 bp) and t was the time (in generations) since expansion (generation time taken as two years)

model estimates, our data suggest that African and Oriental *Otomops* lineages diverged around 4.2 (2.8–6.1) Myr, Malagasy and African lineages split around 1.5 (0.9–2.2) Myr, whilst the south-east-central-west (Clade 2) and north-east (Clade 1) clades split around 1.2 (0.7–1.8) Myr. The two Oriental species diverged 1.9 (1.1–3.2) Myr (Table 4).

Potential Distribution and Habitat Suitability

The algorithm converged after 620 iterations with a regularized training gain of 1.633. Model performance as assessed by the area under the curve (AUC) was very high (0.927) and thus had a strong discriminatory power. Threshold a) gave a value of 7.553 and resulted in a fractional predicted area of 31.4% (training omission rate: 8.5%), threshold b) had a value of 20.764 and resulted in a fractional predicted area of 16.4% (training omission rate: 16.9%), and threshold c) had a value of 50.888 with a fractional predicted area of 4.5% (training omission rate: 27.1%). The two environmental variables with the highest explanatory power when used in isolation were mean maximum temperature in October (Tmax10) and topographic roughness (log STRM30 contrast), i.e. these variables contained the most useful information by themselves. The environmental variables that decreased the overall model most when omitted were two land cover data sets (SPOT VGT unclassified Red-spectral channel and VCF% tree cover), which therefore had the most information that was not present in the other variables. The MaxEnt model (Fig. 3) predicted a potential distribution for *O. martiensseni* largely coinciding with mesic to humid savannas, woodlands, and forests, particularly in areas of rough topography (e.g., Ethiopian Highlands, Albertine Rift, Eastern Arc Mountains, and volcanoes between Kenya and Tanzania). The rainforest region of the central Congo Basin received moderate to very low suitability scores. According to the model, two regions showed patchy and therefore fragmented habitat suitability:

TABLE 3. Results of Analysis of Molecular Variance (AMOVA). Fixation indices for the combined sample (a) were as follows: $F_{SC} = 0.060$, $F_{ST} = 0.090$, $F_{CT} = 0.032$. Based on 10,100 permutations, all these values were significant ($P < 0.01$). Fixation index for Clade 2 (b) was as follows: $F_{ST} = 0.061$ ($P = 0.03$)

Source of variation	*d.f.*	Sum of squares	Variance components	Variance explained (%)
a) All populations				
Among groups	2	1.672	0.0162 Va	3.2
Among populations within groups	13	6.936	0.0291	5.8
Within populations	32	14.600	0.4566 Vb	91.0
Total	47	23.208	0.5016 Vc	
b) Clade 2 (S-E-C-W Africa)				
Among populations	8	4.309	0.0301 Va	6.0
Within populations	13	6.100	0.4692 Vb	94.0
Total	21	10.409	0.4993 Vc	

1) low to moderate suitability from east Nigeria to southwest Sudan, interrupting predicted ranges in west and east Africa, and 2) moderate suitability between Malawi and north Mozambique in the north and south Mozambique and South Africa in the south, separating predicted ranges in east and southern Africa.

Discussion

Taxonomy of Afro-Malagasy Otomops

Three reciprocally-monophyletic groups are discernable from phylogenetic (Fig. 1) and phylogeographic (Fig. 2) analyses. Results from cytochrome *b* and D-loop sequences are congruent, lending further support to this conclusion. The lineage from Madagascar is referable to *O. madagascariensis*. Morphological divergence (Peterson *et al.*, 1995) and geographical isolation provide substantial evidence for recognition of this species. On the other hand, Clade 2 (south-east-central-west Africa) includes localities that are close to the type localities of both *martiensseni* (Magrotto Plantation, west of Tanga, at the foot of the East Usambara Mountains, Tanzania — Matschie, 1897) and *icarus* (Durban, South Africa); hence, *icarus* is the junior synonym of *martiensseni*. If the two African clades represent distinct species, then Clade 1 (north-east Africa + Yemen) would constitute an undescribed taxon. Pending a detailed morphological and morphometric assessment of Afro-Malagasy *Otomops* involving much broader geographic representation (L. Richards, unpublished data), the two African-Arabian lineages are provisionally flagged as evolutionarily significant units (ESU), but are maintained under the name *O. martiensseni*. Although the Clade1-Clade2 divergence of 3.1% (Table 1; excluding comparisons with Ivory Coast) falls below the arbitrary 5% cytochrome *b* divergence threshold for distinct species suggested by Baker and Bradley (2006), it is only slightly below the minimum divergence of 3.3 % reported by them for sister species of bats, based on data from 10 studies, which did not include any test cases within the family Molossidae. There are examples of molossid sister species

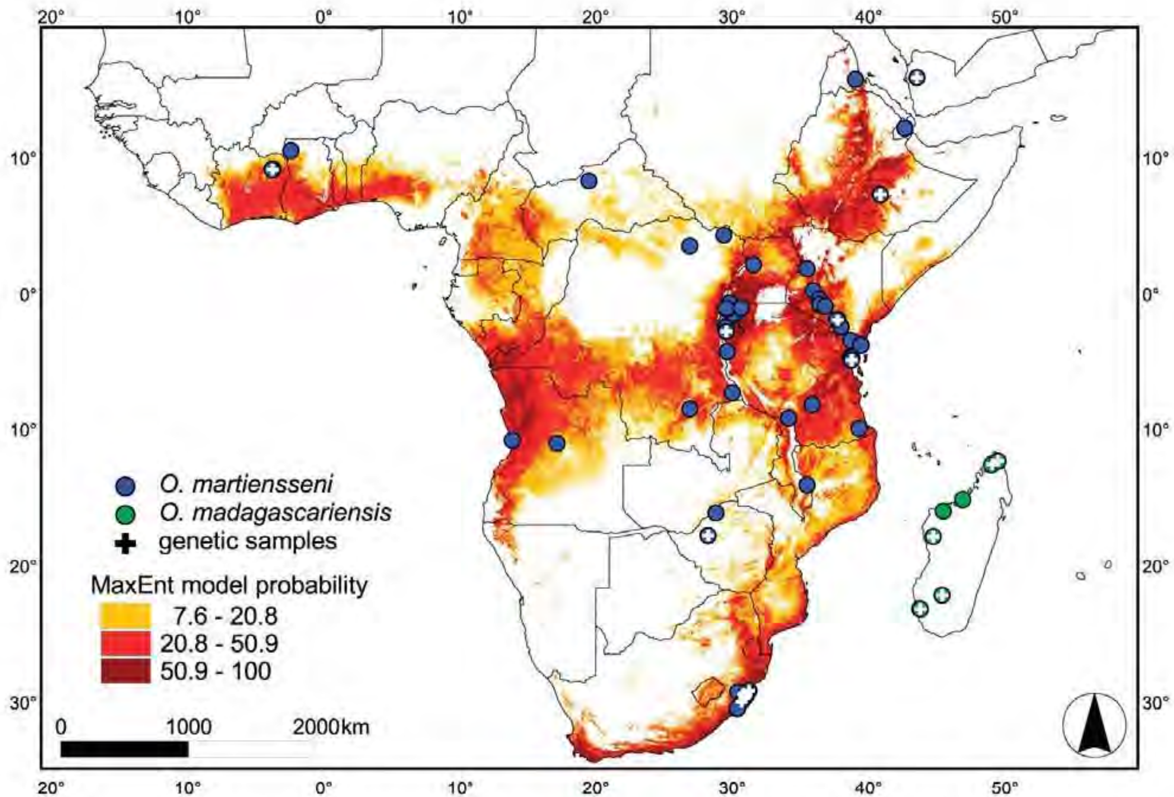

Fig. 3. Modelled potential distribution (MaxEnt) of *Otomops* based on known records of occurrence. Localities sampled for molecular study are indicated with crosses. Note that the Arabian Peninsula and Madagascar were excluded from ecological niche modelling. See text for explanation of different thresholds and distribution probabilities

separated by smaller genetic distances than this 3.3%. For example, the cytochrome *b* genetic distance between *Mops leucostigma* and *M. condylurus* is 2.5% (Ratrimonanarivo *et al.*, In press). Other well-defined bat species have also been reported to be genetically very similar, as shown for four pairs of western Palaearctic vespertilionid bat species (< 2.5% of divergence for the mitochondrial ND1 gene, which evolves at a rate similar to cytochrome *b* — Mayer *et al.*, 2007).

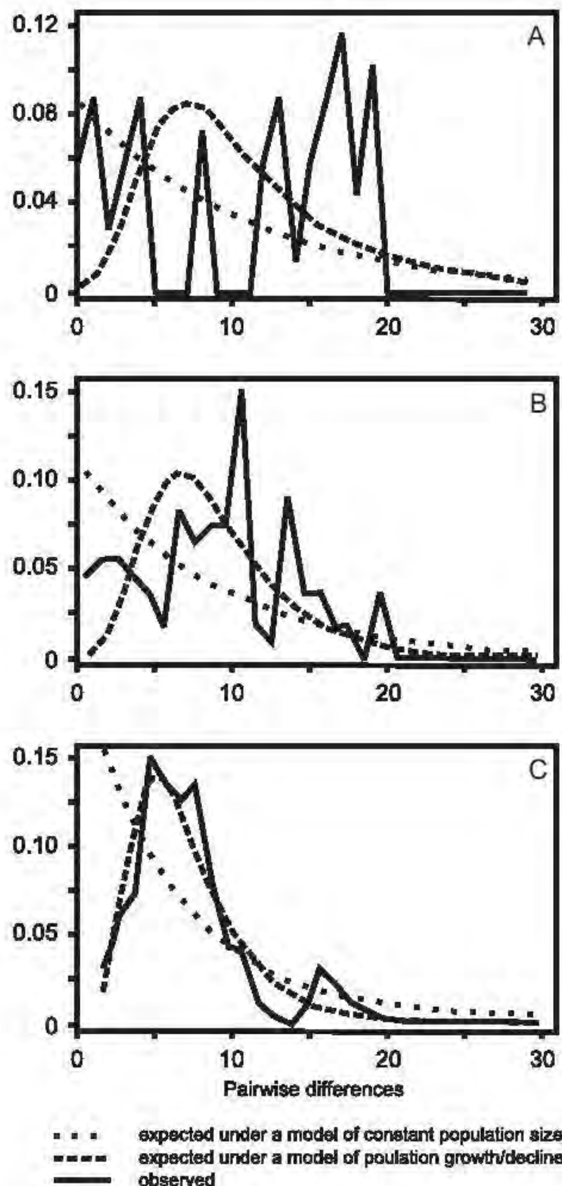

FIG. 4. Mismatch distributions for three phylogenetic groups of *Otomops* based on 1,004 nucleotides of the mitochondrial cytochrome *b* gene (A: south-east-central-west Africa; B: north-east Africa; C: Madagascar)

Phylogeny, Phylogeography and Dispersal of Afro-Malagasy Otomops

Bayesian and other phylogenetic approaches support the monophyly of the four species of *Otomops* studied here, the reciprocal monophyly of the Oriental and Afro-Malagasy species-groups and of the three lineages within the Afro-Malagasy clade. The Oriental species appear to be older, separated by larger genetic distances (cytochrome *b*, GTR-corrected divergence = 6.6%) than the three Afro-Malagasy lineages (3.1–4.4% excluding Ivory Coast, mean 3.8%; Table 1). Although only one west African population (Ivory Coast) was sampled, it is clear that, in spite of its phylogenetic affinity to south-central-east African populations (Clade 2), it is somewhat unique (divergence from other Clade 2 members from south-central-east Africa = 2.1%) and may represent a distinct lineage. However, more data are required to disentangle phylogeographic relationships within the south-east-central-west clade, where genetic sampling of populations from Angola, northern Democratic Republic of the Congo, and the Central African Republic would be particularly critical.

Since the Malagasy lineage is genetically largely equidistant between the south-east-central-west (Clade 1) and north-east (Clade 2) lineages from the mainland, it is difficult to establish the pattern of dispersal and speciation within the Afro-Malagasy clade. If one assumes an Asian origin for the genus (based on higher species richness and older divergences at least for the two species sampled), then the D-loop network indicates a scenario whereby the Oriental ancestor dispersed independently to Madagascar and South Africa, followed by northwards dispersal from South Africa. Another possibility is that Madagascar was occupied first from the Oriental Region, and that one or two subsequent independent colonisations took place, giving rise to the two African clades.

Prevailing easterly (Trade; throughout year) and strong south-easterly (Monsoon; during the austral winter) winds between Madagascar and lesser islands and the African mainland (Jury, 2003) could have aided the dispersal to Africa from Madagascar by fast and high-flying bats such as *Otomops*. Easterly trade winds affect southern Madagascar and southern Africa and could plausibly explain the origin of the south-east-central-west lineage from Madagascar, whilst the strong south-easterly Monsoon winds blow over northern Madagascar in austral winter towards the coast of Ethiopia and

TABLE 4. Estimate absolute ages of nodes (Bayesian analysis – BEAST program) for uncorrelated relaxed clock models with log-normal distribution of branch rates, and fixed mean mutation rate of 0.02 substitution/site/Myr

Node	Relaxed lognormal molecular clock		
	Myr	95% confidence limits	
		Lower	Upper
Otomops	4.20	2.76	6.07
Oriental *Otomops*	1.92	1.06	3.15
Africa + Madagascar	1.51	0.94	2.25
Africa (Clades 1 & 2)	1.16	0.68	1.77
Mops spp.	4.59	2.45	7.00
Tadarida-Mops-Otomops	9.78	6.66	14.70

could account for the origin of the north-east clade.

Recent analyses of the biogeographic affinities of the Malagasy bat fauna indicate that the majority of taxa are of Afrotropical origin (Eger and Mitchell, 2003). More recent work indicates that several species groups colonized the island on different occasions and from different directions. An excellent example can be found in pipistrelloid bats; Bates *et al*. (2006) described a new species of *Pipistrellus* from Madagascar that has close affinity to Oriental species, while other members of this group occurring on the island are of Afrotropical origin. Other genera such as *Emballonura* are rather speciose on western Pacific Ocean islands and southeastern Asia and have their western limit on Madagascar (Goodman *et al*., 2006). Hence, although more limited than from Africa, there are clear examples of bats colonizing Madagascar from Oriental origins, which is in parallel with one scenario presented here for *Otomops*.

Population Genetics and Demographic History

Based on our analyses of the three lineages of *Otomops* occurring in the Afro-Malagasy region, only the lineage from Madagascar shows evidence of exponential demographic expansion. Based on the τ-value obtained from mismatch coefficients, this expansion was estimated to have occurred some 27,000–52,000 years BP. While few data are available on life history traits of *O. madagascariensis*, including dispersal and seasonal movements, a few observations can be made. Bat fossils recovered from breccia deposits in the karst system of Anjohibe (Samonds, 2007), at the northern end of the Mahajanga Province and 400 km from Bemaraha, have been dated to a range from about 80,000–10,000 years ago, using uranium series (^{230}Th/^{234}U). A wide assortment of bat taxa was recovered from the deposits, which did not include *O. madagascariensis*, even though it currently inhabits this cave system (Goodman *et al*., 2005). This information can be construed as corroboration of this species' recent colonization of the Anjohibe Cave system. If this is indeed correct, it would support the hypothesis of recent or ongoing range expansion in this species, as found in the genetic data. During the late Quaternary, there was very significant climate change in Madagascar (see Burney *et al*., 2004 and citations therein), which might be associated with range expansion in this taxon, as has been shown for bats in the Holarctic region (Ruedi and Castella, 2003).

Until recently, there was no evidence of *O. madagascariensis* from the central highlands of Madagascar, other than at Isalo at the western edge of this zone or its occurrence in a synanthropic setting. However, in February 2007 a sub-adult female was captured in a building in a residential neighbourhood of Antananarivo. Whether this individual represents a local resident population or a dispersing individual is unknown. However, this record demonstrates that current information on the distribution of *O. madagascariensis* is incomplete and further fieldwork is needed.

Determinants of Phylogeographic and Population Genetic Structure

In general, genetic structure has been mostly documented in bats with low dispersal capabilities (Worthington-Wilmer *et al*., 1994; Burland *et al*., 1999), but not typically in more wide-ranging species (McCracken *et al*., 1994; Webb and Tidemann, 1996; Russell *et al*., 2005). Wing shape and predicted flight capability suggest that *O. martiensseni* is capable of long-range dispersal (Long, 1995; Rydell and Yalden, 1997) and we predicted little or no phylogeographic structure at local or continental scales. However, social factors such as high female philopatry can promote genetic structure in maternally

inherited mtDNA of mobile species such as bats and other mammals (e.g., McCracken, 1987; Chesser, 1991; Miller-Butterworth *et al.*, 2003; Ruedi and Castella, 2003). Thus, postulated stable harem formation in Durban colonies (Fenton *et al.*, 2002) could encourage female philopatry, leading to structuring at the local level in maternally-inherited mitochondrial sequences but not in biparentally-inherited nuclear genes.

Notwithstanding the fact that some large populations (e.g., from cave colonies in Kenya and Ethiopia) were undersampled, the current data for both mitochondrial sequences of *Otomops* do not show any evidence for strong female philopatry, e.g., clustering of 'matrilines' according to known colonies (e.g., in Durban, east Africa or Madagascar where specific samples are from colonies). Rather, most individuals have unique haplotypes, and haplotypes do not group genetically according to specific colonies. However, more complete sampling of individuals from known colonies, and the use of additional markers such as microsatellites, is clearly required before we can make any conclusions about harem structure.

Little information is available on the group composition of *O. madagascariensis*. In a cave near Antsalova, 15 individuals of this species were captured with a hand net at a single day-roost site, of which 10 (66%) were adult females (SMG, unpublished data). At another nearby site, one roosting colony consisted of 57 pregnant females and five adult males (Andriafidison *et al.*, 2007). In a cave near Sarodrano, 11 individuals of this species were captured during the dusk exit, seven (63%) of which were females (SMG, unpublished data). Hence, social groupings may show parallels to the African species discussed above.

What is surprising is the genetic association between the Durban population and geographically widely separated populations from Ivory Coast (5,700 km), Burundi (3,000 km), Tanzania (2,800 km) and Zimbabwe (1,300 km). These genetic data, combined with scattered records of *O. martiensseni* throughout Africa and from Yemen, and the largely uninterrupted habitat suggested by the MaxEnt model (Fig. 3) indicate a population with continuous distribution and/or one capable of large-scale migrations (as suggested by Mutere, 1973; Kock *et al.*, 2005 based on observations of seasonal and periodic cave evacuations in large Kenyan breeding colonies). Information on wing morphology and diet reflects the fact that these bats fly high and potentially long distances in search of airborne prey (mostly moths — Fenton and Griffin, 1997; Rydell and Yalden, 1997). It is possible that seasonal migrations of these large, fast and high-flying insectivorous bats may be triggered by documented insect migrations (Pedgley *et al.*, 1995), which track movements of the Inter-Tropical Convergence Zone (ITCZ), in much the same way as *Tadarida brasiliensis* (Geoffroy, 1824) has been documented to exploit mass migrations of breeding insects from Mexico to the southern United States (Lee and McCracken, 2005). Supporting such a scenario in Africa, seasonal migrations and mass outbreaks have been well documented in the African armyworm *Spodoptera exempta* (Lepidoptera: Noctuidae — Tucker, 1984, 1994; Wilson and Gatehouse, 1993). The movements of the ITCZ and associated insect biomass from central to southern (and northern) Africa could possibly explain the high degree of relatedness between haplotypes from Burundi, Tanzania, Zimbabwe and South Africa, and their relative genetic distinctiveness from north-east lineage haplotypes, suggesting migratory behaviour or regular long-range dispersal of *O. martiensseni*.

If this is linked to insect migration, we would expect opposite patterns of population movements in the northern and southern hemisphere. Apart from the Ivory Coast sample that is phylogenetically linked to the south-east-central-west lineage, all other samples of this clade come from the southern hemisphere (Burundi, Tanzania, Zimbabwe, South Africa). The north-east lineage (Yemen, Ethiopia, Kenya), on the other hand, is found in the northern hemisphere. If we suppose that the south-east-central lineage is tracking insect phenology and migration with an austral cycle (migrating from the equator southwards) and the north-east lineage is tracking insect phenology with a boreal cycle (migrating from the equator northwards), there might be sufficient geographical isolation leading to the observed phylogeographic pattern, despite the fact that both lineages are found in geographic proximity around the equator. Hockey (2000) presented an illuminating synthesis of intra-African migratory behaviour of birds, showing that it is mainly the insectivorous birds feeding on aerial insects (such as swift, swallows and nightjars) that follow insect phenology from tropical to more temperate latitudes with the onset of the wet seasons. According to this observation, we would expect high-flying bats such as molossids to show a similar pattern.

The potential distribution model did not reveal any apparent gap in habitat suitability between records belonging to the north-east lineage and

south-east-central-west lineage, hence suggesting that factors other than habitat suitability under current climatic conditions have resulted in the observed phylogeographic structure. Possibly, past climatic fluctuations were more important and might have resulted in the phylogeographic structure of continental populations of *O. martiensseni* observed today. Our record from Ivory Coast represents the first for the country and the second for west Africa. Decher *et al*. (1997) speculated that the single specimen from Ghana might have been a disoriented vagrant. However, *O. martiensseni* was regularly captured with elevated mist nets during recent fieldwork in Comoé National Park, Ivory Coast (JF, unpublished data). It is very likely that the lack of records within several regions, which were predicted to offer suitable habitat by the distribution model, is mainly due to inappropriate sampling techniques or a lack of surveys in these regions. According to the distribution model, there is a high probability that *O. martiensseni* should occur in large parts of west Africa (from south-west Ivory Coast to south-central Nigeria), throughout large parts of the Ethiopian Highlands, and throughout a broad region in southern Democratic Republic of the Congo and north-west Angola. The distribution model also predicts a narrow strip of very high habitat suitability along the southeastern coast of South Africa down to the Cape Region. Further fieldwork in these areas is required to validate and refine the actual distribution of *O. martiensseni*, particularly in the light of pronounced phylogeographic structure within its distribution range.

Since phylogeographic structure may result from both current and historical processes, it is important to consider past climates. Past bottlenecks can also influence current genetic structures; in *Myotis myotis* (Borkhausen, 1797) from Europe, genetic structure varied geographically due to the historical effects of the Last Glacial Maximum (Ruedi and Castella, 2003). It is widely acknowledged that Africa underwent a drastic aridification around 1 Mya (deMenocal 2004), which has been invoked to explain vicariance events affecting montane-adapted taxa in the 'Montane Circle' of Africa (e.g., Bowie *et al*., 2006; Carleton *et al*., 2006). Since this date coincides with the estimated date (0.7–1.8 Myr) of the genetic divergence between Clade 1 (which extends along the Albertine or Western Rift and into the Eastern Arc Mountains of Tanzania as far north as the Usambara Massifs) and Clade 2 (from the Kenyan Rift northwards, including the Ethiopian Rift), it is plausible to assume that aridification could have led to population contractions due to low food availability, and restriction of populations occupying distinct mountain ranges associated with the Rift Valley and Eastern Arc Mountains. Indeed, in studies on a montane forest passerine, using molecular data and coalescence approaches, Bowie *et al*. (2006) identified vicariance events separating Albertine Rift from Kenyan Rift populations, and Eastern Arc Mountains from the Kenyan Rift populations. Estimated dates for these events coincided very closely with the range of dates estimated for divergence of Clades 1 and 2 (0.7–1.8 Myr — Table 4).

Why should profound aridification result in vicariance of African Montane Circle populations but not to the same extent between far-flung localities from Durban, Ivory Coast and Tanzania belonging to Clade 1? *Otomops martiensseni* is clearly a species capable of long-range dispersal. Perhaps more importantly, individuals show adaptability in being able to exploit diverse roosting situations, such as tree hollows in lowland rain forest habitats and even man-made sites such as buildings in the metropolis of Durban, South Africa. These life history traits must be important in maintaining gene flow where alternative (non-cavernicolous) roosts were available in abundance. On the other hand, in higher altitude, often treeless mountainous habitats associated with the Rift Valley of east Africa, it is possible that past aridification and/or colder temperatures may have played a vital role in local extinctions of the relative few and sparsely located *Otomops* cave colonies, perhaps forcing them (through low temperatures or low food availability) to move into lower valleys where fewer cave roosts were available.

Acknowledgements

We are grateful to the Direction des Eaux et Forêts and Association National pour la Gestion des Aires Protégées for issuing permits to conduct faunal surveys on Madagascar and Prof. Olga Ramilijaona and Dr. Daniel Rakotondravony of the Université d'Antananarivo for assistance with numerous administrative details. Field research on Madagascar has been generously supported by John D. and Catherine T. MacArthur Foundation and the Volkswagen Foundation. We are grateful to Fanja Ratrimomanarivo for allowing us to cite the unpublished record of *O. madagascariensis* from Antananarivo. Fieldwork in Ivory Coast and GIS analysis was funded by the BIOLOG-program of the German Ministry of Education and Science (BMBF; project W09 BIOTA-West, 01 LC 0411). Dr. Günter Barnikel, Ulm University, substantially contributed to ecological niche modelling, and derived topographic data were kindly processed by Martin Wegmann (German Space Agency [DLR] and University of Würzburg). Sequencing work was supported by a Thuthuka grant from the National Research Foundation, South

Africa, and the University of KwaZulu-Natal. PJT received funding for this project from the National Research Foundation, South Africa. Larry Heaney and Julian Kerbis from the Field Museum of Natural History, Chicago, kindly donated samples for molecular analysis. Alexei J. Drummond and Andrew Rambaut provided welcome and ready advice with the application of their BEAST program. For comments on an earlier draft of this manuscript, we are grateful to two anonymous reviewers.

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Received 15 December 2007, accepted 07 May 2008

APPENDIX.

Locality and specimen details and Genbank accession numbers of sampled individuals of *Otomops* and outgroups. RSA — Republic of South Africa; DM — Durban Natural Science Museum; FMNH — Field Museum of Natural History, Chicago; HZM — Harrison Zoological Museum, Sevenoaks; NMP — National Museum of the Czech Republic, Prague; NMK — National Museums of Kenya, Nairobi; ROM — Royal Ontario Museum, Toronto; SMF — Senckenberg Museum, Frankfurt a. M.; SMG — Université d'Antananarivo, Département de Biologie Animale, Antananarivo (uncatalogued specimen from SMG); Pb: collectors' numbers of Petr Benda (not accessioned in a museum)

Species	Geographic origin	Co-ordinates	Museum no.	Sample label	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops martiensseni s.l.	RSA: Ballito, 20 km N of Durban	29.533°S, 31.211°E	N/A	Ballito6	?	EF216411	
– ״ –	– ״ –	– ״ – – ״ –	N/A	Ballito7	?	EF216412	
– ״ –	RSA: 473 Silverglen Dve, Chatsworth, Durban	29.928°S, 30.900°E	N/A	Silverglen1	?	EF216406	
– ״ –	– ״ –	– ״ – – ״ –	N/A	Silverglen2	?	EF216407	EF216451
– ״ –	– ״ –	– ״ – – ״ –	N/A	Silverglen3	?	EF216408	
– ״ –	– ״ –	– ״ – – ״ –	N/A	Silverglen4	?	EF216409	EF216452
– ״ –	– ״ –	– ״ – – ״ –	N/A	Silverglen5	?	EF216410	EF216453
– ״ –	RSA: 31 Ann Arbour Rd, Scottburgh, 25 km S of Durban	30.300°S, 30.745°E	N/A	Scottburgh6	♀	EF216418	
– ״ –	– ״ –	– ״ – – ״ –	N/A	Scottburgh7	♂	EF216419	
– ״ –	RSA: Ocean View Farm, Park Rynie, 30 km S of Durban	30.339°S, 30.731°E	DM 8031	Durban324	♀	EF216426	EF216446
– ״ –	– ״ –	– ״ – – ״ –	DM 8032	Durban323	♂	EF216427	
– ״ –	RSA: Brynderyn Flats, Morningside, Durban	29.864°S, 31.040°E	DM 7909	Durban321	♂	EF216424	EF216444
– ״ –	RSA: Kingsway School, Amanzimtoti, Durban	30.039°S, 30.894°E	DM 7914	Durban322	♂	EF216425	EF216445
– ״ –	RSA: 8 Buys Rd, Pinetown, Durban	29.757°S, 30.639°E	DM 8421	Pinetown1	♀	EF216413	EF216447
– ״ –	– ״ –	– ״ – – ״ –	N/A	Pinetown2	♀	EF216414	EF216448
– ״ –	– ״ –	– ״ – – ״ –	N/A	Pinetown3	♀	EF216415	EF216449
– ״ –	– ״ –	– ״ – – ״ –	N/A	Pinetown4	♀	EF216416	EF216450
– ״ –	– ״ –	– ״ – – ״ –	N/A	Pinetown5	♂	EF216417	
– ״ –	Ethiopia: S of Omar Caves, 40 km west Ginir, Bale Province	6.90°N, 0.850°E	NMP 91203	EthiopiaA1	♀	EF216429	EF216461
– ״ –	– ״ –	– ״ – – ״ –	NMP 91203	EthiopiaA2	?	EF216430	EF216462
– ״ –	– ״ –	– ״ – – ״ –	Pb2512	EthiopiaB1	♀	EF216431	EF216463
– ״ –	– ״ –	– ״ – – ״ –	Pb2512	EthiopiaB2	?	EF216432	EF216464
– ״ –	– ״ –	– ״ – – ״ –	NMP 91202	EthiopiaC1	♀	EF216433	EF216465
– ״ –	– ״ –	– ״ – – ״ –	NMP 91202	EthiopiaC2	?	EF216434	EF216466
– ״ –	– ״ –	– ״ – – ״ –	NMP 91201	EthiopiaD1	♀	EF216435	EF216467
– ״ –	– ״ –	– ״ – – ״ –	NMP 91201	EthiopiaD2	?	EF216436	EF216468
– ״ –	Kenya: Ithundu Caves, Chyulu Hills, Kiboko, Makuena District	2.358°S, 7.717°E	NMK 15462	Kenya1	♂	EF216428	EF216455
– ״ –	– ״ –	– ״ – – ״ –	NMK 15461	Kenya2	♀	EF216438	

APPENDIX. Continued

Species	Geographic origin	Co-ordinates	Museum no.	Sample label	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops martiensseni s.l.	Kenya: Ithundu Caves, Chyulu Hills, Kiboko, Makuena District	2.358°S, 7.717°E	NMK 15464	Kenya3	♀	EF216439	EF216456
– " –	– " –	– " – – " –	NMK 15463	Kenya4	♂	EF216440	EF216457
– " –	– " –	– " – – " –	NMK 15459	Kenya5	♂	EF216441	EF216458
– " –	– " –	– " – – " –	NMK 15465	Kenya6	♂		EF216459
– " –	– " –	– " – – " –	NMK 15460	Kenya7	♀	EF216442	EF216460
– " –	Burundi: 2.3 km N, 0.7 km W Teza, Kibira	3.200°S, 0.55°E	FMNH 137633	Burundi415	♀	EF216423	EF216443
– " –	Zimbabwe: Hostes Nicholle Institute, Sengwa	18.167°S, 28.217°E	ROM 83979	Zimbabwe	♀	EF216421	
– " –	Yemen: Hud Sawa Cave, Al-Mawheet	15.483°N, 43.533°E	SMF 87650	Yemen	♂?	EF216437	EF216469
– " –	Ivory Coast: Comoé NP	8.715°N, 3.797°W	SMF 92049	Ivory Coast	♂	EF216420	EF216454
– " –	Tanzania: Tongwe F.R, Tanga, Muheza District	5.317°S, 38.733°E	SMF 79542	Tanzania	♀	EF216422	
Otomops madagascariensis	Madagascar: Parc National de Bemaraha, Province de Mahajanga	18.245°S, 44.717°E	FMNH 169667	Mahajanga2	♀	EF216373	EF216384
– " –	– " –	– " – – " –	FMNH 169694	Mahajanga3	♂	EF216374	EF216385
– " –	– " –	– " – – " –	FMNH 169695	Mahajanga4	♂	EF216375	EF216386
– " –	Madagascar: Parc National de Isalo, Province de Fianarantsoa	22.540°S, 45.380°E	FMNH 166073	Fianarantsoa1	♀	EF216372	EF216383
– " –	– " –	– " – – " –	SMG 10996	Fianarantsoa2	♀		EF216401
– " –	Madagascar: Grotte d'Ambanila, Province de Toliara	23.540°S, 43.746°E	FMNH 172938	Toliara5	♀	EF216376	EF216387
– " –	– " –	– " – – " –	FMNH 172940	Toliara6	♂	EF216377	EF216388
– " –	– " –	– " – – " –	FMNH 172934	Toliara7	♂	EF216378	EF216389
– " –	– " –	– " – – " –	FMNH 172936	Toliara12	♂		EF216394
– " –	– " –	– " – – " –	FMNH 172942	Toliara13	♂		EF216395
– " –	Madagascar: Grotte de Bishihiko, Province de Toliara	23.549°S, 43.767°E	FMNH 172944	Toliara8	♀	EF216379	EF216390
– " –	– " –	– " – – " –	FMNH 172948	Toliara9	♂	EF216380	EF216391
– " –	– " –	– " – – " –	FMNH 172951	Toliara10	♂		EF216392
– " –	– " –	– " – – " –	FMNH 172953	Toliara11	♀		EF216393
– " –	– " –	– " – – " –	FMNH 172947	Toliara14	♂		EF216396
– " –	Madagascar: Réserve Spéciale d'Ankarana, Province d'Antsiranana	12.942°S, 49.055°E	FMNH 176354	Ankarana15	♀	EF216381	EF216397
– " –	– " –	– " – – " –	FMNH 176355	Ankarana16	♂		EF216398
– " –	– " –	– " – – " –	FMNH 176356	Ankarana17	♂		EF216399
– " –	– " –	– " – – " –	FMNH 176357	Ankarana18	♂	EF216382	EF216400
– " –	Madagascar: Réserve Spéciale d'Analamerana, Province d'Antsiranana	12.712°S, 49.474°E	FMNH 178849	Antsiranana20	♀		EF216402

APPENDIX. Continued

Species	Geographic origin	Co-ordinates	Museum no.	Sample label	Sex	GenBank #	
						Cytochrome *b*	D-loop
Otomops madagascariensis	Madagascar: Réserve Spéciale d'Analamerana, Province d'Antsiranana	12.712°S, 49.474°E	FMNH 178850	Antsiranana21	♀		EF216403
– " –	– " –	– " – – " –	FMNH 178851	Antsiranana22	♀		EF216404
– " –	– " –	– " – – " –	FMNH 178852	Antsiranana25	♂		EF216405
Otomops wroughtoni	Cambodia: Chhep District, Preah Vihear Province	13.59°N, 105°16°E	HZM 3.33440	*Otomops wroughtoni*	♂	EF504251	EF504253
Otomops cf. *formosus*	Philippines: Barangay Balbalasang, Kalinga Province, Luzon Is	17.458°S, 121.0683°E	FMNH 167240	*Otomops* cf. *formosus*	♂	EF504252	EF504254
Tadarida fulminans	Madagascar					EU760911	
Mops midas	Madagascar					EF474049	
Mops leucostigma	Madagascar					EF474029	
Mops condylurus	KwaZulu-Natal, South Africa					EF474030	
Pipistrellus abramus						NC005436	

Acta Chiropterologica, 13(1): 1–16, 2011
PL ISSN 1508-1109 © Museum and Institute of Zoology PAS
doi: 10.3161/150811011X578589

Toward a molecular phylogeny for the Molossidae (Chiroptera) of the Afro-Malagasy region

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We present phylogenetic information based on nuclear Rag2 and mitochondrial cytochrome *b* sequence data for six genera of Molossidae (*Chaerephon*, *Mops*, *Mormopterus*, *Otomops*, *Sauromys*, *Tadarida*) and 18 species, primarily from Africa and the Malagasy region (Madagascar and neighbouring islands), and further include sequences of 12 New World and African taxa sourced from GenBank. There is strong support for the monophyly of the Molossidae included in this study. The Malagasy region taxa *Mormopterus jugularis* and *M. francoismoutoui* are supported as a basal clade with an age of ≈ 31.2 MYR, and are not monophyletic with the South American *M. kalinowskii*. Asian *Otomops wroughtoni* and *O. formosus* and Afro-Malagasy *O. martiensseni* and *O. madagascariensis* form a strongly-supported ≈ 19.8 MYR-old clade, whose broader relationships among Molossidae are not clearly defined. There is strong support for a ≈ 17.2 MYR-old combined *Chaerephon*/*Mops* clade, in which members of these genera show some paraphyly. The monophyly of the genus *Tadarida*, represented in our analyses by *T. brasiliensis* from the New World and *T. fulminans*, *T. aegyptiaca* and *T. teniotis* from the Old World, is not upheld, although there is good support for a geographically-disjunct ≈ 9.8 MYR-old grouping which includes *C. jobimena* (Madagascar), *T. aegyptiaca* (Africa) and *T. brasiliensis* (America). *Sauromys* is maintained as a monotypic genus, although there is moderate support for its association with *T. fulminans* and the *Chaerephon*/*Mops* clade, the latter of which comprises *M. midas*, *M. leucostigma*, *M. condylurus*, *M. bakarii*, *C. pumilus*, *C. pusillus*, *C. leucogaster* and *C. atsinanana*. An ≈ 8.4 MYR-old New World clade comprising representatives of *Eumops*, *Nyctinomops* and *Molossus* was well-supported.

Key words: Molossidae, Rag2, cytochrome *b*, Africa, Madagascar, Western Indian Ocean, phylogeny

INTRODUCTION

The family Molossidae (Chiroptera), commonly known as free-tailed or mastiff bats, is part of the suborder Vespertilioniformes and, with the Cistugidae (Lack *et al*., 2010), Vespertilionidae, Natalidae and Miniopteridae (as defined by Hoofer and Van Den Bussche, 2003; Miller-Butterworth *et al*., 2007) forms the superfamily Vespertilionoidea (Hoofer *et al*., 2003; Van Den Bussche and Hoofer, 2004; Eick *et al*., 2005; Teeling *et al*., 2005; Lack *et al*., 2010). The Molossidae comprise 17 genera and about 100 species (Simmons, 2005). These generally robust bats are often strong flyers, with long narrow wings, and catch their insect prey in flight. They are widespread, occurring on every continent except Antarctica.

On the basis of a recent taxonomic treatment (Simmons, 2005), the subfamily Molossinae comprises the following genera: *Chaerephon* Dobson, 1874; *Cheiromeles* Horsfield, 1824; *Cynomops* Thomas, 1920; *Eumops* Miller, 1906; *Molossops* Peters, 1865; *Molossus* Geoffroy, 1805; *Mops* Lesson, 1842; *Mormopterus* Peters, 1865; *Myopterus* Geoffroy, 1818; *Nyctinomops* Miller, 1902; *Otomops* Thomas, 1913; *Platymops* Thomas, 1906; *Promops* Gervais, 1856; *Sauromys* Roberts, 1917 and *Tadarida* Rafinesque, 1814 (Simmons, 2005). The subfamily Tomopeatinae includes the genus *Tomopeas* Miller, 1900 (Sudman *et al*., 1994).

Eight molossid genera (*Chaerephon*, *Mops*, *Mormopterus*, *Myopterus*, *Otomops*, *Platymops*, *Sauromys* and *Tadarida*) are found in Africa, its offshore islands (Zanzibar and Pemba), and the Malagasy region, including Madagascar, Mayotte, Anjouan, Grande Comore, Mohéli, Aldabra, La Réunion and Mauritius. Of these, the genera *Myopterus*, *Platymops* and *Sauromys* are endemic to mainland Africa, whereas the others are distributed across portions of the Afro-Malagasy region. Many of the genera have previously been considered as subgenera of others: *Mormopterus* has been regarded as a subgenus of *Tadarida* (Hayman and Hill, 1971) and as a valid genus that includes *Platymops* and *Sauromys* as subgenera (Freeman, 1981; Legendre, 1984); *Chaerephon* and *Mops* have also been placed as subgenera of *Tadarida* (Hayman and Hill, 1971; Meester *et al.*, 1986).

We have been engaged in a series of phylogenetic and phylogeographic studies, primarily of Malagasy region Molossidae, but also including mainland Africa congeners. These studies focused on *Chaerephon leucogaster* (A. Grandidier, 1869) (Ratrimomanarivo *et al.*, 2009*a*); the *C. pumilus* Cretzschmar, 1830–1831 group from southern Africa (Taylor *et al.*, 2009) and the Malagasy region (Goodman *et al.*, 2010); *Mops condylurus* (A. Smith, 1833), *M. leucostigma* (G. M. Allen, 1918) and *M. midas* Sundevall, 1843 (Ratrimomanarivo *et al.*, 2007, 2008); *Mormopterus jugularis* (Peters, 1865) (Ratrimomanarivo *et al.*, 2009*b*); and *Otomops formosus* Chasen, 1939, *O. madagascariensis* Dorst, 1953, *O. martiensseni* (Matschie, 1897) and *O. wroughtoni* (Thomas, 1913) (Lamb *et al.*, 2006, 2008). Another study of the *Mormopterus* occurring on the Mascarene Islands, based on morphology and molecular genetics, found that the populations occurring on Mauritius and La Réunion were distinct from one another and animals from the latter were described as a new species, *M. francoismoutoui* Goodman, van Vuuren, Ratrimomanarivo, Bowie, 2008. We have accumulated mitochondrial cytochrome *b* and nuclear Rag2 sequence data, and are in a position to contribute to the phylogeny, as well as inter- and intra-generic relationships, of the Molossidae occurring in the Malagasy region and mainland Africa. Our dataset for 17 species comprises sequences from six genera (*Chaerephon*, *Mops*, *Mormopterus*, *Otomops*, *Sauromys* and *Tadarida*) and is augmented with data downloaded from the NCBI GenBank for 12 members of the genera *Eumops*, *Chaerephon*, *Molossus*, *Nyctinomops*, *Promops* and *Tadarida.*

Current systematic arrangement of the family Molossidae is based largely on traditional morphological data (Freeman, 1981; Legendre, 1984; Taylor, 1999; Simmons, 2005). The genus *Nyctinomus* Geoffroy St. Hilaire, 1813, which was used extensively in the early taxonomic classification of molossids (Rosevear, 1965), is today considered a junior synonym of *Chaerephon* (Simmons, 2005). In 1814, Rafinesque suggested a new genus name, *Tadarida*, for certain taxa placed in *Nyctinomus* (Dobson, 1878). Freeman (1981) divided the African members of the genus *Tadarida* into four genera (*Mormopterus*, *Tadarida*, *Chaerephon* and *Mops*). Peterson *et al.* (1995) regarded *Chaerephon* as a subgenus of *Tadarida* based on certain morphological characters that are shared by a few species of both *Chaerephon* and *Mops* (Bouchard, 1998). Currently, Simmons (2005) has adopted the generic classification proposed by Freeman (1981), whereby *Chaerephon* is given generic status. The *Chaerephon* occurring on Aldabra in the western Seychelles was named as a distinct taxon, *C. pusillus* (Miller, 1902), and was not recognized by different authorities (Hayman and Hill, 1971; Simmons, 2005). Recent work has shown that this is indeed a distinct species shared with islands in the Comoro Archipelago and Aldabra (Goodman and Ratrimomanarivo, 2007; Goodman *et al.*, 2010).

Nyctinomus is considered variously as a valid genus (McFarland, 1998), or included in *Tadarida* (Koopman, 1993; Grubb *et al.*, 1998), *Chaerephon* (Simmons, 2005) or *Mormopterus* (Jacobs and Fenton, 2002). Mahoney and Walton (1988) considered that the name *Nyctinomus* was published in 1813 and therefore had priority over *Tadarida* Rafinesque, 1814 (Grubb *et al.*, 1998). Van Cakenberghe and Seamark (2009) support Grubb *et al.* (1998), who oppose changing the long-established usage of *Tadarida*; these authors feel that the use of *Nyctinomus* may be more appropriate than the more recent *Chaerephon* Dobson, 1874.

The taxonomic status of *Sauromys* remains unclear, and is discussed by Van Cakenberghe and Seamark (2009). This monotypic genus has been variously described as a subgenus of *Platymops*, the South American flat-headed free-tailed bat (Roberts, 1917); a subgenus of *Mormopterus* (Freeman, 1981; Koopman, 1993, 1994; Jacobs and Fenton, 2002); a subgenus of *Tadarida* (Koopman, 1975); or a distinct genus (Peterson, 1965, 1985; Meester *et al.*, 1986; Simmons, 2005). The generic status of *Sauromys* is favoured by Van Cakenberghe and Seamark (2009) based on its unique ecology and morphology.

Freeman (1981) indicates that *Tadarida aegyptiaca* (E. Geoffroy, 1818) and *T. brasiliensis* (I. Geoffroy St.-Hilaire, 1824) are phenetically similar to *Mormopterus*, explaining this grouping as convergence of shape due to similar life styles.

Some species of *Otomops* were originally placed in *Nyctinomus* (Chubb, 1917). *Otomops* was divided into seven species (Hayman and Hill, 1971; Freeman, 1981), one of which comprises four subspecies. *Otomops martiensseni* from mainland Africa and Yemen, *O. wroughtoni* from southern India, *O. formosus* from Java and *O. madagascariensis* from Madagascar are included in this study.

Materials and Methods

Taxonomic Sampling

In this study, we have used samples of Molossidae from the Afro-Malagasy region, and augmented these with other molossid sequences available on the NCBI GenBank (Appendix). Overall, the molossid nuclear Rag2 dataset comprised samples of 6 genera and 17 species (Appendix). We sequenced the Rag2 region of multiple representatives per species and, as there was little variability within species, we included between one and three representative haplotypes in the final dataset. The original cytochrome *b* dataset was reduced to a dataset of haplotypes; members of a taxonomic unit, which formed a monophyletic group, were reduced to a single representative where appropriate.

The Rag2 gene was sequenced in two parts. Both parts amplified for most of our samples, although we were not able to amplify and sequence the 3'end of the gene for a few samples, including *Tadarida fulminans* (Thomas, 1903) and *Sauromys petrophilus* (Roberts, 1917). Rag2 sequences downloaded from the GenBank comprised either the complete sequence, or the 5' fragment. Although we had complete sequences of the cytochrome *b* gene for all of our samples, many of the sequences on GenBank comprised only the 5' fragment. We carried out analyses based on the following datasets: (1) the (almost) complete Rag2 gene (1262 nt), (2) Rag2 concatenated with the (almost complete) cytochrome *b* gene (2031 nt), (3) the 3'end of the Rag2 gene (634 nt) and (4) the 5'end of cytochrome *b* (325 nt) (Table 1). Fig. 1 is based on analyses of dataset (1) above, with information included from dataset (3) on *S. petrophilus* and *T. fulminans*. Fig. 2 is based on analysis of dataset (2), and Fig. 3 on analysis of dataset (4).

DNA Sequencing and Analysis

Tissue samples used in this study included wing punches, liver, heart, kidney or muscle tissue stored in 80% ethanol or a lysis buffer. DNA was isolated from tissue samples using a DNeasy® DNA isolation kit (QIAGEN Inc.). Trees were rooted on non-molossid sequences, *Natalus stramineus* Gray, 1838 and *Myotis daubentonii* (Kuhl, 1817) or *Mormoops blainvillei* Leach, 1821, derived from the GenBank (Figs. 1 and 2; Appendix).

For most samples, the cytochrome *b* gene was PCR-amplified (Saiki *et al.*, 1988) as two overlapping double-stranded fragments using primer pairs: L14723 (5'-ACCAATGCAATG AAAAATCATCGTT-3') and H15553 (5'-TAGGCAAATAG GAAATATCATTCTGGT-3'); L15146 (5'-CATGAGGACAAA TATCATTCTGAG-3') and H15915 (5'-TCTCCATTTCTGG TTTACAAGAC-3') (Irwin *et al.*, 1991). In order to obtain a complete sequence, primers L14723 and L46RC (5'-CTCAG AAAGATATTTGTCCTCATG-3'), as well as H53RC (5'-AC CAGAATGATATTTCCTATTTGCCTA-3') and H15915 were used to obtain additional data on the first and last 400 bp of sequence, respectively. The Rag2 gene was amplified and sequenced in two parts, using the following combinations of primer pairs: 179F (5'-CAGTTTTCTCTAAGGAYTCCTGC-3') and 968R (5'-CCCATGTTGCTTCCAAACCATA-3'); F2 (5'-TTTGTTATTGTTGGTGGCTATCAG-3') and R2 (5'-GRA AGGATTTCTTGGCAGGAGT-3') (Baker *et al.*, 2000; Stadelmann *et al.*, 2007).

PCR-amplifications were performed in 25 µl volumes. Each reaction contained 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Roche Diagnostics), 0.2 µl *Taq* polymerase (5 U/µl) (Super-Therm) and 4 µl of each primer (6 µM) (forward and reverse) per reaction. The thermal cycling parameters used were as follows: cytochrome *b* — 94°C for 4 min, followed by 36 cycles of (94°C for 40 s, 50°C for 45 s and 72°C for 40 s) and followed by 72°C for 10 min; Rag2 — 95°C for 5 min, followed by 39 cycles of (95°C for 30 s, 55°C for 30 s and 72°C for 2 min) and

Table 1. Characteristics of the datasets used in this study

Dataset	Rag2	Rag2 + cyt *b*	Rag2 3' end	cyt *b* 5' end
Length (nt)	1262	2031	634	325
Nucleotide substitution model	GTR + I + G	GTR + I + G	TrN + G	GTR + I + G
Maximum parsimony parameters				
Variable characters	178	412	79	114
Parsimony-informative characters	95	302	46	100
MP tree length	211	907	113	641
Equally-parsimonious trees	2	1	6	72
Homoplasy index (HI)	0.128	0.406	0.123	0.702
Retention index (RI)	0.908	0.828	0.896	0.594
Maximum likelihood				
Number of ML trees	1	1	1	2
-log likelihood of most likely tree(s)	3056	7357	1581	3043

followed by 72°C for 10 min. Target fragments were purified from excised gel bands using the QIAquick® Gel Extraction Kit (QIAGEN Inc.) and sequenced at InqabaBiotec, Hatfield, Pretoria, South Africa. All fragments were sequenced in both directions to allow reconciliation of ambiguous positions. Sequences were deposited in GenBank (see Appendix). They were aligned using the CLUSTAL W option (Thompson *et al.*, 1994) of the BioEdit program (ver. 5.0.9 for Windows 95/98/NT) and by visual inspection.

Sequence Analyses

We used jModelTest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) applying the AKAIKE information criterion to determine the most appropriate evolutionary model to use in maximum parsimony, maximum likelihood and Bayesian analyses (Table 1). We analysed all datasets using likelihood, maximum parsimony and neighbour-joining methods in PAUP 4.0b10 (Swofford, 2002) and Bayesian Inference as implemented in MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001). Incongruence between datasets was evaluated by determining whether there were any nodes, which were consistently strongly supported (70% bootstrap support, ≥ 95% Bayesian posterior probability) in one dataset that conflicted with strongly-supported nodes in another (De Queiroz, 1993; Eick *et al.*, 2005). The absence of such instances allowed for the concatenation of sequences.

In maximum likelihood analyses, starting trees were obtained by neighbour-joining followed by TBR branch swapping. For parsimony analyses, starting trees were obtained by stepwise addition. The addition sequence was random, with 1 tree held at each step and with 10 replicates. The tree-bisection-reconnection branch-swapping algorithm was used. One thousand bootstrap replicates were carried out using a heuristic search. Bayesian analyses were run using four Markov chains for five million generations each, sampling every 100 generations. The chains were heated with the temperature scaling factor T = 0.02. We discarded the first 50,000 trees as burn-in, in each case having checked in a preliminary run that this was more than sufficient to achieve stationarity, and constructed a 50% majority rule consensus tree from the remaining trees (Table 1).

Dating

Our Rag2 data were used to estimate nodal dates since they provided good node support at the deeper nodes of interest within Molossidae. Recent studies have attempted to calibrate molecular dates of deeper (family-level) nodes of the chiropteran phylogeny based on a robust higher phylogeny and compilation of data from earliest fossil occurrences of bats (Jones *et al.*, 2005; Teeling *et al.*, 2005). To estimate dates of major supported clades within Molossidae, we used the estimated date of 65 MYA for the crown divergence of bats (Teeling *et al.*, 2005) and the crown divergence dates of 35–38 MYA and 47–49 MYA estimated for Molossidae and Vespertilionidae respectively (Jones *et al.*, 2005). Our analysis was based on 600 nucleotides of the 5'end of the Rag2 gene, for which there was a good dataset available, including non-Molossid members of the Vespertilionoidea and *Equus caballus* (AF447533.1), which we used as an outgroup. Bayesian analysis (Markov Chain Monte Carlo [mcmc] algorithm, with length 20,000,000, sampled every 1,000 iterations with burn-in of 100,000) was used to estimate mean and 95% confidence limits of specified nodal dates within Molossidae. We used the uncorrelated relaxed clock model assuming that branch-specific rates followed a lognormal distribution, which gives more biologically reasonable results than the exponential model (A. Rambaut, personal communication). The analysis was achieved using the program BEAST v.1.5.4 (Drummond and Rambaut, 2010) in conjunction with the programs BEAUti v.1.5.1 (Drummond and Rambaut, 2009) and Tracer v.1.51 (Rambaut and Drummond, 2009). The log files from two independent BEAST runs were combined in Tracer as recommended in the programme manual. Based on the results of jMODELTEST 3.7 mentioned above, we specified the HKY + G substitution model. Based on the estimated crown divergence dates, we calibrated the three nodes, Chiroptera, Molossidae and Vespertilionidae, using normal distribution priors, with means set to 65 MYA for Chiroptera and the midpoints of ranges given for Molossidae (35–38 MYA) and Vespertilionidae (47–49 MYA). Standard deviation was set at 0.5 for Chiroptera and, in the case of Molossidae and Vespertilionidae, adjusted so that 95% upper and lower limits matched the maximum and minimum date estimates.

RESULTS

Sequence Data

High retention indices (RI) and low homoplasy indices (HI) (Table 1) indicate that there was little homoplasy in the complete Rag2 dataset (RI 0.908, HI 0.128) or the 3'end of the Rag2 dataset (RI 0.896, HI 0.123). For the cytochrome *b* (5'end) dataset, the RI values were lower (0.594), and the HI values higher (0.702) (Table 1), consistent with a moderate amount of homoplasy. The concatenated Rag2/cytochrome *b* dataset was characterized by RI = 0.828 and HI = 0.406 (Table 1).

A single maximum parsimony tree was recovered from the concatenated Rag2/cytochrome *b* dataset (2031 nt), which had the best resolving power. Two MP trees were recovered from the complete Rag2 dataset, and 6 MP trees from the Rag2 - 3'end dataset. The cytochrome *b* - 5'end dataset had the lowest resolving power, yielding 2 ML trees and 72 MP trees. Where more than one ML or MP tree was obtained, the differences between trees were located in unresolved and unsupported terminal branches.

Monophyly of the Molossidae and Relationship to Other Members of the Superfamily Vespertilionoidea

In all analyses, the Molossidae included in this study formed a very strongly- to moderately-supported monophyletic group (Node A, Figs. 1, 2 and 3) with respect to non-molossid chiropteran outgroups.

Mormopterus

Analyses place clade B (composed of *Mormopterus jugularis* and *M. francoismoutoui*) basal to the other genera of Molossidae included in this study. The split between these mormopterids and the other molossids included in this study is dated at 31.18 (95% confidence interval; 23.91–38.24) MYA (Table 2). Although clade B is very strongly supported in all analyses (Figs. 1, 2 and 3), the basal position of these taxa is moderately supported only in an analysis based on the complete Rag2 gene (node C, Fig. 1).

Analysis of the complete Rag2 sequence (1262 nt) (Fig. 1) reveals three clade B haplotypes; the *M. francoismoutoui* haplotype is separated from each of the two *M. jugularis* haplotypes by one mutation (*p*-distance 0.08%). The GTR + I + G cytochrome *b* genetic distance between these species ranges from 0.94 to 1.26%. Analysis of the 5' fragment of the cytochrome *b* gene (Fig. 3) shows *Mormopterus*, as currently defined, to be paraphyletic, with the South American form, *M. kalinowskii* (Thomas, 1893) not included in the strongly-supported *M. jugularis/francoismoutoui* clade (B), and separated from it by a genetic distance of 17.93–18.74%. *Mormopterus kalinowskii* occupies an unsupported position as sister to a clade comprising *Nyctinomops laticaudatus* (E. Geoffroy, 1805) and *N. aurispinosus* (Peale, 1848), from which it is separated by a distance of 10.77–14.78%.

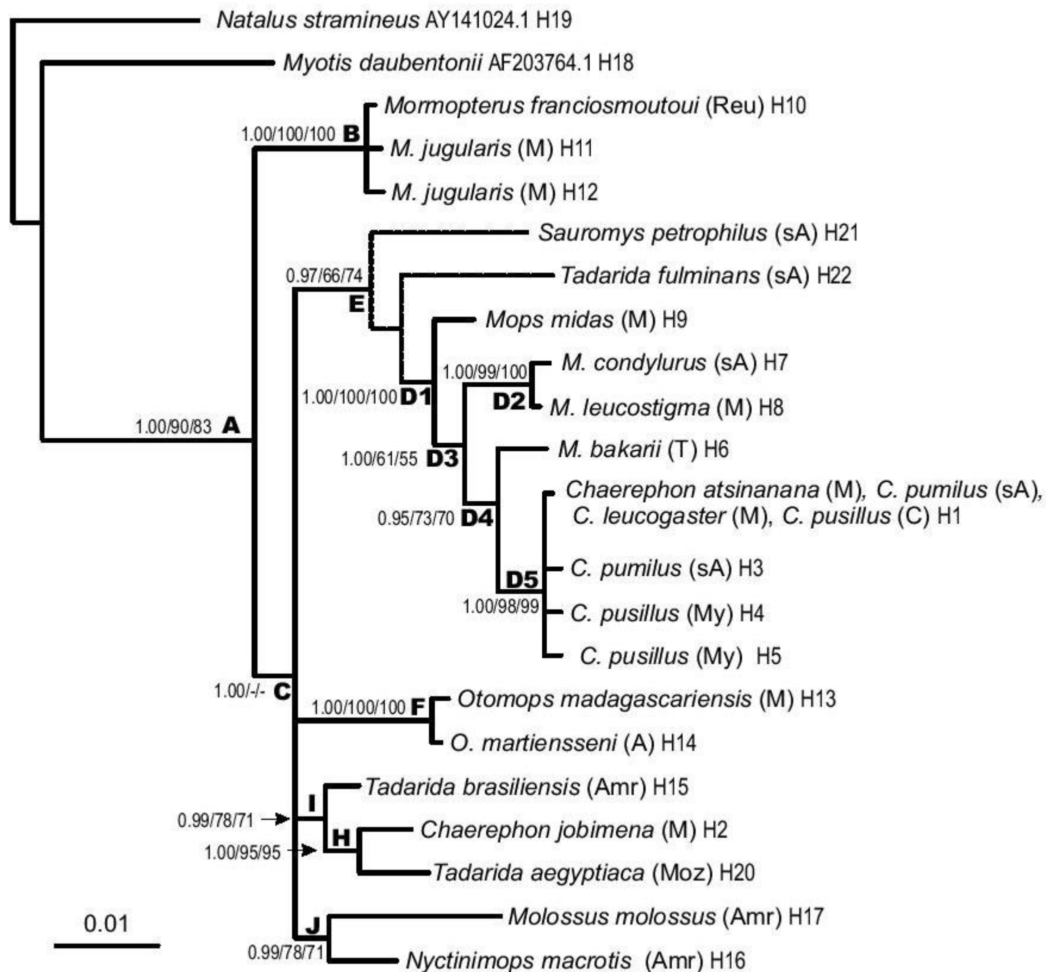

FIG. 1. Bayesian inference tree based on analysis of 1262 nucleotides of the nuclear Rag2 gene illustrating evolutionary relationships between molossid bat haplotypes and non-molossid outgroups. Nodal support values are represented as (Bayesian posterior probability / maximum parsimony bootstrap percent / neighbour-joining bootstrap percent). The positions of *Sauromys petrophilus* and *Tadarida fulminans*, indicated by dotted lines, were based on an analysis of the 3'end of the Rag2 region (634 nt). H = haplotype (see Appendix). A = Africa, Amr = Americas, C = Comoros, M = Madagascar, Moz = Mozambique, My = Mayotte, Reu = La Réunion, sA = southern Africa, T = Tanzania

Mops/Chaerephon

With the exception of *Chaerephon jobimena* Goodman and Cardiff, 2004, all *Chaerephon* and *Mops* taxa form a monophyletic group (node D1) which is very strongly-supported in analyses based on the Rag2 and concatenated Rag2-cyt *b* datasets (Figs. 1 and 2). The split between the groups comprising node D1 and the other Molossidae included in this study is dated at 17.19 (95% confidence interval; 9.95–25.58) MYA (Table 2). In analyses which include Rag2 data (Figs. 1 and 2), the *Mops* taxa

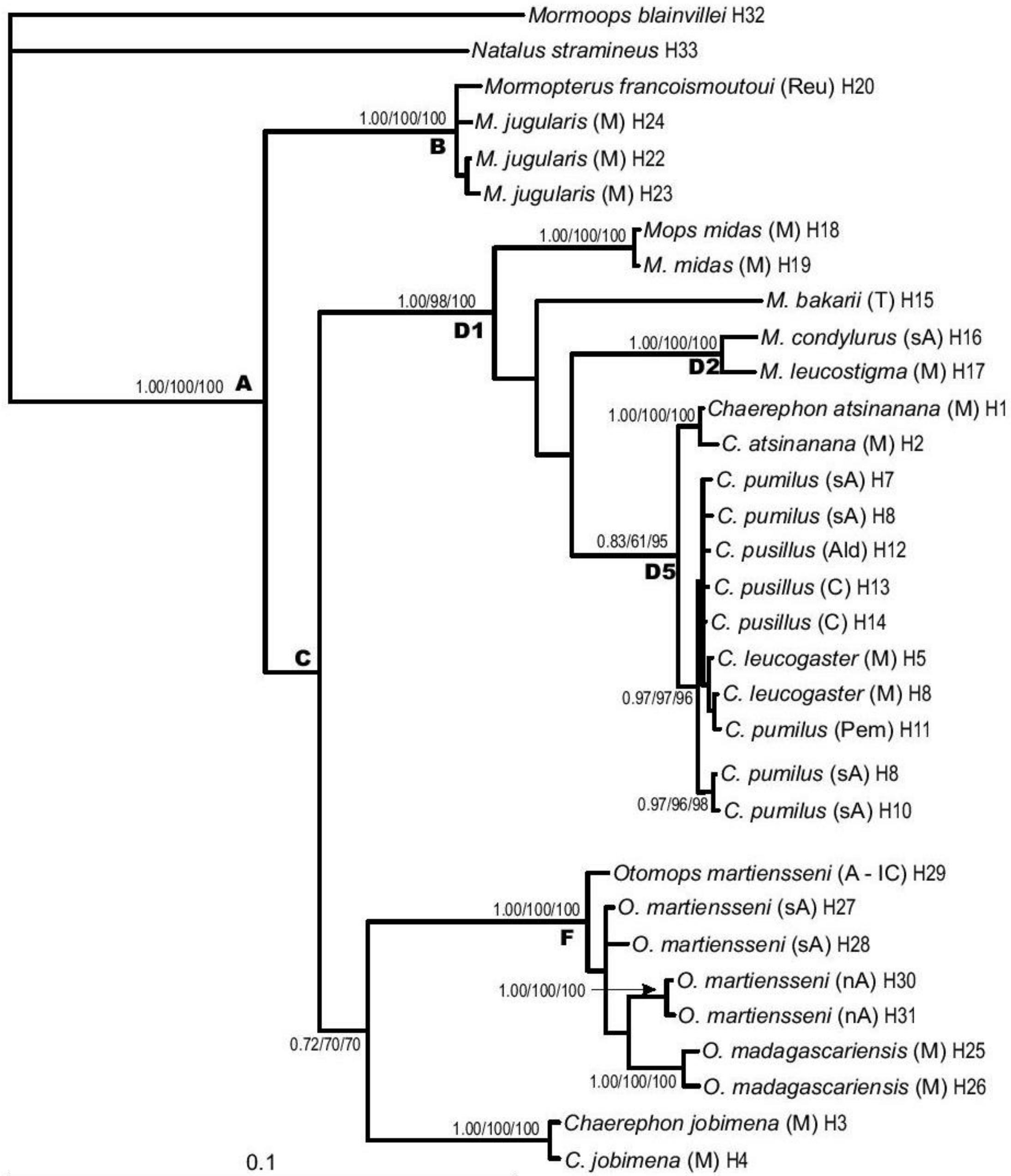

FIG. 2. Bayesian inference tree based on analysis of a concatenated Rag2-cytochrome *b* sequence (2031 nucleotides) illustrating evolutionary relationships between molossid bat haplotypes and non-molossid outgroups. Nodal support values are represented as (Bayesian posterior probability / maximum parsimony bootstrap percent / neighbour-joining bootstrap percent). H = haplotype (see Appendix). A = Africa, Ald = Aldabra, C = Comoros, IC = Ivory Coast, M = Madagascar, nA = northern Africa, Pem = Pemba, Reu = La Réunion, sA = southern Africa, T = Tanzania

(*M. bakarii* Stanley, 2008, *M. midas*, *M. condylurus*, *M. leucostigma*) appear ancestral to a generally more derived *Chaerephon* group. However, analyses based on the 5' fragment of the cytochrome *b* gene (Fig. 3), which includes additional GenBank-derived samples, appear to indicate paraphyly among *Mops* and *Chaerephon* although the nodes are not supported. *Chaerephon chapini* J. A. Allen, 1917, *M. midas*

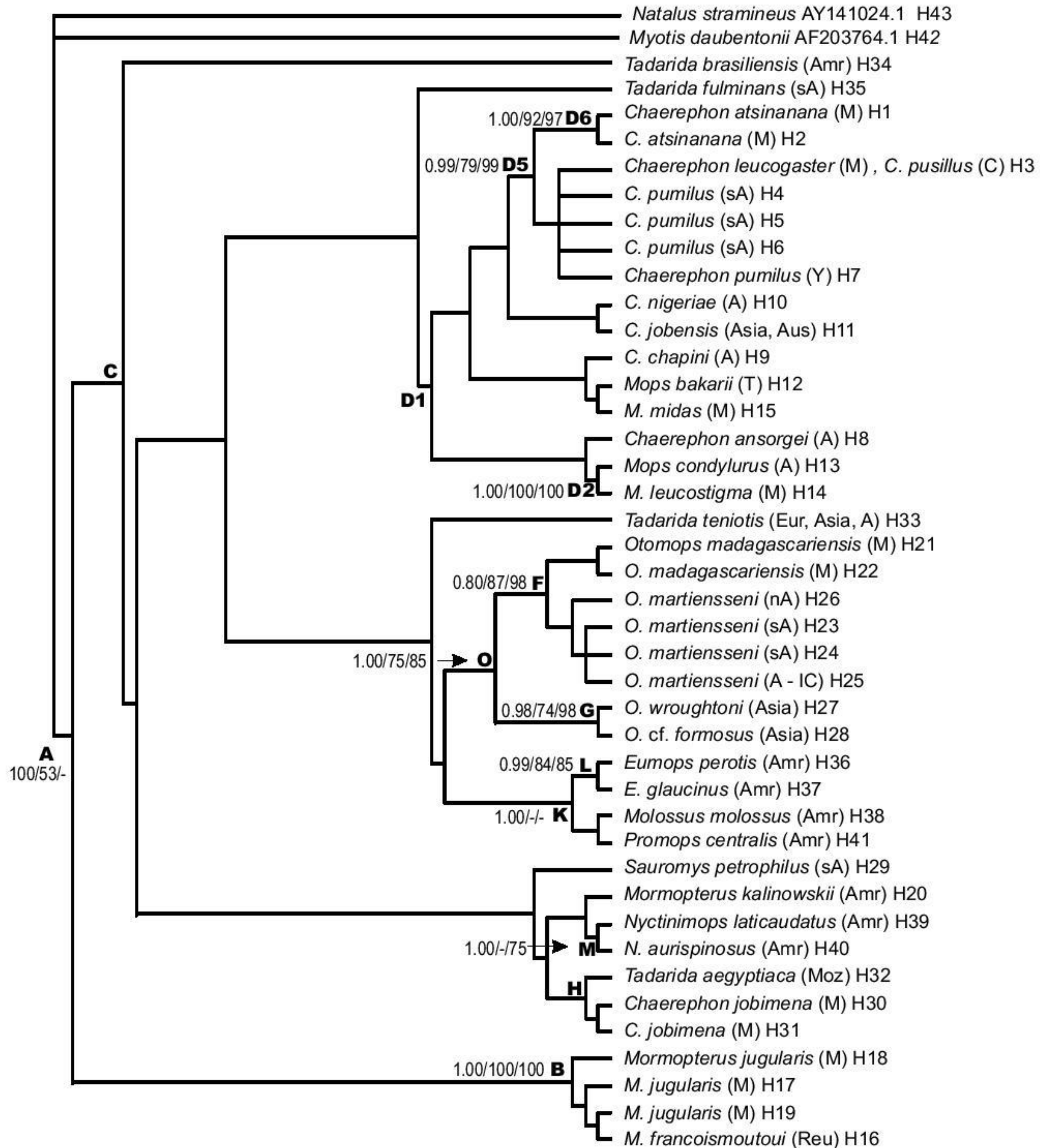

FIG. 3. One of two most likely trees derived from maximum likelihood analysis of 325 nucleotides of the mitochondrial cytochrome *b* gene illustrating evolutionary relationships between molossid bat haplotypes and non-molossid outgroups. Nodal support values are represented as (Bayesian posterior probability / maximum parsimony bootstrap percent / neighbour-joining bootstrap percent). H = haplotype (see Appendix). A = Africa, Amr = Americas, Aus = Australia, C = Comoros, Eur = Europe, IC = Ivory Coast, M = Madagascar, Moz = Mozambique, nA = northern Africa, Reu = La Réunion, sA = southern Africa, T = Tanzania, Y = Yemen

TABLE 2. Estimates of divergence dates (MYA) for selected nodes. TMRCA: time to the most recent common ancestor

Taxon-set	Mean node date	95% credibility interval	Node	Comment
Chaerephon-Mops	17.19	9.95–25.58	D1	Node including all *Mops* and *Chaerephon* (with exception of *C. jobimena*)
Mormopterus	31.18	23.91–38.24	A	Node splitting *Mormopterus* from other molossids
New World	18.37	8.97–27.46	J, K	Node of New World genus clade (excluding *T. brasiliensis*)
Otomops	19.84	8.89–31.20	F	Node dividing *Otomops* from *T. brasiliensis-C. jobimena*
Tadarida	9.80	1.78–18.59	I	Node separating *T. brasiliensis* from *C. jobimena*

and *M. bakarii* form a clade, as do *C. ansorgei* (Thomas, 1913), *M. condylurus* and *M. leucostigma*. In many cases, *Mops* taxa are genetically less distant from *Chaerephon* taxa than they are from other *Mops* species. The mean cytochrome *b* genetic distance among taxa currently placed in *Mops* is 10.65%, whereas the mean distance between *Mops* and *Chaerephon* (excluding *C. jobimena*) is 11.66%. *Mops midas* is separated from *M. bakarii*, *M. condylurus* and *M. leucostigma* by 10.06, 11.07 and 10.82%, respectively, whereas the distances to *C. atsinanana*, *C. leucogaster*, *C. pumilus* (from the KwaZulu-Natal Province of South Africa) and *C. pusillus* are less, being 9.36, 8.62, 9.49 and 8.62%, respectively (Table 3). *Chaerephon jobimena* forms a clade with *Tadarida aegyptiaca* (node H, Figs. 1 and 3) and is not placed in the *Mops*/ *Chaerephon* clade (D1). Node H is very strongly supported in analyses based on the complete Rag2 dataset (Fig. 1).

Sister taxa *M. condylurus* and *M. leucostigma* form a very strongly-supported clade (D2) in all analyses (Figs. 1, 2 and 3). Analysis of the Rag2 dataset reveals a nested set of supported clades (D3, D4 and D5) within clade D1. There is very strong to moderate support for a clade comprising *C. atsinanana*, *C. leucogaster*, *C. pumilus* and *C. pusillus* (node D5, Figs. 1, 2 and 3). Within this clade *C. atsinanana* is strongly supported as sister to the other *Chaerephon* taxa (node D6, Fig. 3 — see Goodman *et al*., 2010 for a discussion of relationships among members of this *Chaerephon* clade). There is good support for a clade (D4) in which *M. bakarii* is basal to clade D5, and moderate support for a clade (D3) in which *M. condylurus*/*M. leucostigma* (D2) are basal to clade D4 (Fig. 1). Analysis of the cytochrome *b* dataset reveals some paraphyly among *Chaerephon* and *Mops* taxa within clade D1, with neither *Mops* nor *Chaerephon* forming monophyletic clades (Fig. 3), although none of the mixed *Mops*/ *Chaerephon* subclades are supported.

Otomops

A clade comprising the sister-species *Otomops martiensseni* and *O. madagascariensis* is very strongly- to well-supported (node F, Figs. 1, 2 and 3). There is also strong support for a clade comprising the Asian forms, *O. wroughtoni* and *O. formosus* (node G, Fig. 3) and good support for the monophyly of all four of the above species (node O, Fig. 3 —

TABLE 3. GTR + I + G genetic distances between *Chaerephon* and *Mops* species included in Fig. 3. KZN: KwaZulu-Natal

Species	1	2	3	4	5	6	7	8	9	10	11	12
1 *C. atsinanana*												
2 *C. leucogaster*	1.25											
3 *C. pumilus* KZN	1.91	0.63										
4 *C. pusillus*	1.25	0.00	0.63									
5 *C. ansorgei*	10.87	11.84	11.87	11.84								
6 *C. chapini*	9.54	8.81	9.24	8.81	13.91							
7 *C. nigeriae*	7.04	7.11	6.73	7.11	13.17	11.99						
8 *C. jobensis*	9.94	10.83	10.05	10.83	10.01	13.52	9.55					
9 *M. bakarii*	12.21	12.33	13.33	12.33	15.58	12.76	12.52	14.10				
10 *M. condylurus*	10.89	11.31	12.25	11.31	12.53	10.98	11.52	12.52	14.16			
11 *M. leucostigma*	10.66	11.08	12.03	11.08	10.55	10.70	13.31	13.12	14.91	2.87		
12 *M. midas*	9.36	8.62	9.49	8.62	14.37	9.06	12.61	9.95	10.06	11.07	10.82	
13 *C. jobimena*	16.44	16.99	17.51	16.99	14.60	18.77	16.25	16.03	15.99	17.71	15.67	19.08

see Lamb *et al.*, 2008 for further discussion). There is no support for any higher-level relationships involving *Otomops*. The split between the *Otomops* clade and clade I comprising *Tadarida brasiliensis*, *Chaerephon jobimena* and *T. aegyptiaca* (Fig. 1) is dated at 19.84 (95% confidence interval; 8.89–31.20) MYA (Table 2).

Tadarida and *Sauromys*

The *Tadarida* species represented in this study are *T. fulminans*, *T. aegyptiaca*, *T. brasiliensis* and *T. teniotis* (Rafinesque, 1814). As previously mentioned, there is very strong support for a *T. aegyptiaca*/*Chaerephon jobimena* clade (H) (Fig. 1). There is also good support from the Rag2 dataset for the association of clade H with *T. brasiliensis* (node I, Fig. 1) to form a group whose age is estimated at 9.80 (95% confidence; 1.78–8.59) MYR. Based on analyses of the 3'end of the Rag2 dataset, *Sauromys petrophilus* and *T. fulminans* are basal in a moderately-supported clade (E), which also contains *Chaerephon*/*Mops* clade D1. There is no support for the monophyly of *Tadarida*, and in particular, *T. teniotis* is not sister to other species placed in the genus *Tadarida* (Fig. 3).

Molossus, *Nyctinomops* and *Eumops*

There is good support for association of *Molossus molossus* (Pallas, 1766) and *Nyctinomops macrotis* (Gray, 1840) (node J, Fig. 1). The age of this clade has been estimated at approximately 18.37 (95% confidence; 8.97–27.46) MYR. The cytochrome *b* dataset (Fig. 3) provides support for the following associations: *Eumops perotis* (Schinz, 1821)/*E. glaucinus* (Wagner, 1843) (node G, strong support), *M. molossus* and *Promops centralis* Thomas, 1915 (node K, moderate support) and for *Nyctinomops laticaudatus*/*N. aurispinosus* (node M, good support).

Discussion

Analyses of the nuclear Rag2 gene have shed some light on basal relationships within the Molossidae of eastern Africa and the islands of the western Indian Ocean, whereas those based on the mitochondrial cytochrome *b* gene have provided insights into relationships within and between genera and species. Some genera (e.g., *Otomops*) are strongly-supported and distinct whereas others (e.g., *Tadarida*) are not supported. These studies, which have a number of taxonomic implications, have revealed paraphyly between closely-related as well as distantly-related genera, and even within genera across continents, which highlights the need for taxonomic approaches based on a combination of morphological and molecular data. The Molossidae form a monophyletic clade in analyses based on the 5'end of the cytochrome *b* gene as well as in analyses of the Rag2 and concatenated Rag2/cytochrome *b* datasets.

This study provides strong support for the monophyly of the *Chaerephon* + *Mops* taxa, but not for either of the genera *Chaerephon* or *Mops* separately, as some paraphyly was recovered, particularly for *C. jobimena*. Further, although there are indications that, within this grouping, certain *Mops* species are ancestral and *Chaerephon* species more derived, lack of resolution and paraphyly among *Chaerephon* and *Mops* species mitigates against proposals that *Chaerephon* as currently described is a subgenus of *Tadarida* (Peterson *et al.*, 1995) or a genus in its own right (Freeman, 1981). Pending the outcome of more complete sampling, consideration might be given to combining *Chaerephon* (with the exception of *C. jobimena*) and *Mops* into a single genus.

Chaerephon jobimena, referred to *Chaerephon* based on morphological similarities to other *Chaerephon* spp. (Goodman and Cardiff, 2004), is genetically more similar to *Tadarida aegyptiaca*, with which it forms a well-supported clade based on analyses of Rag2 sequence data, than to other members of the genus *Chaerephon*. The cytochrome *b* genetic distances are: *C. jobimena* to other *Chaerephon* species, 14.62–18.74%, and *C. jobimena* to *T. aegyptiaca*, 11.15% (Table 3). The former distances are more consistent with the mean distance between currently-established Molossidae genera (18.35%), and the latter with that between molossid species (11.24%) (P. J. Taylor, S. M. Goodman, F. H. Ratrimomanarivo, W. Buccas, and J. M. Lamb, unpublished data). The morphological similarity of *C. jobimena* to *Chaerephon* taxa may be indicative of sometimes problematic parallel evolution in morphological characters in bats (Ruedi and Mayer, 2001). As currently-recognized, the genus *Tadarida* is not a natural group. *Tadarida teniotis* is the type species of this genus, and as *T. aegyptiaca* and *C. jobimena* form a separate clade and *T. aegyptiaca* is the type species of the genus *Nyctinomus* (Van Cakenberghe and Seamark, 2009), a possible resolution to this taxonomic quandary is to place these latter two taxa and sister species subsequently discovered in a separate genus, *Nyctinomus*. We

await a greater taxonomic sampling and resolution of the phylogeny of other African Molossidae before formally making this recommendation.

This study is the first molecular report on the phylogenetic position of the mainland African form, *Mops bakarii*, which forms part of the well-supported *Mops*/*Chaerephon* clade (D1). Within this clade it appears to occupy a derived position relative to *M. midas*, *condylurus* and *leucostigma* and to be ancestral to a *C. pumilus*/*pusillus*/*atsinanana*/*leucogaster* clade (Fig. 1).

The genus *Mormopterus* has a disjunct distribution, being represented in our dataset by *M. jugularis* (Madagascar), *M. francoismoutoui* (La Réunion) and *M. kalinowskii* (New World); the Malagasy region and New World taxa are paraphyletic. *Mormopterus jugularis* and *M. francoismoutoui* form a monophyletic clade, which defines this genus and is basal within the Molossidae. *Mormopterus kalinowskii* is separated from the Malagasy region taxa by a cytochrome *b* genetic distance of 17.93% to 18.74%, consistent with molossid inter-generic distances (mean 18.35%) (P. J. Taylor, S. M. Goodman, F. H. Ratrimomanarivo, W. Buccas, and J. M. Lamb, unpublished data). The position within the Molossidae of *M. kalinowskii*, for which only cytochrome *b* data is available, is not supported, although it appears as sister to the South American forms *Nyctinomops laticaudatus* and *N. aurispinosus* and is separated from them by a mean genetic distance of 12.78%, consistent with the mean interspecific distance for Molossidae of 11.15% (P. J. Taylor, S. M. Goodman, F. H. Ratrimomanarivo, W. Buccas, and J. M. Lamb, unpublished data), which is likely to be an over-estimate as it includes incorrectly-assigned taxa. While more sampling is needed, it is clear that *M. kalinowskii* is not correctly placed in the genus *Mormopterus*. Since *M. jugularis* is the type species of *Mormopterus*, our data clearly show that the New World *M. kalinowskii* must be referred to a different genus (based on future research with wider more taxonomic sampling of New World '*Mormopterus*'). A recent morphological and molecular genetics study (Goodman *et al*., 2008) showed that the species from La Réunion (*M. francoismoutoui*) and Mauritius (*M. acetabulosus*) showed a 5.01% uncorrected sequence divergence from one another in the mitochondrial DNA control region. They were assigned separate species names based on this and other sequence data, which did not include cytochrome *b*. There is no genetic support for a relationship between *T. aegyptiaca*, *T. brasiliensis* and *Mormopterus*, to which they are phenetically (morphologically) similar (Freeman, 1981), supporting the notion that this grouping is due to convergence of shape.

The monophyly of the genus *Tadarida*, represented in this study by the species *T. fulminans*, *T. aegyptiaca*, *T. brasiliensis* and *T. teniotis* is not supported. Further genetic data is needed from a greater number of taxa referred to this genus for a better resolution of the species that should be placed in *Tadarida*. Given that *T. teniotis* is the type species of the genus *Tadarida*, it and demonstrated sister-taxa should be those restricted to this genus.

There is good support for a *C. jobimena*/*T. aegyptiaca*/*T. brasiliensis* clade (node I, Fig. 1). Although *C. jobimena* and *T. aegyptiaca* appear separated by an interspecific-level genetic distance (11.15%), *T. brasiliensis* is separated from *C. jobimena* (17.26%) and *T. aegyptiaca* (15.07%) by larger distances, more consistent with the mean intergeneric distance for Molossidae (18.35%) (P. J. Taylor, S. M. Goodman, F. H. Ratrimomanarivo, W. Buccas, and J. M. Lamb, unpublished data). Thus, although the Afro-Malagasy *C. jobimena* and *T. aegyptiaca* are likely congeners, they are notably distinct from the New World *T. brasiliensis* at the generic level. The type species of the currently unrecognised genus *Nyctinomus* is *N. aegyptiaca*; this generic name would be available for this clade.

There is moderate support for an African clade including *Sauromys petrophilus*, *T. fulminans* and *Chaerephon*/*Mops* clade D1 in an analysis based on the 3' fragment of the Rag2 gene (node E, Fig. 1). The cytochrome *b* genetic distance between the monospecific genus *Sauromys* and *T. fulminans* is 14.4%, intermediate between the mean inter-specific and inter-generic values for Molossidae (11.15% and 18.35% — P. J. Taylor, S. M. Goodman, F. H. Ratrimomanarivo, W. Buccas, and J. M. Lamb, unpublished data). *Sauromys petrophilus* is separated from members of the well-supported *Chaerephon*/*Mops* clade (D1) by a distance of between 14.1 and 19.1%, consistent with its status as a distinct genus, as in Peterson (1965, 1985) and Simmons (2005). There is no evidence that *Sauromys* is a subgenus of *Mormopterus*, as proposed by several taxonomists (e.g., Freeman, 1981; Koopman, 1994). The distance between *T. fulminans* and *Chaerephon*/*Mops* clade D1 (11.5–13.8%) indicates a closer relationship.

The composition of the genus *Otomops* is strongly-supported by both the Rag2 and cytochrome *b* datasets; it appears to be discrete, with no clearly-defined wider relationships among the Molossidae included in this study. A clade comprising the sister-

species *O. martiensseni* and *O. madagascariensis* is moderately supported as sister to, but clearly separate from, a strongly-supported clade comprising the Asian forms, *O. wroughtoni* and *O. formosus*.

The earliest molossid fossil, *Tadarida rusingae* from Rusinga Island, Kenya, dates back to the early Miocene, 17.5 to 18 MYA (Arroyo-Cabrales *et al.*, 2002) whereas the Vespertilionoidea, which include the Molossidae, are estimated to have originated 52 to 50 MYA (Teeling *et al.*, 2005). Our dataset supports mixed clades comprising New World and Old World Molossidae. For example, the genus *Mormopterus*, estimated to have originated 31.18 MYA, and for which the type species is a Malagasy endemic, is basal to a well-supported clade which includes a well-supported American *Molossus*/*Nyctinomops* clade (≈ 18.37 MY-old) and a strongly-supported ≈ 17.19 MY-old Afro-Malagasy *Mops*/*Chaerephon* clade.

Assuming an Old World origin for the Molossidae, and as the split between South America and Africa (100–84 MYA) predates the origin of Molossidae (35–38 MYA — Jones *et al.*, 2005), it is likely that the presence of Molossidae in the Americas required at least two dispersal events, one involving the ancestor of *Molossus*/*Nyctinomops* occurring approximately 8.37 MYA, and the other involving *T. brasiliensis*, which split from the Malagasy taxon, *C. jobimena*, ≈ 9.8 MYA. The position of New World *M. kalinowskii* is equivocal, although analysis of cytochrome *b* sequences (Fig. 3) suggest it may be associated with the New World clade. Trans-Atlantic dispersal would have required island hopping across the widening South Atlantic, or rafting (Parrish, 1993). An alternative route may have involved northward dispersal to Eurasia across the Tethys Sea followed by entry into North America via Beringia or three putative trans-Atlantic land bridges, followed by entry into South America via the islands of the Caribbean (Janis, 1993; Sanmartin *et al.*, 2001). Clearly, these hypotheses require testing from much wider taxonomic sampling from New World molossids.

On the basis of current systematics, the disjunct distribution of certain genera, such as *Mormopterus* or *Tadarida*, on either side of the Atlantic may either be a result of a secondary dispersal event or morphological convergence in certain classically-used taxonomic characters (see Hoofer and Van Den Bussche 2003 for further discussion). In the case of *Tadarida*, which is genetically rather diverse and does not appear to be a natural group based on conservative Rag2 sequences, it would appear that the latter is true, although greater insight can only be obtained by more complete taxonomic and genomic sampling.

Taxonomic Conclusions

The data from this study reaffirm that the taxonomic use of morphological characters alone can be misleading. For example, the generic placement of the recently-described *C. jobimena* was based on morphological characters (Goodman and Cardiff, 2004), whereas genetic estimates based on both the nuclear Rag2 and mitochondrial cytochrome *b* genes place it as closest to *Tadarida aegyptiaca*. This is likely to be a case of homoplasy resulting from convergent morphological evolution.

The name *Mormopterus* should be reserved for the western Indian Ocean island forms *M. jugularis*, *M. francoismoutoui*, *M. acetabulosus* and other taxa that can be demonstrated to be members of this clade, whereas the South American *Mormopterus kalinowskii* appears to be incorrectly placed within this genus. *Sauromys petrophilus* is supported as a monotypic genus allied to the *Chaerephon*/*Mops* clade and *Tadarida fulminans*. *Otomops* is strongly supported as a discrete genus.

The monophyly of a combined *Chaerephon*/*Mops* group is strongly supported, but not the integrity of the genera *Chaerephon* or *Mops*, which show some paraphyly in a combined clade in which *Mops* taxa are generally ancestral to more-derived *Chaerephon* species. Our molecular data places *M. bakarii* within the well-supported *Mops*/*Chaerephon* clade.

The genus *Tadarida* is not monophyletic. Pending wider taxonomic sampling, it is recommended that the genus name *Tadarida* be reserved for *T. teniotis*, the type species, and any yet-to-be discovered closely-related taxa. Further, the genus name, *Nyctinomus*, might be applied to *T. aegyptiaca*, the type specimen, *C. jobimena* and other close relatives.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from the Volkswagen Foundation, The John D. and Catherine T. MacArthur Foundation and the National Research Foundation (South Africa). We also acknowledge, with thanks, the comments of the reviewers of this manuscript.

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Received 18 August 2010, accepted 02 March 2011

APPENDIX

Molossid and outgroup taxa included in this study. Museum numbers include the Durban Natural Science Museum (DM), Field Museum of Natural History (FMNH), Harrison Zoological Museum (HZM), National Museum of Kenya (NMK), National Museum of Prague (NMP), the Senckenberg Museum, Frankfurt a. M. (SMF), and Université d'Antananarivo, Département de Biologie Animale, Antananarivo (UADBA). Uncatalogued specimens collected by S. M. Goodman are denoted with the collector number SMG = Steven M. Goodman, RSA = Republic of South Africa, NA = not available

Species	Geographic origin	Co-ordinates		Museum no./ Field no.	Rag2 Hap Tree1	Rag2 +Cyt*b* Hap Tree2	Cyt*b* Hap Tree 3	GenBank #	
								Cyt*b*	Rag2
Chaerephon atsinanana	Madagascar: Vohipeno, Fianarantsoa Province	22.35°S,	47.84°E	FMNH 185294	1	1	1	HQ 384479	HQ 384487
C. atsinanana	Madagascar: Vangaindrano, Fianarantsoa Province	23.36°S,	47.60°E	FMNH 185229	1	2	2	HQ 384480	HQ 384488
C. leucogaster	Madagascar: Toliara, Toliara Province	23.40°S,	43.72°E	FMNH 184237	1	6	3	HM802905	HM631634
C. leucogaster	Madagascar: Toliara, Toliara Province	23.40°S,	43.72°E	FMNH 184239	1	6	3	EU 716036	HM631635
C. leucogaster	Madagascar: Toliara, Toliara Province	23.40°S,	43.72°E	FMNH 184240	1	6	3	EU 716037	HM631636
C. leucogaster	Madagascar: Toliara, Toliara Province	23.40°S,	43.72°E	FMNH 184245	1	6	3	HM802900	HM631629
C. leucogaster	Madagascar: Mahajanga, Mahajanga Province	15.71°S,	46.31°E	FMNH 184608	1	5	3	HM802901	HM631630
C. leucogaster	Madagascar: Antanimbary, Mahajanga Province	17.19°S,	46.86°E	FMNH 184899	1	5	3	HM802902	HM631631
C. leucogaster	Madagascar: Nosy Be, Antsiranana Province	13.37°S,	48.32°E	FMNH 187756	1	6	3	HM802903	HM631632
C. leucogaster	Tanzania: Pemba Island	4.97°S,	39.71°E	FMNH 192887	1	6	3	HM802904	HM631633
C. pusillus	Seychelles: Aldabra	9.39°S,	46.20°E	FMNH 205318	1	12	3	GQ 489134	HM631643
C. pusillus	France: Mayotte	6.00°S,	39.39°E	FMNH 194031	4	13	3	HQ 384481	HM631644
C. pusillus	France: Mayotte	6.00°S,	39.39°E	FMNH 194032	5	14	3	HQ 384482	HM631645
C. pumilus	Tanzania: Pemba Island	5.13°S,	39.44°E	FMNH 192823	3	11	3	HQ 384483	
C. pumilus	RSA: Athlone Park, KwaZulu-Natal	30.05°S,	30.88°E	DM 7377	1	7	4	HM802906	HM631637
C. pumilus	RSA: Athlone Park, KwaZulu-Natal	30.05°S,	30.88°E	DM 7401	1	8	5	HM802907	HM631639
C. pumilus	RSA: Mkuze Game Reserve, KwaZulu-Natal	27.58°S,	32.22°E	DM 7373	1	8	5	FJ 415815	HM631641
C. pumilus	RSA: Mkuze Game Reserve, KwaZulu-Natal	27.58°S,	32.22°E	DM 7374	1	10	5	FJ 415816	HM631642
C. pumilus	RSA: Hell's Gate, KwaZulu-Natal	28.00°S,	32.30°E	DM 7367	1	8	5	FJ 415814	HM631638
C. pumilus	RSA: Hell's Gate, KwaZulu-Natal	28.07°S,	32.42°E	DM 7371	1	9	6	HM802908	HM631640
C. jobimena	Madagascar: Isalo National Park, Fianarantsoa Province	45.38°S,	22.54°E	FMNH 175992	2	3	30	HM802932	HM631627

APPENDIX. Continued

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	Rag2 Hap Tree1	Rag2 +cyt*b* Hap Tree2	Cyt*b* Hap Tree 3	GenBank # Cyt*b*	GenBank # Rag2
M. bakarii	Tanzania: Pemba Island	4.97°S, 39.71°E	FMNH 192898	6	15	12	HM802911	HM631646
M. condylurus	RSA: Hell's Gate, KwaZulu-Natal	26.00°S, 32.92°E	DM 6291	7	16	13	HM802912	HM631647
M. condylurus	RSA: Phinda Game Reserve, KwaZulu-Natal	27.86°S, 32.31°E	DM 6332	7	16	13	HM802913	HM631648
M. leucostigma	Madagascar: Mahajanga, Mahajanga Province	15.71°S, 46.31°E	FMNH 184698	8	17	14	HM802914	HM631649
M. leucostigma	Madagascar: Ampitabe, Toamasina Province	19.00°S, 48.53°E	FMNH 188009	8	17	14	HQ 384484	HQ 384489
M. midas	Madagascar: Sakaraha, Toliara Province	22.91°S, 44.53°E	FMNH 184306	9	18	15	HM802915	HM631650
M. midas	Madagascar: Ankazomborona, Mahajanga Province	16.12°S, 46.07°E	FMNH 185187	9	19	15	HM802916	HM631652
Mormopterus francoismoutoui	La Réunion: Saint Clotilde	20.92°S, 55.48°E	FMNH 194015	10	20	16	HM802917	HM631653
M. francoismoutoui	La Réunion: Saint Clotilde	20.92°S, 55.48°E	FMNH 194016	10	20	16	HM802918	HM631654
M. jugularis	Madagascar: Sakaraha, Toliara Province	22.92°S, 44.53°E	FMNH 184347	11	22	17	HM802919	HM631655
M. jugularis	Madagascar: Andasibe, Toamasina Province	18.90°S, 48.42°E	FMNH 184576	11	23	18	HM802920	HM631656
M. jugularis	Madagascar: Fianarantsoa, Fianarantsoa Province	21.46°S, 46.08°E	FMNH 184445	12	24	19	HM802921	HM631657
Otomops madagascariensis	Madagascar: Bisihiko Cave, Toliara Province	23.54°S, 43.77°E	FMNH 172944	13	25	21	HM802922	HM631658
O. madagascariensis	Madagascar: Isalo National Park, Fianarantsoa Province	22.54°S, 45.38°E	UADBA SMG 10996	13	26	22	HQ 384485	HQ 384490
O. martiensseni	RSA: Brynderyn Flats, Morningside, KwaZulu-Natal	29.86°S, 31.04°E	DM 7909	14	27	23	HM802923	HM631659
O. martiensseni	RSA: Kingsway School, Amanzimtoti, KwaZulu-Natal	30.04°S, 30.89°E	DM7914	14	28	24	HM802924	HM631660
O. martiensseni	Ivory Coast: Comoe National Park	8.73°S, 3.38°E	SMF 92049	14	29	25	HM802925	HM631661
O. martiensseni	Kenya: Ithundu Caves, Makuena District	2.36°S, 37.72°E	NMK 15461	14	30	26	HM802927	HM631663
O. martiensseni	Kenya: Ithundu Caves, Makuena District	2.36°S, 37.72°E	NMK 15462	14	31	26	HM802926	HM631662
O. wroughtoni	Cambodia: Chhep District, Preah Vihear Province	13.59°S, 105.16°E	HZM 3.33440			27	HM802928	
O. cf. *formosus*	Philippines: Kalinga Province, Luzon Island	17.46°S, 121.07°E	FMNH 67240			28	HM802929	
Sauromys petrophilus	RSA: Cedarberg	32.14'S 19.00'E	DM 8613	21		29	HM802931	HM631664

APPENDIX. Continued

Species	Geographic origin	Co-ordinates	Museum no./ Field no.	Rag2 Hap Tree1	Rag2 +cyt*b* Hap Tree2	Cyt*b* Hap Tree 3	GenBank #	
							Cyt*b*	Rag2
Tadarida aegyptiaca	Mozambique: 40 km west of Ribaue	NA	DM 8617	20		32	HM802930	HM631668
T. fulminans	Mozambique: 40 km west of Ribaue	NA	DM 8619	22		35	HQ 384486	HM631667
Chaerephon ansorgei	NA	NA	NA			8	AY377967	
C. chapini	NA	NA	NA			9	AY591329	
C. jobensis	NA	NA	NA			11	AY591331	
C. nigeriae	NA	NA	NA			10	AY591330	
Eumops glaucinus	NA	NA	NA			37	L19719.1	
E. perotis	NA	NA	NA			36	L19720.1	
Molossus molossus	NA	NA	NA	17		38	L19724.1	AY141017
Mormopterus kalinowskii	NA	NA	NA			20	L19725.1	
Myotis daubentoni	NA	NA	NA	18		42	AB106589.1	AF203764.1
Natalus stramineus	NA	NA	NA	19	33	43	AY621019.1	AY141024.1
Nyctinomops aurispinosis	NA	NA	NA			40	L19728	
N. laticaudatus	NA	NA	NA			39	L19729	
N. macrotis	NA	NA	NA	16				AY141018.1
Promops centralis	NA	NA	NA			41	L19732.1	
T. brasiliensis	NA	NA	NA	15			L19734.1	AY141019.1
T. fulminans	NA	NA	NA			35	EU760911.1	TBI
T. teniotis	NA	NA	NA			33	DQ120910.1	
Mormoops blainvillei	NA	NA	NA		32		AY604462.1	AF338701

Phylogeography of southern and northeastern African populations of *Otomops martiensseni* (Chiroptera: Molossidae)

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Summary

Lamb, J.M., Abdel-Rahman, E.H., Ralph, T., Fenton, M.B., Naidoo, A., Richardson, E.J., Denys, C., Naidoo, T., Buccas, W., Kajee, H., Hoosen, N., Mallett, D., & Taylor, P.J. Phylogeography of southern and northeastern African populations of *Otomops martiensseni* (Chiroptera: Molossidae). *Durban Museum Novitates* 31: 42-53. The rare, large-eared free-tailed bat *Otomops martiensseni* is sparsely distributed throughout sub-Saharan Africa and Yemen. Whilst currently considered conspecific, South African populations from the Durban metropolitan region were previously regarded to be distinct (*O. icarus*) from populations elsewhere in Africa (*O. martiensseni*) and Madagascar (*O. madagascarensis*). We used DNA sequencing of the cytochrome *b* gene (1034 base pairs, n=17), the mitochondrial control region, or d-loop (208 base pairs, n=16), and random amplification of polymorphic DNA fragments (PCR-RAPDs; n=74), to investigate phylogeographic structure in geographically distant (>3000 km) populations from Kenya (one colony), Ethiopia (one colony) and South Africa (Durban: seven colonies). Phylogenetic analysis of cytochrome *b* sequences revealed two well-supported (100% bootstrap), but genetically similar (2.5% divergence) lineages, from northeastern and South Africa. The cytochrome *b* haplotype network showed a minimum of 22 mutational steps between northeastern and southern clades, and, within each clade (particularly Durban), a high number of unique haplotypes with multiple mutational steps (4-12) between sequential haplotypes. Based on phylogenetic analysis of d-loop sequences, colonies from Ethiopia and Kenya formed one clade, whereas other colonies sampled formed distinct and widely separated clades; one Durban colony (Pinetown) was phylogenetically intermediate between another Durban colony 30 km away (Ballito), and the Kenyan/Ethiopian clade (>3000 km apart), suggesting multiple north-south or south-north dispersal events. Grouped-colony PCR-RAPD data revealed slight nucleotide divergence between East and South African populations (0.9%), with considerable overlap of individuals between colonies and high intra-colony divergences (0.19% for Utundu Cave in Kenya, and 0.54-2.16% for five Durban colonies). Both nucleotide and haplotype diversity was higher in the southern than in the northeastern clade, possibly in part due to differences in roosting biology (numerous dispersed house roosts in Durban versus single large cave colonies in northeastern Africa). Genetic data were explained by a combination of high-altitude, long-distance flight capabilities of the species, migration events, and female philopatry resulting from stable harems, at least in the Durban population.

KEYWORDS: *Chiroptera, cytochrome b, d-loop, DNA sequences,* Otomops martiensseni, *PCR-RAPDs, philopatry.*

Introduction

Taxonomic status can be central to level of protection under endangered species legislation and there are examples of "taxonomic mischief", situations where endangered taxa have lost their protected status (e.g. O'Brian & Mayr 1991). Today in some jurisdictions, protected status has been conferred on distinct populations, not just those named as species (e.g. Kaiser 2001; Friedmann & Daly 2004) and identification of evolutionarily significant units is an important component of conservation programmes (Moritz 1994; Brown & Houlden 2000).

Worldwide, bats represent a significant proportion of mammal diversity (Wilson & Reeder 2006), and new taxa are often described (Kingston *et al.* 2001; Mayer & Von Helversen 2001; Cotterill 2002; Bates *et al.* 2004a, b; Goodman & Cardiff 2004; Goodman *et al.* 2005; 2006; Jacobs *et al.* 2006). Paucity of data about distribution and habitat requirements of many species makes it difficult to assess their taxonomic and/or conservation status. For example, *Otomops martiensseni* (Matschie 1897) is globally listed as "Vulnerable" by the International Union for Conservation of Nature (IUCN: Hutson *et al.* 2001). This bat is widespread in Africa and Madagascar, but represented by few specimen records (Al-Jumaily 1999; Fenton *et al.* 2002). *Otomops martiensseni sensu lato* could include at least three distinct taxa: *Otomops martiensseni* from sub-Saharan Africa excluding South Africa, *Otomops madagascarensis* Dorst (1953) from Madagascar, and *Otomops icarus* Chubb (1917) from Durban, South Africa. Peterson *et al.* (1995) used morphometric evidence to support the view that *O. madagascariensis* was distinct from *O. martiensseni*, a position accepted by Simmons (2006). Fenton *et al.* (2002) found no significant differences in size (forearm length) between *Otomops* from Madagascar and Durban, but animals from these two populations were significantly smaller than those from East Africa. Today *O. icarus* is not viewed as a distinct species (Harrison 1957; Meester *et al.* 1986; Koopman 1993; Bronner *et al.* 2003; Simmons 2006).

In northeast Africa, *O. martiensseni* have usually been found in large (hundreds to tens of thousands of individuals) cave colonies (Mutere 1973; Kock *et al.* 2005), where apparent substantial declines in populations have been used to justify the IUCN listing (Hutson *et al.* 2001; but see Kock *et al.* 2005 for a contrasting view). Around Durban and elsewhere, colonies are smaller and roosts are located in buildings or tree hollows (Fenton *et al.* 2002). Roosts in Durban typically comprise up to 30 individuals, an adult male and 1 to >10 adult females with young, suggesting a harem social structure (Richardson & Taylor 1995; Taylor 1998, 2000; Fenton *et al.* 2002). In the province of KwaZulu-Natal, *O. martiensseni* has the same conservation status ("Specially Protected") as *Diceros bicornis*, the black rhinoceros (Taylor 2000). In South Africa, due to its restricted distribution, *O. martiensseni* has recently been assigned a Vulnerable IUCN rating (Friedmann & Daly 2004).

Separation by >3000 km, different habitats, threats and conservation implications, significant differences in size of individuals, and in colony size between east African and South African populations, as well as the taxonomic history make it important to resolve the taxonomic status of African populations of *Otomops*.
genetic data (mitochondrial cytochrome *b* and d-loop sequences and random amplification of polymorphic DNA fragments, or PCR-RAPDs) to assess the taxonomic status of northeastern African (Kenya, Ethiopia) and South African populations of *Otomops* and the extent of phylogeographic structure at continental (>3000 km) and local (<100 km; within the Durban region) scales. Genetic structure occurs in bats with low dispersal abilities (Worthington-Willmer *et al.* 1994; Burland *et al.* 1999), but not typically in more mobile species (McCracken *et al.* 1994; Webb & Tidemann 1996, Russel *et al.* 2005). Wing shape and predicted flight capability suggest that *Otomops martiensseni* is capable of long-range dispersal (Rydell & Yalden 1997) and we predict little or no phylogeographic structure at local or continental scales. However, social factors such as high female philopatry can promote genetic structure in mobile species of bats and other mammals (e.g. McCracken 1987; Chesser 1991; Miller-Butterworth *et al.* 2003; Ruedi & Castella 2003). Thus, postulated stable harem formation in Durban colonies (Fenton *et al.* 2002) could promote female philopatry, leading to structuring at the local level in maternally inherited mitochondrial sequences but not in biparentally inherited PCR-RAPDs. In nursery colonies of *Moyotis myotis* in Europe, Ruedi & Castella (2003) found evidence of historical gene flow restrictions due to past glaciation in mitochondrial sequences but not in nuclear markers.

Materials and methods

Sample collection

We investigated genetic variation in *O. martiensseni* using mitochondrial cytochrome *b* gene sequencing (n=17), d-loop sequencing (n=16) and random-amplification of polymorphic DNA (PCR-RAPDs) (n=74) (Fig.1). For the PCR-RAPD analysis we sampled five colonies in the Durban metropolitan region of South Africa and an additional colony from Kenya (Fig. 2). The Kenyan colony sampled for PCR-RAPDs was also sampled for cytochrome *b* and d-loop sequences. An additional Ethiopian cave colony (Sof Omar) was also sampled for cytochrome *b* and d-loop sequences. For one Durban colony (Ballito), data were available for RAPDs, cytochrome *b* and d-loop sequences, but, for logistical reasons, additional Durban colonies were sampled for cytochrome *b* (Silverglen) and d-loop sequences (Pinetown) (Fig. 2). Sample details are summarised in Table 1.

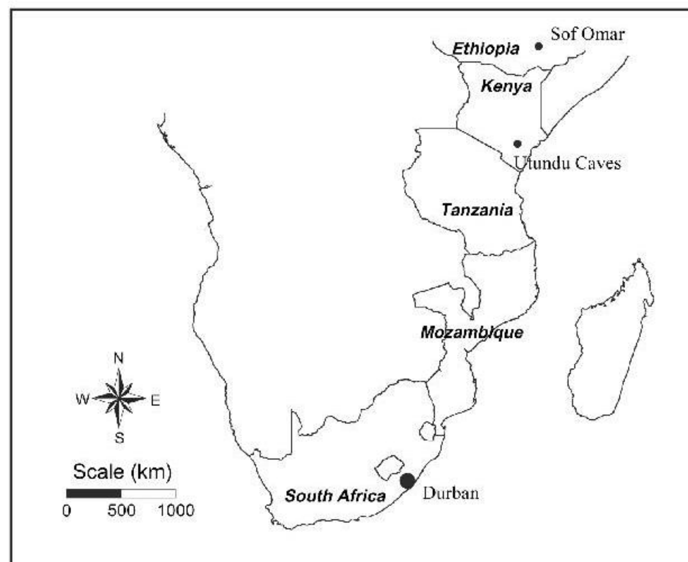

Fig. 1 Map of central and southern Africa showing the three primary sampling sites.

DNA isolation

Otomops DNA was isolated from liver, heart, kidney, or muscle tissues preserved in 80% ethanol by the CTAB method of Winnepenninckix *et al.* (1993) or using a DNeasy® DNA isolation kit (QIAGEN). DNA concentrations and purities were assessed spectrophotometrically according to Sambrook *et al.* (1989) or using a Hoefer DyNAQUANT® 2000m fluorometer (Amersham, Pharmacia).

Amplification and sequencing of the mitochondrial cytochrome b gene

The cytochrome *b* gene was PCR-amplified as two overlapping double stranded fragments (Saiki *et al.* 1988) using primer pairs: L 14723 (5'- ACC AAT GCA ATG AAA AAT CAT CGT T-3') and H 15553 (5'- TCT CCA TTT CTG GTT TAC AAG AC-3'); L15146 (5'-CAT GAG GAC AAA TAT CAT TCT GAG-3') and H15915 (5'-TCT CCA TTT CTG GTT TAC AAG AC-3') (Irwin *et al.* 1991). Amplifications were performed in 50 µl reaction volumes. Each reaction included 0.6 µl primers (50 pM μl^{-1}), 2.5 µl of DMSO, 2 µl of dNTP mixture (10mM), 5 µl of reaction buffer (X10) (Appligen) and 0.3 µl of Taq DNA polymerase (Appligen). The following thermal cycling parameters were used: 4 minutes at 94° C, 36 cycles (40 s at 94° C, 45 s at 50 °C, 40 s at 72 °C).

The d-loop region was amplified as a single fragment using primers P (S'-TCCTACCATCAGCACCCAAAGC) and F (S'-GTTGCTGGTTTCACGGAGGTAG) (Wilkinson and Chapman, 1991). Reactions were performed in 25µl reaction volumes containing 4mM $MgCl_2$, 0.1mM dNTP's, 1 unit *Taq* DNA polymersase (Promega), 2.5µl 10X Promega Buffer, 20 ng DNA and 14pmol of each primer. Target fragments were purified from excised gel bands using a MinElute® Kit (QIAGEN).

Otomops material was sequenced directly from purified PCR products using the primers used for the initial amplifications. Sequencing was carried out using the Big-Dye™ Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems) in a 10 µl reaction containing 3 µl of ready reaction mix, 7 pmol of primer and 50 – 100 ng of purified PCR fragment. The following thermal cycling parameters were used: 25 cycles of 10 s at 94° C, 5 s at 50°C, and 40 s at 72 °C. The sequencing amplification products were cleaned of unincorporated nucleotides by precipitation and resuspension of amplification products. All fragments were sequenced in both directions. Sequences were deposited in Genbank (Accession numbers: AY591532 – AY591536 and AY59137-AY5139).

Analysis of sequence data

Sequences were aligned using the CLUSTAL W option (Thompson *et al.* 1994) of the BioEdit programme (Version 5.0.9 for Windows 95/98/NT) and by visual inspection. Sequences were cropped to a common length of 1030 nt for the cytochrome b gene and 208 nt for the d-loop. For both the cytochrome *b* and d-loop regions, sequences obtained from the Molossid genera *Chaerephon pumila* and *Mops leucostigma* were used as outgroups.

The possibility of obtaining a nuclear cytochrome b pseudogene during PCR amplification was assessed using criteria for a functional mtDNA protein-coding gene (Smith *et al.* 1992; Esposti *et al.* 1993; Arctander 1995). The cytochrome *b* alignments were verified by converting sequences to 380 amino acids using the mtDNA code in MacClade (version 3, Maddison & Maddison 1992).

Table 1. Details of bats sampled in this study.

Species	Country	Colony/sample locality	Co-ordinates	n=	Technique	Source/GenBank #
Otomops martiensseni	South Africa (Durban)	Marian Rd, Ballito,	29°32'20"S; 31°13'3"E	17	RAPD	this study
		Jacqueline Rd, Ballito,	29°31'59"S; 31°12'40"E	18	RAPD	this study
				3	Cytochrome b	this study
				9	D-loop	this study
		Marine Drive, Bluff,	29°54'53"S; 31°1'26"E	15	RAPD	this study
		Casuarina Circle, Durban North	29°45'30"S; 31°2'10"E	7	RAPD	this study
		Milky Way, Amanzimtoti	30°5'9"S; 30°51'29"E	12	RAPD	this study
		Silverglen, Chatsworth Pinetown, Durban	29°55'40"S; 30°54'00"E	5	Cytochrome b	this study
				4	D-loop	this study
	Kenya	Ithundu Caves, Kiboko District	2°20'S; 37°44'E	5	RAPD	this study
				7	Cytochrome b	this study
				1	D-loop	this study
	Ethiopia	Sof Omar Caves, Bale Province	6°54'N; 40°51'E	2	Cytochrome b	this study
				2	D-loop	this study
Mops leucostigma	Madagascar	Antanimbary	17°11'S; 46°51'E	1	Cytochrome b	this study
				1	D-loop	this study
Chaerephon pumila	South Africa	Durban		1	Cytochrome b	this study
				1	D-loop	this study
	Unknown	Unknown		2	D-loop	Genbank AY347954, AY347955

* FMNH- Field Museum of Natural History; NMCR- National Museum of the Czech Republic

We estimated nucleotide divergence among genotypes with Kimura two parameter genetic distances (Kimura 1980). We used Maximum Parsimony (MP) and Neighbor-Joining (NJ; Saitou & Nei 1987) for tree reconstructions using PAUP 4.0b10 for Macintosh PPC/Altivec (Swofford 1993). We used the random additions sequence option (n=10) for discrete, unordered characters. The shortest tree was searched for with the heuristic search option using the tree bisection-reconnection (TBR) branch swapping option. The degree of character support for each node of the resulting tree was estimated by using bootstrap re-sampling analysis (Felsenstein 1985; Felsenstein & Kishino 1993; Hillis & Bull 1993). Separate bootstrap analysis was run with 1000 replicates in PAUP for phylogenetic tree reconstruction. The analysis was checked for goodness-of-fit by calculating the consistency index (CI), Retention Index (RI) and Rescaled Index (RC).

In some interspecific analyses a hierarchical tree format may be inappropriate for representing relationships among haplotypes because the period of time over which the samples have evolved is so short that ancestral and descendant haplotypes exist concurrently or because sexual reproduction or recombination can lead to reticulate relationships among haplotypes (Posada and Crandall, 2001, Kratysberg *et al.* 2004). In such instances, a haplotype network is more appropriate to illustrate relationships among the sampled haplotypes by using multiple pathways to illustrate possible recombination, homoplasy or reverse mutations. We constructed a cytochrome b haplotype network using TCS 1.21 (Clement *et al.* 2000).

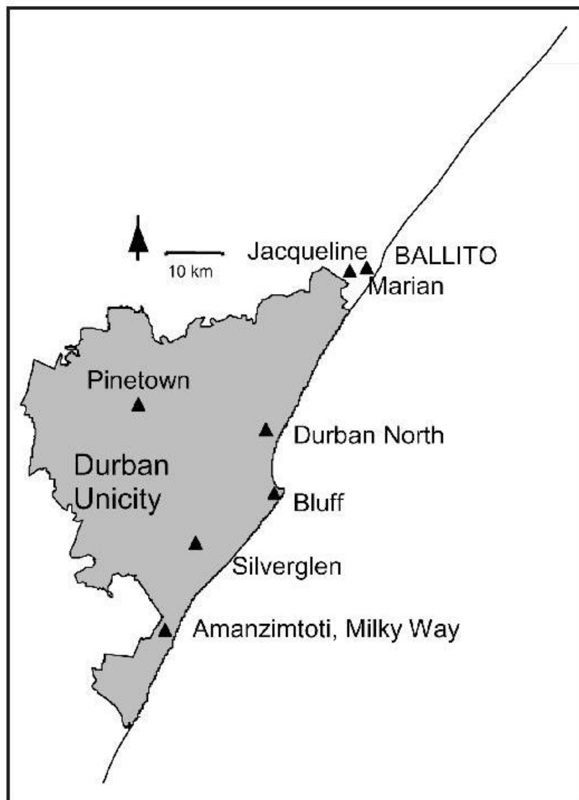

Fig. 2. Map showing colonies of Durban (South Africa) *Otomops martiensseni* sampled for PCR-RAPD analysis and mitochondrial cytochrome b and d-loop sequence analysis.

PCR-RAPDs

We optimized the concentrations of the components of the PCR-RAPD reactions prior to sample analysis as the quality of amplification is a function of template, primer, enzyme, dNTP and $MgCl_2$ concentration (Carlson *et al.* 1991; Devos & Gale 1992). In addition, we performed four separate amplifications using the same DNA samples to check for repeatability. RAPD reactions were set up in sterile 200 l thin-walled tubes by addition of 8 l of diluted DNA template (25ng) and 16 l of master mix to give a final volume of 24 l. Each reaction contained primers (Operon Technologies, Kit A, numbers 1 - 20) (0.68μM), deoxynucleoside-triphosphate mixture (10mM of each), $MgCl_2$ (4mM), 1X Stoffel reaction buffer and 1.6U of *Taq* DNA polymerase (Stoffel Fragment). We used the following thermal cycling parameters $\{94°,180s/ 39°,120s/ 72°,180s\}_1$ / $\{94°,120s/ 39°,120s/ 72°,180s\}_{40}$ /{72°,420s}. Primers from Operon Kit A were tested and chosen for inclusion in the analysis if the banding pattern was clear and the bands could be assessed across all 74 sample animals. One negative control (containing no template DNA) was performed for each set of amplifications. Amplification products were separated electrophoretically on 8% polyacrylamide gels using TBE buffer (0.89M Tris, 0.89M boric acid, 0.11M EDTA, pH 8.3) and stained using the silver staining protocol of Mitchell *et al.* (1996).

PCR-RAPD analysis

PCR-RAPD bands were classified as intense, medium or faint, based on resolution and degree of amplification (Weeden *et al.* 1992). Only bands classified as intense or medium were included in the analysis. Each band was treated as a unit character. The presence of a specific amplified DNA band was scored as '1', while the absence of such a band was scored as '0'. Pairwise similarities between all individuals were calculated using the SimQual algorithm (Similarity for Qualitative Data) and DICE option (see Nei & Li 1979) of NTSYS-pc version 1.5 (Numerical Taxonomy and Multivariate Analysis System: Rohlf 1998).

Cluster analysis of the pairwise similarities was conducted using the SAHN (Sequential, Agglomerative, Hierarchical and Nested Clustering) option of NTSYS-pc. The results were displayed as a UPGMA phenogram. The nucleotide divergence between *Otomops* populations and nucleotide diversity within each population were computed from the presence/absence data matrix using the programme RAPDDIP (Clark & Lannigan 1993). We created a triangular matrix from this output and produced a phenogram showing interpopulation relationships using NTSYS-pc version. 1.5 as described previously. We determined the goodness of fit of the phenogram to the original data from the cophenetic correlation coefficient.

We estimated the degree of cluster support for each node of the resulting UPGMA trees by using the bootstrap re-sampling analysis program RAPDDIST (Blank & Antolin 1997) and PHYLIP3.5-pc (Felsenstein 1989).

Results

Cytochrome b sequences

The unweighted Maximum Parsimony analysis (heuristic search-bisection reconnection with 10 replicates) yielded 94 variable characters (parsimony-uninformative) and 362 parsimony-informative characters. The maximum parsimony analysis resulted in 13 equally parsimonious trees, from which a single consensus tree is presented (tree length = 885, CI = 0.677; RI =0.694 and RC = 0.470, Fig. 3). The monophyletic *Otomops* clade was subdivided into two well-supported lineages (100% bootstrap support) corresponding to *Otomops martiensseni* from Kenya/Ethiopia and South Africa respectively. However, the genetic distances separating these lineages were small (about 0.01 substitutions per site) as indicated by the distance-based, Neighbour-Joining (NJ) tree (Fig. 3).

Genetic distances (Table 2) obtained under the Kimura two-parameter model of evolution (Kimura 1980) were low for *O. martiensseni* (n=17, Table 2). The mean genetic divergence between Kenyan/Ethiopian individuals was 0.57% (range 0.0 - 1.2%), 0.91% (0.0-1.9%) between South African individuals. The mean divergence between Kenyan/Ethiopian and South African populations was 2.50% (1.9-3.3%).

The haplotype network (Fig. 4) showed 19 mutational steps between the ancestral haplotypes from South Africa and northeastern Africa (22 steps between the closest South African and Kenyan individuals). Most haplotypes were unique, but three Kenyan individuals shared one haplotype, whilst two Durban individuals shared another. In general, northeastern haplotypes were more similar to each other than Durban haplotypes (three Durban haplotypes differed by eight or more steps from all others). Ethiopian individuals were very closely related (by two and four steps) to the most common Kenyan haplotype.

Table 2. Individual Pairwise Kimura 2 - parameter distance matrix for Cyt b sequences (1034 bp) of *Otomops* and outgroups.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 *Otomops* Kenya1																		
2 *Otomops* Kenya2	0.008																	
3 *Otomops* Kenya3	0.003	0.011																
4 *Otomops* Kenya4	0.007	0.012	0.008															
5 *Otomops* Kenya5	0.003	0.009	0.004	0.006														
6 *Otomops* Kenya6	0.003	0.009	0.004	0.006	0.000													
7 *Otomops* Kenya7	0.003	0.009	0.004	0.006	0.000	0.000												
8 *Otomops* Ethiopia1	0.004	0.012	0.005	0.007	0.003	0.003	0.003											
9 *Otomops* Ethiopia2	0.003	0.009	0.004	0.006	0.000	0.000	0.000	0.003										
10 *Otomops* Durban1	0.027	0.032	0.030	0.024	0.028	0.028	0.028	0.027	0.028									
11 *Otomops* Durban2	0.026	0.031	0.027	0.021	0.025	0.025	0.025	0.024	0.025	0.011								
12 *Otomops* Durban3	0.028	0.033	0.029	0.023	0.027	0.027	0.027	0.026	0.027	0.019	0.016							
13 *Otomops* Durban4	0.022	0.025	0.025	0.023	0.021	0.021	0.021	0.022	0.021	0.009	0.008	0.018						
14 *Otomops* Durban5	0.022	0.025	0.025	0.023	0.021	0.021	0.021	0.022	0.021	0.009	0.008	0.018	0.000					
15 *Otomops* Durban6	0.027	0.032	0.028	0.022	0.026	0.026	0.026	0.025	0.026	0.008	0.007	0.015	0.007	0.007				
16 *Otomops* Durban7	0.024	0.029	0.025	0.019	0.023	0.023	0.023	0.022	0.023	0.011	0.008	0.008	0.010	0.010	0.007			
17 *Otomops* Durban8	0.025	0.030	0.026	0.020	0.024	0.024	0.024	0.023	0.024	0.006	0.005	0.013	0.005	0.005	0.002	0.005		
18 *Chaerephon pumila*	0.178	0.178	0.177	0.180	0.174	0.174	0.174	0.177	0.174	0.184	0.183	0.186	0.178	0.178	0.182	0.181	0.179	
19 *Mops leucostigma*	0.178	0.180	0.179	0.179	0.179	0.179	0.179	0.180	0.179	0.177	0.177	0.180	0.175	0.175	0.176	0.175	0.174	0.130

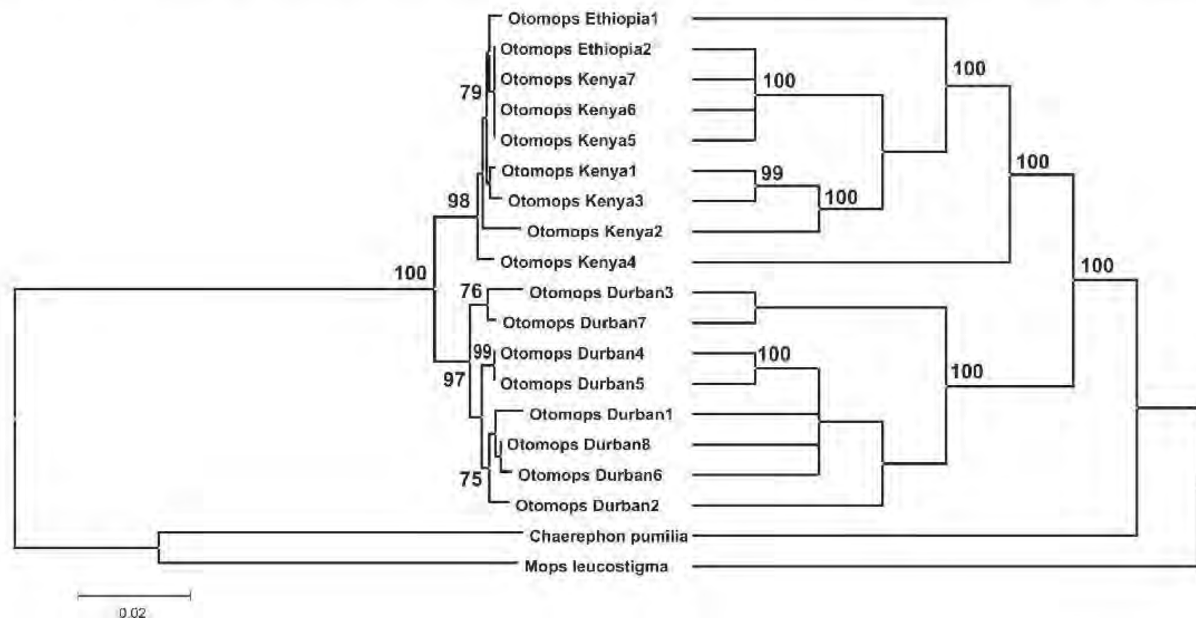

Fig. 3. Neighbour-joining (left) and Maximum parsimony (right) trees based on nucleotide substitutions in the *Otomops* mitochondrial cytochrome b gene (1034 bp): The tree was retrieved from PAUP 4.0b10 for Macintosh PPC/Altivec (Swofford, 1993). *Chaerephon pumilia* and *Mops leucostigma* were used as outgroups. Values at nodes represent bootstrap support (1000 iterations).

D-loop sequences

Neighbour-Joining and Parsimony trees of D-loop sequences (Fig. 5) revealed significant phylogeographic structure at the local (Durban region) and continental scales. Specifically, both trees revealed two well supported clades, one representing individuals from the coastal Ballito colony of Durban and the other combining Kenyan/Ethiopian and the inland Pinetown (Durban) colonies. Within the latter clade, Pinetown and Kenya/Ethiopia form two distinct and well supported sub-clades (bootstrap values of 82-100%).

PCR-RAPDs

All *Otomops* DNA samples gave a discrete high molecular weight band when subjected to agarose gel electrophoresis. The average A260/A280 ratio of the DNA was 1.68 $\pm$ 0.09. A concentration of 20 ng of DNA per reaction, 0.4M primers, 4.0 M Mg^{2+} ions, and 0.23 mM dNTPs produced consistent high-level amplification patterns. Furthermore, repeated amplifications using the same DNA samples showed very little or no variation. Hot start PCR eliminated the production of primer side products and non-specific amplification products from the negative controls, which contained no bands.

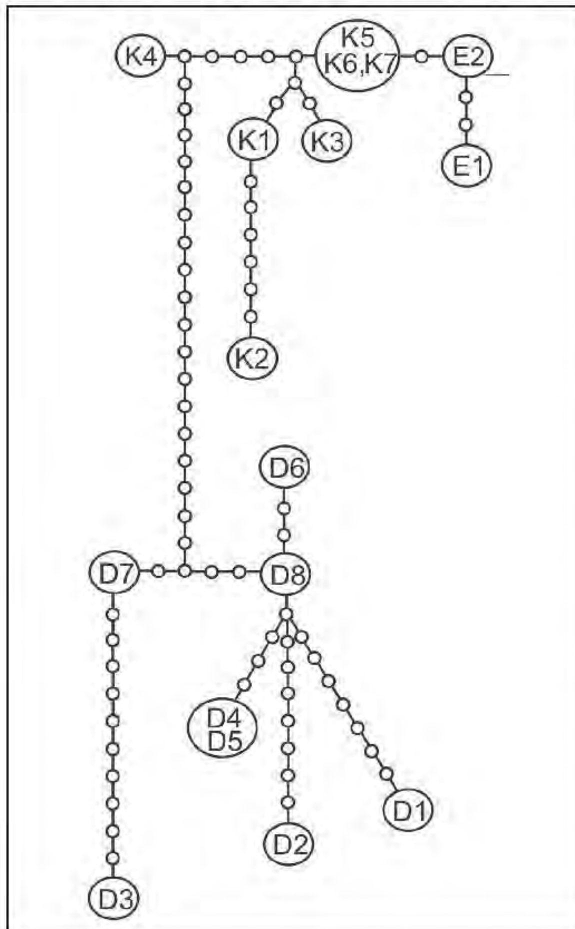

Fig. 4. Statistical parsimony network of all *Otomops* cytochrome b haploypes. Large circles represent existing haplotypes. Small circles represent ancestral or unsampled haplotypes. D – Durban, K – Kenya, E – Ethiopia.

Of 79 RAPD fragments included in the analysis, six (4.6%) were monomorphic. Twenty bands were exclusive to Kenya, and two bands were common to the South African (Durban) population. The numbers of bands exclusive to each Durban colony were as follows: Ballito, Marian Road - 2, Ballito, Jacqueline Road -1, Bluff - 2, Amanzimtoti - 3, Durban North - 3.

A phenogram generated by clustering of similarities between *Otomops* individuals (Fig. 6) showed genetic similarity ranging from 55.9% to 97.5%. Major clusters in this phenogram show mixing of individuals from different colonies, with bootstrap support at <50% for any cluster. However, *Otomops* from Kenya were more closely related to each other than those in South Africa, and always clustered together. A cluster analysis of divergences of six *Otomops* colonies (estimated using the programme RAPDDIP; Fig. 7) revealed a clear separation between the Kenya colony and the South African colonies, with an estimated nucleotide divergence of 0.92%. Three Durban colonies (Bluff, Durban North and Ballito (Jacqueline Rd.)) separated from one another with 100% bootstrap support, although the genetic distances between them were very low (divergences between 0.2% and 0.4%).

Discussion

Phylogeographic structure

Our cytochrome *b* sequencing and grouped RAPD results indicate shallow structuring of northeast African (Kenya, Ethiopia) and South African populations of *Otomops martienssseni* (2.5 % divergence for cytochrome *b* and 0.9 % for RAPDs). These divergences fall well below the arbitrary 11% cytochrome *b* divergence threshold for "good species" suggested by Bradley & Baker (2001), and are exceeded by an order of magnitude by the inter-specific divergence values for the two molossid outgroup species (17.4 – 18.6 %; Table 2). Thus, under a genetic species concept, the present cytochrome *b* data do not support the specific status of *Otomops icarus*. Nevertheless, use of genetic distances to infer taxonomic status should be tempered with caution, especially since rates of speciation may vary between and within lineages; major shifts in rates of diversification have been inferred in several mammalian orders including bats, particularly between vespertilionid and molossid lineages (Jones *et al.* 2005).

The cytochrome *b* haplotype network distinguished southern and northeastern African colonies (minimum of 22 steps between closest Durban and Kenyan individuals). Within each population, very few haplotypes were shared and sequential haplotypes were separated by multiple mutations (2-8 for northeast Africa and 5-12 for Durban). A similar pattern of unique haplotypes with multiple mutations between sequential haplotypes was also found in *T. brasiliensis* where it was attributed to very large population sizes (Russell *et al.* 2005). Unlike *O. martiensseni*, no geographic structuring between haplotypes was found in *T. brasiliensis*.

The d-loop data are congruent with the cytochrome *b* data in: 1) the genetic near-identity of Ethiopian (Sof Omar Cave) and Kenyan (Utundu Cave) individuals (from caves separated by some 1000 km), and 2) genetic separation of Kenyan/Ethiopian and Durban populations. However, d-loop trees reveal deeper complexity in that one Durban colony

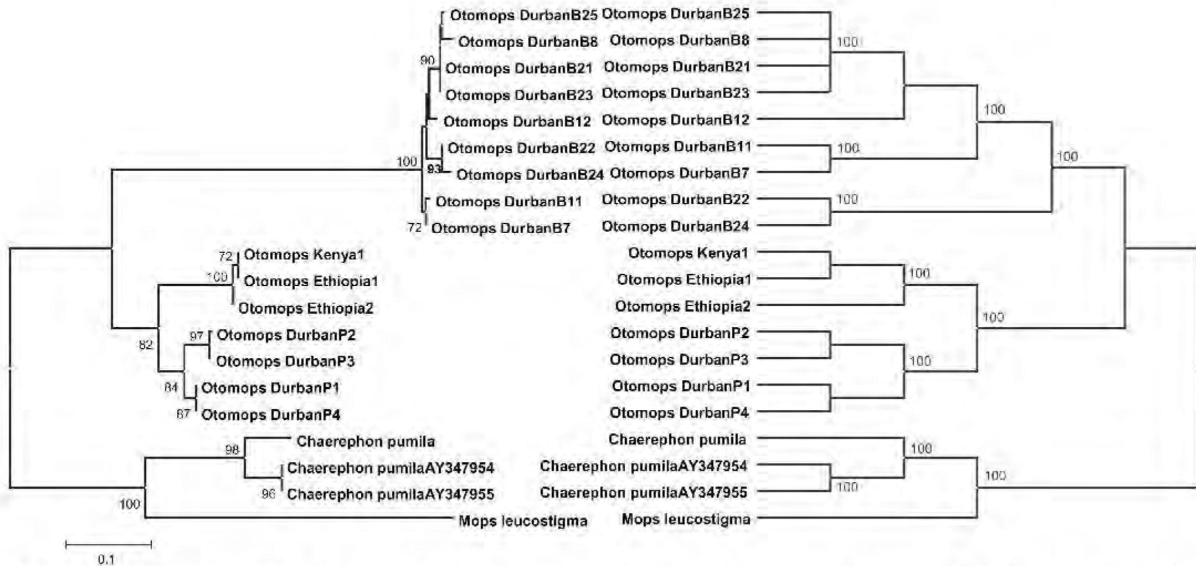

Fig. 5. Neighbour-joining (left) and Maximum parsimony (right) trees based on nucleotide substitutions in the *Otomops* mitochondrial d-loop region (208 bp). The tree was retrieved from PAUP 4.0b10 for Macintosh PPC/Altivec (Swofford, 1993). *Chaerephon pumila* and *Mops leucostigma* were used as outgroups. Values at nodes represent bootstrap support (1000 iterations). "DurbanP" = Pinetown colony; "DurbanB" = Ballito colony.

(Pinetown) shares a closer cladistic and distance relationship with Kenya/Ethiopia (separated by 3000-4000 km) than with a neighbouring colony from Ballito, Durban (separated by <30 km). Since the mitochondrial d-loop is maternally inherited, the intermediate position of the Pinetown colony suggests a relatively recent dispersal event of matriline(s) from East to South Africa (or vice versa), suggesting that the coastal Ballito colony (and possibly other Durban colonies) represents an earlier colonisation from north to south or vice versa.

Individual RAPD data also show a closer relationship between some Durban animals (mostly from the Bluff colony) and Kenyan animals, but colonies are not differentiated as is the case with the d-loop sequence analysis. Since RAPD variation reflects random mutations throughout the genome (i.e. it is bi-parentally inherited), the lack of complete RAPD differentiation between Kenyan and South African populations, in contrast to their differentiation using mitochondrial (maternally inherited) cytochrome b and d-loop sequences, may suggest wider dispersal and migration of males compared with females.

The complete d-loop differentiation of two Durban colonies separated by <30 km suggests that female philopatry may play a part in phylogeographic structure at the local level. Thus, whilst the Pinetown colony is genetically linked to Kenya/Ethiopia, and may represent a recent immigration event into South Africa, it is possible that behavioural factors such as female philopatry, or perhaps latent mate recognition, may play a part in maintaining genetic differences, at least between matrilines. Long-term fidelity of matrilines to particular males over many generations has been demonstrated in British populations of greater horseshoe bats (Rossiter *et al.* 2005).

The present genetic data, combined with scattered records of *O. martiensseni* throughout Africa and from Yemen (Fenton *et al.* 2002), suggest a taxon with a continuous distribution and/or one capable of large-scale migrations (as suggested by Kock *et al.* 2005 based on observations of seasonal and periodic cave evacuations in large Kenyan breeding colonies). Information on wing morphology and diet reflects the fact that these bats fly high and potentially long distances in search of airborne prey (mostly moths) (Rydell & Yalden 1997). They are rarely caught in nets; in spite of their relative abundance around Durban, netting effort on the ground by PJT, has produced only a single capture in mist nets set away from colonies. Extensive sampling with mist nets elsewhere in Africa (South Africa and Zimbabwe – MBF) presents a similar picture about the distribution of this bat. It is possible that this large, fast and high-flying insectivorous bat may be able to follow documented insect migrations (Cotterill & Fergusson 1999) which track movements of the Inter-Tropical Convergence Zone (ITCZ) from central to southern Africa, in much the same way as *Tadarada brasiliensis* has been documented to exploit mass migrations of breeding insects from Mexico to the southern United States (McCracken 1996). Such dynamic weather patterns may at least partly explain north-south migrations such as those implied from the d-loop data for *Otomops martiensseni* in this study.

Absence of genetic structuring at the continental geographic scale occurs in the American molossid species, the migratory *Tadarida brasiliensis* (McCracken *et al.* 1994; Russell *et al.* 2005), as well as in Australian pteropodids (*Pteropus spp.*), which also cover large distances (Webb & Tidemann 1996). In contrast, small-scale geographical genetic isolation by distance emerged from microsatellite data in *Plecotus auritus* from north-east Scotland, in an area equivalent in size to the Durban area (Burland *et al.* 1999). *P. auritus* forages by gleaning and its wings are adapted for slow, manoeuvrable flight. Movements of ringed individuals and radio-tagging confirm low vagility in this species (foraging distances of 0.5 km (females) to up to 2.8 km (males)). Similarly, extreme genetic structuring occurs in the non-migratory *Macroderma gigas*,

another species expected to have low vagility (Worthington-Wilmer *et al.* 1994). The situation in *O. martiensseni* seems to be somewhat intermediate between the two extremes, depending on the genetic marker used, with no local structuring (RAPDs, cytochrome *b*) to deep local structuring (d-loop) and shallow (RAPDs, cytochrome *b*) to deep (d-loop) structuring at the continental scale.

Social factors such as female philopatry and high variance in reproductive success of at least one sex can promote genetic structuring even in mobile species (McCracken 1987; Chesser 1991). Ecological divergence and/or female philopatry (restricted gene flow) associated with stable maternity roosts may explain regional genetic differentiation based on microsatellite and mitochondrial cytochrome *b* sequences (and morphology) in Schreiber's long-fingered bat (*Miniopterus schreibersii*) in South Africa (Miller-Butterworth *et al.* 2003). The fact that *O. martiensseni* colonies appear to represent stable harems with one reproductive male (Fenton *et al.* 2002) suggests the possibility of genetic structuring between colonies. This prediction is supported by the present d-loop data which indicate the possibility of female philopatry accounting for differentation of neighbouring (<30 km) Durban colonies. On the other hand, the absence of any obvious genetic structuring inthe biparentally inherited RAPD data, coupled with evidence for movements between roosts (Fenton et al. 2002), suggests extra-colony copulations by males and/or dispersal of juveniles that homogenize genetic variation.

DNA diversity

Historical bottlenecks, geographical isolation and/or small or fluctuating population size may theoretically, and sometimes in practice, result in the loss of genetic heterozygosity, along with other negative consequences of inbreeding in wild animal populations (Caughley & Gunn 1996). In small, isolated plains zebra *Equus quagga* herds from KwaZulu-Natal, heterozygosity declines predicted by population size and duration of isolation (from VORTEX modelling) were confirmed by RAPD but not allozyme data (Bowland *et al.* 2001). In the present study, higher RAPD and cytochrome *b* nucleotide diversity within Durban colonies (cyt b = 0.91%; RAPD = 0.54% – 2.16%, mean 1.37%) compared with the Utundu Cave colony of Kenya (cyt b = 0.55%; RAPD = 0.19%) is consistent with the localised abundance and dispersed distribution (in numerous widespread house roosts) of *O. martiensseni* in the greater Durban area and the known potential for individuals to switch roosts (Fenton *et al.* 2002). In contrast, the Utundu Cave provides the only suitable roost for a large area and supports a large colony of tens of thousands of individuals, which fluctuates dramatically, seasonally and periodically, possibly due to migration (Mutere 1973; Kock *et al.* 2005). However, caution should be exercised with the RAPD results as the Kenyan sample size is much smaller than that of the Durban colonies (Table 1). Thus, whilst levels of genetic variation in the Utundu Cave colony from Kenya are only 14% (RAPDs) to 60% (cytochrome *b*) of that found in Durban colonies, the differences are not profound enough to suggest a bottleneck event such as may have been expected with apparent population declines in Kenyan cave colonies in the past few decades (Hutson *et al.* 2001).

In the present study, while 20 of 79 RAPD bands scored were exclusive to the Kenyan population (i.e. "private alleles"), only two bands were exclusive to the South African group of colonies, and none of the five Durban colonies had more than

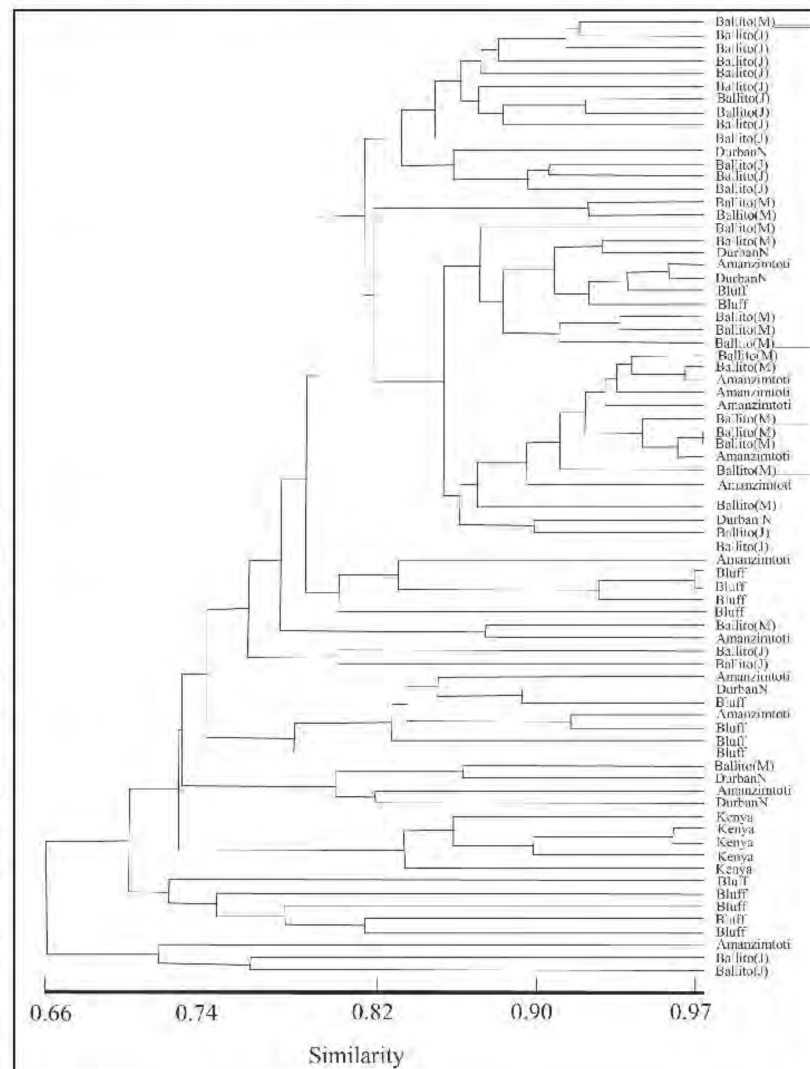

Fig. 6. Cluster analysis of genetic similarities between individual *Otomops martiensseni* from South Africa and Kenya. "Ballito(J)" = Jacqueline Road colony; "Ballito (M)" = Marian Road colony.

three exclusive bands (private alleles). The number of private alleles in a population can provide a useful estimation of levels of gene flow (Slatkin & Barton 1989). Thus, these data support our genetic diversity data in suggesting greater genetic isolation and higher levels of inbreeding in the Kenyan colony. Clearly, further studies, with larger samples and the use of additional population genetic markers such as microsatellites, are required to investigate correlations between genetic variation, migration, and historical population declines in East African populations of this species.

Utility of PCR-RAPD data

Once reproducibility has been demonstrated over a wide range of conditions (see Materials and methods), RAPD data can be used to estimate nucleotide diversity and divergence. They complement sequence data in that they sample large numbers of unlinked or loosely linked sites scattered throughout the genome. RAPD data can yield robust population estimates of genetic diversity relatively unbiased by local genomic variation, compared to estimators derived from sequence data (Borowsky 2000). The major limitation of RAPD data for population genetic analysis stems from the fact that they are dominant markers and cannot identify heterozygotes (Lynch & Milligan 1994). This limitation does not preclude the use of simple but powerful computational analyses of RAPD markers (e.g. Nei & Li 1979; Clark & Lanigan 1993; Bowland *et al.* 2001), in conjunction with independent markers, to assess genetic diversity and divergence of populations (Owuor *et al.* 1999; Li *et al.* 2001). The congruence in divergence estimates we found based on PCR-RAPDs and cytochrome *b* sequences supports our main conclusions.

Conservation implications

In the Durban area, *O. martiensseni* is one of the most common house bats, with individuals thriving in a landscape dominated by sugar cane and urban settlement (Fenton *et al.* 2002). Indeed, the ability of this bat to prosper in the face of extensive human disruptions is a good-news story for conservation (Fenton *et al.* 2002). Meanwhile, possible declines in the East African populations raise concern and justify, on a precautionary basis, the IUCN status ("Vulnerable") for the species. *Otomops martiensseni* may be yet another example of a species thriving in some parts of its range and struggling in others. While genetic differences may not underlie the observed life-history and morphological differences between Durban and Kenyan populations (although further studies with other high-resolution molecular markers may prove otherwise), clearly different regional conservation strategies are required to protect the species in different parts of its wide range in Africa.

Basic survey studies are still required to clarify the distribution and regional abundances and habitats of this species. Fortunately, the fact that this species possesses distinctive vocalizations audible to humans (Fenton *et al.* 2002, 2004) provides a potentially very useful field acoustic method for conducting surveys of the presence of the species in new locations. Further studies are also necessary to quantify accurately the level of genetic heterozygosity in different populations of *O. martiensseni* and to test the hypothesis that observed population declines in cave colonies in East Africa and elsewhere could have led to reduced heterozygosity and/or inbreeding depression. Studies employing additional molecular markers (microsatellites) and larger samples which include Madagascar and hitherto under-sampled regions of Africa, are required to address these questions as well as basic taxonomic questions.

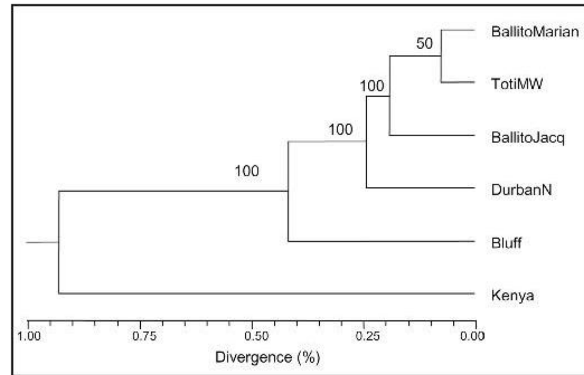

Fig. 7. Cluster analysis (COMPLETE option) of divergences between six *Otomops* colonies estimated using the programme RAPDDIP. Bootstrap values (1000 iterations) were obtained using PHYLIP. "BallitoJacq" = Jacqueline Road colony; "BallitoMarian" = Marian Road colony.

Table 3. Group-based Kimura 2 - parameter distance matrix for Cyt b sequences (1034 bp) of *Otomops* and outgroups.

	1	2	3	4
1 Durban				
2 Kenya	0.018			
3 Ethiopia	0.018	0.000		
4 *C. pumila*	0.177	0.174	0.176	
5 *M. leucostigma*	0.172	0.177	0.177	0.130

Table 4. Percent divergences between six Otomops colonies. The data are based on values from RAPD data analysed by RAPDDIP (standard errors are above the diagonal).

	Ballito (Marian)	Amanzim toti	Durban North	Bluff	Kenya	Ballito (Jacq.)
Ballito (Marian)		0.01	0.03	0.07	0.10	0.13
Amanzimtoti	0.11		0.05	0.08	0.11	0.15
Durban North	0.17	0.15		0.07	0.14	0.18
Bluff	0.41	0.34	0.40		0.09	0.14
Kenya	0.9	0.82	0.92	0.71		0.23
Ballito (Jacq.)	0.26	0.23	0.21	0.47	0.83	

Acknowledgements

Yakub Dahiye and Issa Aggundey (National Museums of Kenya) are thanked for procuring samples of *Otomops martiensseni* from Ithundu Caves, under a permit of the Kenyan Wildlife Service obtained by PJT. The Service of Molecular Systematics of the Museum National d'Histoire Naturelle, Paris (SSD), and E. Pasquet, G. Lecointre and A. Tuiller, are thanked for providing laboratory facilities for some of the cytochrome *b* sequences. We thank Enrico Bernard, Sylvie Bouchard, Karrianne DeBaeremaeker, Steve Goodman, Hannah ter Hofstede, Lydia Hollis, Joanna Lister, Angela Milne, Daniela Rambaldani, Fanja Ratrimonarivo, John Ratcliffe, Liz Reddy and Cori Wright for assisting with the collection of genetic samples. The financial support of the National Research Foundation to PJT is gratefully acknowledged, along with that of the Natural Sciences and Engineering Research Council of Canada to MBF.

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