Genetic diversity of the *Chaerephon leucogaster/pumilus* complex from mainland Africa and the western Indian Ocean islands

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ABSTRACT

Chaerephon (Dobson, 1874), an Old World genus belonging to the family Molossidae, is part of the suborder Vespertilioniformes. Members of this genus are distributed across mainland Africa (sample sites; Tanzania, Yemen, Kenya, Botswana, South Africa and Swaziland), its offshore islands (Zanzibar, Pemba and Mozambique Island), Madagascar and the surrounding western Indian Ocean islands (Anjouan, Mayotte, Moheli, Grande Comore, Aldabra and La Reunion). A multifaceted approach was used to elucidate the phylogenetic and population genetic relationships at varying levels amongst these different taxa.

Working at the subspecific level, I analysed the phylogenetics and phylogeography of *Chaerephon leucogaster* from Madagascar, based on mitochondrial cytochrome *b* and control region sequences. Cytochrome *b* genetic distances among *C. leucogaster* samples were low (maximum 0.35 %). Genetic distances between *C. leucogaster* and *C. atsinanana* ranged from 1.77 % to 2.62 %. Together, phylogenetic and distance analyses supported the classification of *C. leucogaster* as a separate species. D-loop data for *C. leucogaster* samples revealed significant but shallow phylogeographic structuring into three latitudinal groups (13º S, 15 - 17º S, 22 - 23º S) showing exclusive haplotypes which correlated with regions of suitable habitat defined by ecological niche modelling. Population genetic analysis of D-loop sequences indicated that populations from Madagascar have been expanding since 5 842 - 11 143 years BP.

At the infra-generic level, I carried out analyses of sequences of the mitochondrial cytochrome *b* gene and control region, and the nuclear RAG2 region, to resolve the evolutionary history and taxonomy of the *C. pumilus* species complex from Africa and the western Indian Ocean islands. The

nominate form comprised *C. pumilus* from Massawa, Eritrea, and this was genetically distinct from all other forms of *Chaerephon*. Our molecular evidence does not support that the syntype of *C. limbatus* and the holotypes of *C. elphicki* and *C. langi* and topotype of *C. naivashae* are specifically distinct from *C. pumilus* s.s. There is evidence of introgression of both *C. pusillus* and *C. pumilus* s.l. (south eastern Africa) mitochondrial haplotypes into *C. leucogaster*. The *C. pumilus* species complex has several attributes of a ring species, but appears to differ from this model in some important respects. It occurs on the African mainland and western Indian Ocean Islands, including Madagascar, ringing a potential barrier to gene flow, the Mozambique Channel. The taxa within the species complex form a ring in which the differentiated terminal forms, *C. pusillus* and *C. leucogaster*, occur in sympatry on Mayotte (Comoro Islands). Although there is evidence of isolation by distance around the ring, there is also a relatively high degree of genetic structure and limited gene flow. It appears that the island-based component species may have differentiated in allopatry, with some gene flow by over water dispersal, whereas the African mainland species may have differentiated through isolation by distance.

A further study was aimed at re-examining the phylogeny of *C. pumilus* sensu lato from south eastern Africa based on a considerably larger sample set with a wider geographic range; I confirmed the previously-reported phylogenetic structure, and identified an additional strongly-supported control region clade. Discriminant Function Analysis based on four echolocation parameters could not discriminate between these clades. The hypothesised existence of cryptic species with distinct echolocation characteristics was not supported. Indices of diversity and neutrality, combined with a ragged multimodal

mismatch distribution, are inconsistent with demographic expansion of a single *C. pumilus* south eastern African population and suggest that the control region lineages are stable populations at demographic equilibrium that were established during the late Pleistocene between 60 000 and 13 000 years ago.

Further, more variable markers (microsatellites) were employed for finer-scale resolution of population genetic structure among the five genetic lineages of *C. pumilus* sensu lato found in the Durban area of KwaZulu-Natal, and to search for hybridization between these lineages. We recovered strong mitochondrial genetic structure, with 90% of the molecular variance occurring among four phylogenetically-defined groups, and a high significant Fst (0.897). Microsatellite data recovered three admixed populations with 3% of the nuclear variance occurring among populations, and global (Fst=0.037) and pairwise Fst values among populations were low and not significant. This is indicative of little genetic structure among the groups of *C. pumilus* s.l., which appear to comprise a single interbreeding population. Such high levels of mitochondrial genetic structure in the absence of significant nuclear structure are consistent with social isolation mechanisms such as female philopatry, and may reflect introgression of mitochondrial genes due to past hybridisation events with mitochondrially-distinct forms from outside the sampled area.

PREFACE

The experimental work described in this thesis was carried out at the School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban. This was carried out under the supervision of Prof. Jenny M. Lamb, Dr. M.C. Schoeman, Dr. Peter J. Taylor and Dr. Steven M. Goodman.

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

Signed: _____ **Name: Miss. T. Naidoo** **Date:**

DECLARATION 1 – PLAGARISM

I, Theshnie Naidoo 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.
    (b) Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

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 section.

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DECLARATION 2 – PUBLICATIONS

PLEASE NOTE: CHAPTER 1 OF THIS THESIS COMPRISES MY MSC THESIS. IT WAS EXAMINED AND PASSED IN 2008.

Publication 1 (Chapter 2)

NAIDOO, T., M. CORRIE. SCHOEMAN, PETER .J. TAYLOR, STEVEN. M. GOODMAN, AND JENNIFER. M. LAMB. 2013. Stable Pleistocene-era populations of *Chaerephon pumilus* (Chiroptera: Molossidae) in south eastern Africa do not use different echolocation calls. African Zoology, 48: 125-142.

The ideas were conceived by Naidoo T, Lamb J and Schoeman M. The data were collected and analysed by Naidoo T. The writing was led by Naidoo T.

Publication 2 (Chapter 3)

NAIDOO, T., M. CORRIE. SCHOEMAN, STEVEN. M. GOODMAN, PETER .J. TAYLOR, AND JENNIFER. M. LAMB. Discordance between mitochondrial and nuclear genetic structure in the bat, *Chaerephon pumilus* s. l. (Chiroptera: Molossidae) from South Africa

Submitted to *Biological Journal of the Linnean Society*

The ideas were conceived by Naidoo T, Lamb J and Schoeman M. The data were collected and analysed by Naidoo T. The writing was led by Naidoo T.

Publication 3 (Chapter 4)

NAIDOO, T., STEVEN. M. GOODMAN, M. CORRIE. SCHOEMAN, PETER .J. TAYLOR, AND JENNIFER. M. LAMB. The *Chaerephon pumilus* species complex (Chiroptera: Molossidae) from south eastern Africa and the western Indian Ocean islands is not a classical ring species.

Submitted to *Acta Chiropterologica*

The ideas were conceived by Naidoo T, Lamb J and Goodman S. The data were collected by Goodman S, Taylor P, Schoeman M and Naidoo T. The data were analysed by Naidoo T. The writing was led by Naidoo T.

Publication 4

RATRIMOMANARIVO, F.H., S.M. GOODMAN, W.T. STANLEY, T. NAIDOO, P.J. TAYLOR, and J. LAMB. 2009. Patterns of geographic and phylogeographic variation in *Chaerephon leucogaster* (Chiroptera: Molossidae) of Madagascar and the western Indian Ocean islands of Mayotte and Pemba. Acta Chiropterologica, 11: 25-52.

This is multi-authored report on a collaborative project involving morphometric and genetic assessments of phylogenetic and phylogeographic relationships in *Chaerephon leucogaster*.

Sample collection and the morphological work, which comprises half of the paper, was carried out by Ratrimomanarivo, F.H., Goodman, S.M. and Stanley, W.T.

The molecular work, which comprises the other half of the paper, was carried out by Naidoo, T. who was supervised by Lamb, J., Taylor, P.J and Goodman, S.M.

Publication 5

TAYLOR, P.J., J. LAMB, D. REDDY, T. NAIDOO, F. RATRIMOMANARIVO, E. RICHARDSON, and S.M. GOODMAN. 2009. Cryptic lineages of little free-tailed bats, *Chaerephon pumilus* (Chiroptera: Molossidae) from southern Africa and the western Indian Ocean islands. African Zoology, 44: 55-70.

This work was carried out and formed the basis of the MSc thesis of Devendran Reddy. Further sample collection, morphological work and the molecular work on *Chaerephon pumilus* was carried out by Taylor, P.J and Reddy, D.

The molecular work and analysis on *Chaerephon leucogaster* was carried out by Naidoo, T. who was supervised by Lamb, J., Taylor, P.J and Goodman, S.M.

Publication 6

LAMB, J.M., T.M.C. RALPH, T. NAIDOO, P.J. TAYLOR, F. RATRIMOMANARIVO, W.T. STANLEY, and S.M. GOODMAN. 2011. Toward a molecular phylogeny for the Molossidae (Chiroptera) of the Afro-Malagasy region. Acta Chiropterologica, 13: 1-16.

Molecular work of all *Chaerephon* samples together with a few other molossids was carried out by Naidoo, T.

Publication 7

GOODMAN, S.M., W. BUCCAS, T. NAIDOO, F. RATRIMOMANARIVO, P.J. TAYLOR, and J.M. LAMB. 2010. 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. Zootaxa, 2551: 1-36.

Molecular work for all *Chaerephon* samples was carried out by Naidoo, T supervised by Lamb, J.M.

Sample collection and the morphological work, which comprises half of the paper, was carried out by Ratrimomanarivo, F.H., Goodman, S.M.

Publication 8

LAMB, J.M., T. NAIDOO, P.J. TAYLOR, M. NAPIER, F. RATRIMOMANARIVO, and S.M. GOODMAN. 2012. Genetically and geographically isolated lineages of a tropical bat (Chiroptera: Molossidae) show demographic stability over the late Pleistocene. Biological Journal of the Linnean Society, 106: 18-40.

Molecular work was carried out by Naidoo, T and Napier, M. supervised by Lamb, J.M.

Publication 9

NAIDOO, T., A. MCDONALD, and J. LAMB. 2013. Cross-genus amplification and characterisation of microsatellite loci in the little free tailed bat, *Chaerephon pumilus* s.l. (Molossidae) from South Eastern Africa. *African* Journal of Biotechnology, 12: 3143-3147.

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

Signed:_____

“It is interesting to contemplate a tangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent upon each other in so complex a manner, have all been produced by laws acting around us. . .

There is grandeur in this view of life, with its several powers, having been originally breathed by the Creator into a few forms or into one; and that, whilst this planet has gone circling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved.”

- *Charles Darwin*

ACKNOWLEDGEMENTS

I am extremely indebted to my supervisor, Prof. Jenny Lamb. Thank you for your time, support and guidance in shaping me as a scientist. You have been a true inspiration with your wealth of knowledge and patience throughout the years.

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My sincere appreciation to the National Research Foundation and the University of KwaZulu Natal for their financial assistance over these years.

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My achievements thus far are a heartfelt gift of thanks to my family: my grandmother (the matriarch of the family), my parents (Pinky and Danny) and my brother and sister in law (Preshen and Raksha) for their love and unwavering support during these years. Mum, Dad thank you for letting me choose a very uncertain path in life, something I sincerely loved, and for providing me with an education.

To husband, Indhrasen, thank you for the unconditional love and support during this journey and thank you for being a part of it. You mean more to me than words can ever describe....

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LIST OF ABBREVIATIONS

AMOVA	Analysis of Molecular Variance
AUC	Area under curve
bp	base-pair/s
BP	before present
˚C	degrees Celsius
dNTPs	deoxynucleoside triphosphates
ESU	evolutionary significant unit
g	grams
GTR	general time reversible
Iss	index of substitution saturation
Iss.c	critical value of index of substitution saturation
mg	milligram
ml	millilitre
mM	millimolar
MP	maximum parsimony
mtDNA	mitochondrial DNA
myr	million years
ng ml^{-1}	nanogram per millilitre
ng ul^{-1}	nanogram per microlitre
ng	nanogram
NJ	neighbour-joining
PCR	polymerase chain reaction
Taq	*Thermus aquaticus*
V	volts
w/v	weight per volume
µM	micromolar
µl	microlitre

GENERAL INTRODUCTION

The measure of biodiversity is typically based on the fundamental and central unit of species (O'Brien 1994; Stearns and Hoekstra 2000), which is essential to future conservation efforts. Understanding biodiversity, more specifically genetic diversity, and how it is distributed is central to the resolution of taxonomic uncertainties surrounding species complexes (Cooper *et al.* 1998; Cardinal and Christidis 2000), including the identification of genetically distinct populations. Additionally, these measures have important conservation management implications.

Bats belong to the order Chiroptera and are one of the most diverse mammalian groups. Huge strides have been made recently in our understanding of chiropteran systematics even though gaps remain. Previous studies based on morphological characters suggested that the Order Chiroptera is comprised of two suborders: Megachiroptera and Microchiroptera (Simmons 1995; Simmons and Geisler 1998). In contrast, Megachiroptera was suggested to be more closely related to primates than to microchiroptera (Smith and Madkour 1980; Pettigrew 1986, Pettigrew *et al.* 1989; Pettigrew and Kirsch 1995) thus challenging the monophyletic status of bats (Smith and Madkour 1980; Pettigrew *et al*. 1989; Goodman 1991; reviewed in Simmons 1994). Over the past decade, molecular analyses strongly supported the monophyly of bats (Miyamoto *et al.* 2000; Teeling *et al.* 2000; Jones *et al.* 2002; Eick *et al.* 2005). However, the monophyly of groups that are part of the suborder Microchiroptera has also been controversial. Some authors suggest based on morphological analyses that Microchiroptera have a common ancestor with all extant taxa and have the defining feature of a complex laryngeal echolocation system (Simmons 1998; Simmons and Geisler 1998) and that and Megachiroptera comprise all other non-echolocating bats. Recent molecular studies confirmed microbat paraphyly using multiple nuclear gene sequences (Teeling *et al*. 2000, 2002, 2005, Springer *et al*. 2001). Monophyletic Chiroptera was systematically divided into Yinpterochiroptera (Springer *et al*. 2001), grouping megabats and Rhinolophoidea and Yangochiroptera (Simmons and Geisler 1998), containing all other microbats.

However, under the new proposed nomenclature Chiroptera is divided into two sub-orders, Pteropodiformes (formerly referred to as Yinpterochiroptera) and Vespertilioniformes (formerly referred to as Yangochiroptera) (Hutcheon and Kirsch 2006). Pteropodiformes consists of the families Pteropodidae, Hipposideridae, Rhinolophidae, Megadermatidae and Craesonycteridae

(Hutcheon and Kirsch 2006). Vespertilioniformes consists of the remaining bat families and includes the families Vespertilionidae, Miniopteridae, Molossidae, Emballonuridae, and Nycteridae (Hutcheon and Kirsch 2006). The family Molossidae Gervais, 1856, comprises 16 genera (Simmons 2005); seven Old World genera (*Mops*, *Chaerephon*, *Platymops*, *Sauromys*, *Cheiromeles*, *Otomops*, and *Myopterus*), seven New World genera (*Promops*, *Molossus*, *Eumops*, *Nyctinomops*, *Molossops*, *Cynomops*, and *Tomopeas*), and two genera with members found in both the Old World and New World (*Mormopterus* and *Tadarida*). These genera comprise about 100 species. The species delimitations of African, Arabian Peninsula and western Indian Ocean island members of *Chaerephon pumilus* (Cretzschmar, 1830-1831), a molossid species-complex, remain largely unresolved. Most species within this genus have been defined by traditional morphological approaches. However, genetic markers provide an important tool for unravelling phylogenetic and phylogeographic patterns in species complexes such as *C. pumilus* (Taylor 1999; Aspetsberger *et al*. 2003; Fenton *et al*. 2004; Jacobs *et al*. 2004; Bickford *et al*. 2006; Pfenninger and Schwenk 2007; Taylor *et al*. 2009; Goodman *et al*. 2010). Molecular approaches have been successfully applied to the resolution of other taxonomically complicated bat groups occurring on western Indian Ocean islands (e.g., Ratrimomanarivo *et al*. 2008, 2009a; Weyeneth *et al*. 2008; Goodman *et al*. 2009a) and other regions (Campbell et *al*. 2004; Juste *et al*. 2004; Miller-Butterworth *et al*. 2005; Ibanez *et al*. 2006). The discovery of cryptic species has also shown to have profound implications on biodiversity (Bickford *et al*. 2006). Despite an exponential increase in discovery of cryptic species over the past few decades, there is still uncertainty on the number of cryptic species that remain un-described; for this reason, efforts to catalogue and explain biodiversity need to be prioritized (Pfenninger and Schwenk 2007).

My thesis encompasses a multifaceted approach to elucidate phylogenetic and population genetic relationships amongst members of the *C. pumilus* species complex from eastern and southern Africa, Arabia, Madagascar and western Indian Ocean islands including the Comoros Archipelago (Grande Comore, Mohéli, Mayotte, Anjouan), Seychelles (Aldabra), Pemba, Zanzibar and Mozambique Island. A further aim was to assess the phylogenetic position of *Chaerephon* within the family Molossidae.

The *Chaerephon pumilus* species complex and *C. pumilus sensu lato*

The taxonomic arrangement of the genus *Chaerephon* currently includes 21 species, based on both morphological and genetic data. Simmons (2005) recognised 18 species and recent studies described an additional three species (Goodman and Cardiff 2004; Goodman *et al*. 2010). Six species (*C. major*, *C. chapini*, *C. pumilus*, *C. ansorgei*, *C. nigeriae* and *C. bivittatus*) occur in the southern African subregion (Monadjem *et al*. 2010) and four (*C. atsinanana*, *C. leucogaster*, *C. pusillus* and *C. jobimena*) across Madagascar and on some western Indian Ocean islands. On the African mainland, there are a number of geographically diverse forms of *Chaerephon* that are grouped in a single species complex, *C. pumilus.* Animals placed in the genus *Chaerephon* found in western portions of Madagascar were assigned to *C. leucogaster* (Ratrimomanarivo *et al*. 2009b), and a recent study by Goodman *et al*. (2010) referred forms occurring in the eastern portion of the island and previously identified as *C. pumilus* to new species, *C. atsinanana* (Appendix 4). Further, Goodman *et al*. (2010) assigned *C. pumilus* from western Seychelles and Comoros Archipelago to *C. pusillus*. Goodman *et al*. (2010) obtained DNA from a 120 year old specimen of the nominate form, *C. p. pumilus*, from the type locality (Massawa, Eritrea) and, based on a portion of the mitochondrial cytochrome *b* gene, defined this individual as the nominate *pumilus* (Appendix 4). By implication, all other genetically distinct forms which bore the name '*C. pumilus*' are referred to herein as *C. pumilus sensu lato*. This includes *C. leucogaster*, which has been shown by Taylor *et al*. (2009) to be included in a paraphyletic *C. pumilus sensu lato* clade from south eastern Africa.

ORGANISATION AND STRUCTURE

Sub-specific level studies

Many molecular studies of the interrelationships among genera and species have made use of mitochondrial DNA. Over the last two decades, the cytochrome *b* gene has widely used molecular marker (Bradley and Baker 2001; Avise 2004; Baker and Bradley 2006), useful in elucidating relationships at various systematic levels (Porter and Baker 2004). For a number of bat families, this marker has been used at intra-generic level to define species, for example within the genera of the following families: Molossidae (Baker *et al*. 2009), Phyllostomidae (Van de Bussche and Baker 1993; Hoffman and Baker 2001; Porter and Baker 2004; Larsen *et al*. 2007), Vespertilionidae

(Hulva *et al*. 2004; Rodriguez and Ammerman 2004), Miniopteridae (Tian *et al*. 2004; Goodman *et al*. 2009b) and Rhinolophidae (Li *et al*. 2006).

My work has been undertaken within the context of a research group including Prof. P. J. Taylor, Dr. C. Schoeman, Dr. S. Goodman and Prof. J. Lamb, in a molecular lab at the School of Life Sciences at the University of KwaZulu-Natal. This group is engaged in a series of ongoing phylogenetic and phylogeographic studies on molossid bat taxa, primarily from Madagascar and western Indian Ocean, but also including congeners from mainland Africa. These studies, based on mitochondrial DNA markers, focused on several different taxa, namely: *C. leucogaster* (Ratrimomanarivo *et al*. 2009b); the *C. pumilus* 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; *Otomops madagascarensis* Dorst, 1953 and *O. martiensseni* Matchie, 1897 (Lamb *et al*. 2006, 2008).

My MSc thesis focused on the genetic diversity of *C. leucogaster* from Madagascar and the western Indian Ocean islands of Mayotte and Pemba from a phylogenetic and phylogeographic perspective. It was examined and passed in 2008, and I took the option of upgrading my MSc to a PhD. This meant that I would not graduate with a MSc, but would incorporate the MSc thesis into my PhD thesis; hence, Chapter 1 of this thesis comprises my MSc thesis. This work was published jointly with the morphological data of Fanja Ratrimomanarivo and is included as Appendix 1 (Ratrimomanarivo *et al*. 2009).

Devendran Reddy, a fellow student at the University of KwaZulu Natal, carried out a study in a similar vein focusing on *C. pumilus* from southern Africa, referred to in this thesis as *C. pumilus sensu lato*, because subsequent work (Goodman *et al.* 2010) has shown that *C. pumilus sensu stricto* refers to a genetically distinct form from the type locality of Massawa in Eritrea. Work carried out by Devendran Reddy reported genetically distinct lineages within *C. pumilus sensu lato* from south eastern Africa and neighbouring Swaziland. Analyses of the mitochondrial cytochrome *b* and control regions revealed at least four clades from the greater Durban (South Africa) area, separated by inter-clade cytochrome *b* genetic distances of 0.6 - 0.9%. Taylor *et al*. (2009) extended this study with a greater sample set, which included *C. leucogaster* samples from western Madagascar, and hypothesised that the southern African *C. pumilus* clades may represent cryptic species with distinct echolocation characteristics (Appendix 2).

The examination of molecular data has demonstrated that many morphologically cohesive species harbour genetically distinct cryptic species (Mayr 1996; Lincoln *et al*. 1998; Pfenninger and

Schwenk 2007). Molecular phylogenetic techniques have proven to be a powerful tool in revealing cryptic forms across different biological groups (Birungi and Arctander 2000; Omland *et al*. 2000; Peppers and Bradley 2000; Olson *et al*. 2004; Ravaoarimanana *et al*. 2004; Vences and Glaw 2005; Yoder *et al*. 2005; Brambilla *et al*. 2008; Brown *et al*. 2007; Pfenninger and Schwenk 2007; Towes and Irwin 2008). Bioacoustic information combined with genetic data has been widely used to provide additional insight into taxonomic delimitations in bat species complexes (Russo and Jones 2000; Rydell *et al*. 2002; Kingston and Rossiter 2004; Thabah *et al*. 2006; Ramasindrazana *et al*. 2011).

Using bioacoustic and genetic information, I extended the study by Taylor *et al*. (2009) with an increased sample size and expanded geographic representation to test the hypothesis that the south eastern African *C. pumilus sensu lato* clades represent cryptic species with distinct echolocation characteristics. This work, which focuses on the molecular ecology (genetic diversity and echolocation characteristics) of a taxon within the *C. pumilus* species complex, forms the second chapter of this thesis.

Population genetic structure of *Chaerephon pumilus sensu lato* from South Africa

Environmental barriers, historical demographic processes and life histories have shaped the genetic structure of populations (Castella *et al*. 2000; Donnelly and Townson 2000; Gerlach and Musolf 2000; Palsson 2000; Tiedmann *et al*. 2000; Burland *et al*. 2001; Balloux and Lougon-Moulin 2002, Salgueiro *et al*. 2008; Bilgin *et al*. 2008; Chinnasamy *et al*. 2011; Dixon 2011). In addition to mitochondrial marker systems, recent research has demonstrated the usefulness at population level of nuclear marker systems, the most conventional being microsatellites. Microsatellites have grown in popularity because these are single-locus, co-dominant markers which are useful for the description and understanding of the social life (e.g. parentage analysis, mating systems, roosting biology and sex-biased dispersal) and population structure in bats (Castella *et al*. 2000; Burland *et al*. 2001; Castella *et al*. 2001; Nagy *et al*. 2007; Salgueiro *et al*. 2008; Campbell *et al*. 2009; Durrant *et al*. 2009; Flanders *et al*. 2009; Chen *et al*. 2010; Hua *et al*. 2011).

The focus of Chapter 3 of this dissertation is the microsatellite analysis of the structure of *C. pumilus sensu lato* populations from South Africa. The aim of this work was to search for finer-scale resolution among the genetic lineages found in the Durban area of KwaZulu-Natal (reported

on in Chapter 1), and to search for hybridization between these lineages. This work had a methodological aspect, as I tested nine primer pairs used by Russell *et al*. (2005) on the genus *Tadarida* for cross-amplification and variability within *C. pumilus sensu lato*.

Position of *Chaerephon* within the Molossidae

Earlier taxonomic studies on the family Molossidae were based on characteristics such as dental and cranial morphology (Freeman 1981; Legendre 1984; Taylor 1999) and a molecular phylogeny on the family was lacking. Ongoing studies on different Afro-Malagasy molossid taxa led to the investigation on the molecular phylogeny of the family for the region (Lamb *et al*. 2011). Mitochondrial DNA markers (such as the cytochrome *b*) evolve too rapidly to provide adequate resolution at deeper nodes, owing to problems with homoplasy (Guillén *et al*. 2003), whereas more slowly-evolving nuclear markers are suitable for resolution at higher taxonomic levels, for example among genera within a family (Lovejoy and Collette 2001; Springer *et al*. 2001; Steppan *et al*. 2004, Rubinoff and Holland 2005). Analyzing single-gene data sets problems arise due to gene trees and species trees which may be conflicting. Species trees reflect the evolutionary history of a group, whereas gene trees reflect the diversification of a group of sequences derived from different gene fragments. Unlike species trees, gene trees are sensitive to the effects of gene duplication, hybridization, introgression, lineage sorting and female biased disperal (Moritz & Hillis 1996; Lyons-Weiler & Milinkovitch 1997), but the congruence of multiple independently segregating markers may provide a more reliable estimate of the species tree (Pamilo & Nei 1988).

Therefore, it is important to use multiple sources of phylogenetic information to overcome the limited ability of a single dataset to reconstruct molecular phylogenies with accuracy (Cao *et al*. 1994; Cummings *et al*. 1995). Thus, phylogenies incorporating mitochondrial cytochrome *b* and nuclear DNA could provide valuable insight into the evolutionary relationships of/among different taxa. Molecular phylogenetic approaches using multiple genes were used to elucidate the evolutionary history of a number of bat families: Vespertilionidae (Hoofer and Van Den Bussche 2003; Roehrs *et al*. 2010), Phyllostomidae (Baker *et al*. 2003), Emballonuridae (Lim *et al*. 2008), Pteropodidae (Colgan and da Costa 2002), Natalidae (Da´valos 2005), and Mormoopidae (Lewis-Oritt *et al*. 2001).

The taxonomic relationship among three genera within the family Molossidae namely, *Mops*, *Chaerephon* and *Tadarida* has been an issue of contention due to their polyphyletic association (Arroyo-Calabres *et al*. 2002; Monadjem *et al*. 2010). Freeman (1981) divided certain African members formerly placed in the genus *Tadarida* into four genera (*Mormopterus, Tadarida*, *Chaerephon* and *Mops*). Peterson *et al*. (1995) regarded *Chaerephon* as a subgenus of *Tadarida* based on morphological characters that are shared by a few species of *Chaerephon* and *Mops*. Simmons (2005) has adopted the generic classification proposed by Freeman (1981), whereby *Chaerephon* is given generic status.

I sequenced the nuclear RAG2 gene of all regional *Chaerephon* taxa from Africa and the western Indian Ocean islands and contributed to a paper in which these sequences were used, together with the mitochondrial cytochrome *b* gene (my *C.leucogaster* cytochrome *b* sequences were included here), to resolve phylogenetic relationships within the *Chaerephon/Mops* grouping of the Molossidae family (Lamb *et al*. 2011) (Appendix 3). We found no variation in RAG2 sequences between the different *Chaerephon* taxa. This study revealed that *Chaerephon* and *Mops* were not clearly distinct genera. *Chaerephon* and *Mops* combined formed a very strongly-supported monophyletic group (1.00 pp and ~100% bootstrap support) in both the RAG2 and concatenated RAG2/cytochrome *b* analyses. This group included all *Chaerephon* samples except *C. jobimena*, which was nested within *Tadarida*. Within the *Chaerephon*/*Mops* clade, it appears that *Mops* maintains the more ancestral position relative to the more derived *Chaerephon* samples. These results were further supported by a study subsequently published by Ammerman *et al*. (2012).

A phylogeny for *Chaerephon pumilus sensu lato*

Chapter 4 of this thesis is a more in depth look at the phylogenetic structure of *C. pumilus sensu lato* by extending the study of Goodman *et al*. (2010) using broader geographic and taxonomic sampling to include specimens from eastern and southern Africa (Appendix 4). I also attempt to resolve the taxonomy of *C. elphicki*, *C. langi*, *C. limbata* and *C. naivashae*, currently considered synonyms of African *C. pumilus sensu stricto*.

AIMS OF THIS STUDY

The overall aim of this study is to resolve phylogenetic and population genetic relationships within the *Chaerephon pumilus* species complex, and the position of *Chaerephon* within the Molossidae.

Specific aims were as follows (objectives are given within the chapters):

(i) Investigate the genetic diversity and phylogeography of *Chaerephon leucogaster* populations from Madagascar and the islands of Mayotte and Pemba in the western Indian Ocean (Chapter 1).

(ii) Revisit the study of Taylor *et al.* (2009) on the phylogeny on *C. pumilus sensu lato* in south eastern Africa by increasing the sample size and expanding the geographic sampling to include a wider area of southern and northern KwaZulu-Natal. Further, to test their hypothesis that the southern African *C. pumilus sensu lato* clades represent cryptic species with distinct echolocation characteristics (Chapter 2).

(iii) Optimise the microsatellite markers used by Russell *et al*. (2005) in a study of population structure in New World *Tadarida brasiliensis* for cross-amplification and variability in *C. pumilus sensu lato*. Use the applicable markers for fine-scale resolution of relationships among the genetic lineages of *C. pumilus sensu lato* found in South Africa (established in Chapter 2), and to search for possible hybridization between these lineages (Chapter 3).

(iv) Establish an inclusive phylogeny for *C. pumilus sensu lato* from mainland Africa, Arabia, Madagascar and western Indian Ocean islands by extending the mitochondrial cytochrome *b* and D-loop dataset of Goodman *et al*. (2010) to include samples from Mozambique and Zanzibar and further samples from the Comoros Archipelago, Pemba Island and southeastern Africa. To include museum specimens of *C. limbata*, *C. langi*,

C. naivashae and *C. elphicki* which are considered synonyms of *C. pumilus* in the current systematic arrangement and comment on the validity of these species designations (Chapter 4).

(v) To assess the position of *Chaerephon* within the Molossidae using nuclear RAG2 and mitochondrial cytochrome *b* sequence analysis (Appendix 3).

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CHAPTER ONE

Analysis of the Genetic Diversity of *Chaerephon leucogaster* (Chiroptera: Molossidae) of Madagascar and from the western Indian Ocean islands of Mayotte and Pemba

ABSTRACT

Chaerephon leucogaster, Grandidier's free-tailed bat, belongs to the family Molossidae. *Chaerephon leucogaster* was regarded as synonymous with *C. pumilus*. Current taxonomic classification ranks *C. leucogaster* and *C. pumilus* as separate species. This study was conducted to provide information on the genetic diversity of *C. leucogaster* and to resolve issues concerning its taxonomic classification.

DNA sequencing of the mitochondrial cytochrome *b* (863 nucleotides, n = 39) and D-loop (338 nucleotides, n = 71) regions was used to estimate the genetic diversity of *C. leucogaster* individuals from Madagascar, Mayotte (Comoros Archipelago) and Pemba (an off-shore island of Tanzania). *Chaerephon leucogaster* from these islands formed a monophyletic clade with respect to the outgroups (*Mops leucostigma* and *Mops midas*). This was supported by congruent results from Bayesian (posterior probability 1.00), maximum parsimony (bootstrap 99 %) and neighbor-joining (bootstrap 100 %) analyses. *Chaerephon pumilus* (Madagascar) formed a sister taxon to the *C. leucogaster* clade (bootstrap 98 %; posterior probability 0.95).

Cytochrome *b* genetic distances among *C. leucogaster* samples were low (maximum 0.35 %). Genetic distances between *C. leucogaster* and *C. pumilus* ranged from 1.77 % to 2.62 %. Together, phylogenetic and distance analyses supported the classification of *C. leucogaster* and *C. pumilus* (Madagascar) as separate species.

For cytochrome *b* and the D-loop, haplotype diversity (*h*) was high, whilst nucleotide diversity (π) was low (cytochrome *b* - 0.718, 0.0011; D-loop - 0.870, 0.00737). D-loop data for Malagasy samples revealed significant but shallow phylogeographic structuring into three latitudinal groups (13º S, 15 - 17º S, 22 - 23º S) showing exclusive haplotypes which correlated with regions of suitable habitat defined by ecological niche modelling. Analysis of Molecular Variance indicated that 40.44 % of the variance occurred among the three groups.

The Mozambique Channel is suggested to be an insignificant barrier to gene flow. Africa is considered to be the origin of dispersal for *C. leucogaster* populations on Madagascar, Mayotte and Pemba. Population genetic analysis of D-loop sequences indicated that populations from Madagascar have been expanding since 5 842 - 11 143 years BP.

1. INTRODUCTION

1.1 BACKGROUND INFORMATION ON *CHAEREPHON*

1.1.1 THE FAMILY MOLOSSIDAE

The family name Molossidae is derived from the Greek term “molossos” for an ancient Greek wolf-dog (Rosevear, 1965). Bats belonging to this family have acquired this name because of the mastiff appearance of the face and head. Other characteristic features are the exceptionally large ears of some species and the tail, which projects well beyond the interfemoral membrane – hence the term free-tailed bat (Rosevear, 1965).

Molossidae are widely distributed in the warmer parts of the world. According to Hill and Smith (1984), members of this family are strong and fast-flying bats which feed largely on insects and other invertebrates. Molossids frequently inhabit human dwellings, outbuildings, caves, tunnels, rocks and hollow trees (Hill and Smith, 1984). These bats are gregarious or semi-gregarious and often live in large colonies. Males and females occasionally live as separate groups.

Molossidae were considered a sub-group of Vespertilionidae or Emballonuridae, until established as a separate family by Gervais in 1856 (Rosevear, 1965). Molossidae is a large family, comprising 16 genera (*Chaerephon, Cheiromeles, Cynomops, Eumops, Molossops, Molossus, Mops, Mormopterus, Myopterus, Nyctinomops, Otomops, Platymops, Promops, Sauromys, Tadarida, Tomopeas*) (Simmons, 2005). In Madagascar, Molossidae comprise five genera and eight species; *Mormopterus jugularis, Otomops madagascariensis, Mops midas, M. leucostigma, Tadarida fulminans, Chaerephon pumilus, C. jobimena* and *C. leucogaster* (Goodman and Cardiff, 2004).

1.1.2 CURRENT TAXONOMIC STATUS

The genus *Nyctinomus* was proposed by Geoffroy St Hilaire (1813) and was used extensively in the taxonomic classification of molossid bats (Rosevear, 1965). In 1814, Rafinesque suggested a new genus name *Tadarida*. *Nyctinomus* was still used by many authors (Dobson, 1878), whilst *Tadarida* was accepted and also used in the literature. Dobson (1874) first proposed *Chaerephon* as a subgenus of *Nyctinomus/Tadarida*. It was later raised to generic status by Andersen in 1907 (McLellan, 1986). In 1951, Ellerman *et al*. once again proposed *Chaerephon* as a subgenus of

Tadarida. Freeman (1981) divided the African members of the genus *Tadarida* into four genera (*Mormopterus*, *Tadarida*, *Chaerephon* and *Mops*), thus ranking *Chaerephon* as a genus – this is a conclusion not followed by several other bat taxonomists (e.g. Corbet and Hill, 1986; Legendre, 1984; Meester *et al.,* 1986; Bouchard 1998). Peterson *et al*. (1995) regarded *Chaerephon* as a subgenus of *Tadarida* on the basis of 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.

There has also been much debate on the taxonomic status of *C. leucogaster* (Allen 1939; Hill and Carter, 1941; Rosevear, 1965; Hayman and Hill, 1971; Ansell, 1978; Freeman, 1981; Happold, 1987; Koopman, 1993; Peterson *et al.,* 1995; Bouchard, 1998; Grubb, *et al.,* 1998; Hutson *et al.,* 2001; Russ *et al.,* 2001). This species of *Chaerephon* was initially considered to be distinct and endemic to Madagascar (Type locality: Madagascar, Menabe). It was first named by Grandidier in 1869 as *Nyctinomus leucogaster*. In the 1960's *C. leucogaster* and various other west African forms of this bat were suggested to be synonyms of *C. pumilus* (Rosevear, 1965). *C. leucogaster* was included in *C. pumilus* by Koopman (1993) and Bouchard (1998). More recently, Peterson *et al*. (1995) gave an account of morphological and biological characteristics and Russ *et al.* (2001) of echolocation frequencies; both ranked *leucogaster* as a species in the genus *Tadarida*. Hutson *et al.* (2001), Goodman and Cardiff (2004), Lavrenchenko *et al.* (2004) and Simmons (2005) adopted the classification whereby *leucogaster* is considered to be a valid species in the genus *Chaerephon*.

1.1.3 PREVIOUS TAXONOMIC STUDIES ON *CHAEREPHON LEUCOGASTER*

Hutson *et al.* (2001) reported that *C. leucogaster* is endemic to Madagascar. This is inaccurate given that there are various geographical forms recorded from the African continent (Mali, Ghana, Congo and Ethiopia). The following review is based on previous taxonomic studies conducted on African and Malagasy forms to illustrate the aetiology of the current taxonomic status of *C. leucogaster*.

Allen (1939) listed the small west African forms of *Chaerephon* as distinct species – *C. frater* (Allen, 1917), *C. gambianus* (De Winton, 1901), *C. leucogaster* (Grandidier, 1869), *C. limbatus* (Peters, 1852), *C. pumilus* (Cretzschmar, 1826) and *C. websteri* (Dollman, 1908). He therefore considered the smallest west African forms to be conspecific with the smallest Malagasy

Chaerephon. In his study of the bats of west Africa, Rosevear (1965) focused on two African species, *C. pumilus* and *C. limbata*. Aellen (1952) considered *limbata* as a possible synonym of *C. pumilus* as these two species co-existed and differed primarily in external morphology (colour). Rosevear (1965) adopted the taxonomic status proposed by Ellerman *et al.* (1951), in which these two forms are regarded as distinct species.

The species *C. websteri* was created by Dollman in 1908 and was thought to differ from *C. gambiana* in size and morphology (Rosevear, 1965). Aellen (1952) suggested that *C. websteri* and *C. gambiana* were identical. Discrepancies in the taxonomic classification of *C. websteri* arose when measurements were later taken of dry '*websteri'* skins and it was found that the forearm length and skull length measurements did not correspond to those made by Dollman (Rosevear, 1965). The external appearance of *C. websteri* alone was not thought to warrant species status. To quote Rosevear (1965) "all small *Chaerephon* forms should be dealt with as separate entities with the exception of *websteri*, which is synonymous with *gambiana*". *Chaerephon gambiana* (which was given species status) was found to be almost identical to *C. pumilus*, and it was suggested by Monard (1939) that these two forms might belong to the same species (Rosevear, 1965). The form *nigri*, although considered to be a distinct species by Hatt (1928), was found to be similar to *gambiana* and *websteri*. Aellen (1952) later included *nigri* with *websteri* (Rosevear, 1965). Rosevear (1965) recognised six species: *pumila*, *limbata*, *gambiana*, *nigri*, *major* and *nigeriae*.

Hayman and Hill (1971) did not recognize *leucogaster* as a separate species, and combined all *Chaerephon* forms under the name *C. pumilus*. These species (*cristata, elphicki, langi, limbata, leucogaster, frater, faini, gambiana, hindei, naivashae, nigri, pusilla* and *websteri*) were considered synonyms of *pumila*. Happold (1987) and subsequently Koopman (1993) adopted this taxonomic classification of *Chaerephon*.

Peterson *et al.* (1995) used a morphometric approach in determining whether *leucogaster* warrants species status. This study included *leucogaster* samples from Madagascar and *chapini, cristata, frater, hindei, pumila, shortridgei* and *websteri* from the African mainland (Ethiopia and Ghana). Results indicated that there were two statistically-different groups based on size. *Tadarida pumila* was present in the group containing the large-sized bats whereas *T. leucogaster* was found amongst the smaller bats. *Tadarida websteri* was found to be closer in size to *T. leucogaster*. Concluding remarks made by Peterson *et al*. (1995) suggested that *leucogaster* be considered as a valid species

in the genus *Tadarida*. *Tadarida websteri* (Dollman, 1908), *cristatus* (Allen, 1917), *frater* (Allen, 1917) and *nigri* (Hatt, 1928) (all West African forms) were regarded as synonymous of *T. leucogaster*.

In 1998, Bouchard reviewed the classification from Freeman (1981) and Koopman (1993; 1994) and recommended that *Chaerephon leucogaster* be considered a synonym of *C. pumilus*. Russ *et al*. (2001) distinguished *C. leucogaster* from *C. pumilus* on the basis of its smaller size and different echolocation calls. *Chaerephon leucogaster* was considered a valid species in the genus *Chaerephon* by Hutson *et al*. (2001), Goodman and Cardiff (2004) and Lavrenchenko *et al*. (2004). Currently, the taxonomic classification by Simmons (2005) recognizes that the genus *Chaerephon* comprises 18 species and ranks *C. leucogaster* as a distinct species.

1.1.4 DESCRIPTION OF *CHAEREPHON LEUCOGASTER*

1.1.4.1 General Morphology

Chaerephon leucogaster is commonly known as Grandidier's free-tailed bat or the Madagascan white-bellied free-tailed bat. *Chaerephon leucogaster* is distinguished morphologically from *C. pumilus* by size, colour, dental and skull characteristics (reviewed by Peterson *et al.*, 1995). Russ *et al.* (2001) distinguished the two species based on echolocation calls. *Chaerephon leucogaster* is the smallest species within the genus. Both the dorsal and ventral pelage are mid-brown whilst the mid-ventral abdominal region is greyish white. The wing membranes are a whitish grey; this is a defining characteristic of this species. *Chaerephon leucogaster* from Pemba have darker pelage colorations and the white portion on the abdominal regions extends mid-ventrally; in this way it differs from members of this species found on Madagascar and Mayotte (Ratrimomanarivo *et al.*, in press).

Ranges of external measurements of *C. leucogaster* are; mass 7 - 11 g, total length 75 - 91 mm, forearm length 33 - 38 mm and wingspan 262 - 280 mm (Ratrimomanarivo *et al.*, in press). As in most bats belonging to this genus, the wings of *C. leucogaster* are long and narrow and may be adapted for fast flight (Hutcheon, 1994). An external phenotypic feature that identifies males from females is the postaural crest, which is less developed in adult females. *C. leucogaster* possesses wrinkled lips, a small square tragus and a large square antitragus (Peterson *et al.*, 1995). The ears

are large and round relative to the size of the head. The muzzle is usually short and broad. Bristles occur on the outer-most toes.

Individuals of the species are high-flying aerial hawkers with distinctive calls (Russ *et al.,* 2001). They produce a long and steep FM call which has a maximum energy of 28 kHz, an inter-pulse interval of 72 ms and duration of 9 ms (Russ *et al.,* 2001).

1.1.4.2 Distribution

Chaerephon leucogaster has been recorded over much of western and eastern Africa (Simmons, 2005). This species has been reported from the north of Nigeria, where it inhabits public buildings and high forests which have undergone degradation to woodlands (Rosevear, 1965). Its range extends to the northwest (Mali) and to the west (Ghana). In Ghana, these bats roosts naturally in crevices of trees and are also found in large numbers under roofs of buildings (Grubb *et al.,* 1998). *Chaerephon leucogaster* is also known to roost in areas of the Congo Basin (Democratic Republic of Congo) and Ethiopia (Dollman, 1908; Allen, 1917; Hatt, 1928; Simmons, 2005). In the DRC they roost in open woodlands and more arid areas, as well as swampy gallery forest (Rosevear, 1965).

Chaerephon leucogaster is distributed along the western portion of Madagascar. It is found mainly at lower elevations (< 900 m). Individuals were obtained from a locality at a higher elevation from the other sampled localities, 870 m above sea level (Sakaraha). One individual was collected from a locality (Manakara) on the eastern side of Madagascar. Its range extends to the northwest offshore islands of Nosy Be and Nosy Komba as well as to the island of Mayotte in the Comoros Archipelago and the offshore Tanzanian island of Pemba in the western Indian Ocean. All source material of *C. leucogaster* from these areas was collected from synanthropic settings (Peterson *et al.,* 1995; Eger and Mitchell, 2003; Goodman *et al.,* 2005; Rakotonandrasana and Goodman, 2007). Goodman and Cardiff (2004) suggested that this colonization and speciation on distant oceanic islands could be due the capability of these bats to disperse over water.

1.1.4.3 Habitat, Ecology, Social Structure and Dietary Requirements

Chaerephon leucogaster is partly synanthropic (Peterson *et al.,* 1995*;* Eger and Mitchell, 2003*;* Goodman *et al.,* 2005; Rakotonandrasana and Goodman, 2007), inhabiting man-made shelters such

as old buildings, as well as natural roosts such as trees and deep canyons (Goodman and Cardiff, 2004). Ratrimomanarivo *et al.* (in press) highlight the physical settings of the synanthropic day roosts of *C. leucogaster* from Madagascar. These bats are found in roofs and attics of old buildings, particularly under metal roofs with false ceilings in schools and hospitals that have been recently constructed but possess the architectural features of older colonial-style buildings. Typically, these buildings are less than 6 meters high. Bats from Pemba were collected from a similar setting, the attic of a hospital (Stanley, in press). In Madagascar, an animal was recorded roosting 5 m off the ground under the exfoliating bark of a dead tree in a sandy valley with agricultural fields and stands of massive baobab trees (Goodman and Cardiff, 2004).

Chaerephon leucogaster may occur in monospecific roosts or may share roosting sites with other molossid bats such as *Mormopterus jugularis*, *C. pumilus* and *Mops leucostigma* (Goodman and Cardiff, 2004). The sizes of roosting colonies of this species vary from a few bats to hundreds. *Chaerephon leucogaster* is an insectivorous bat which has a dietary requirement of small soft-bodied insects.

1.2 CONSERVATION BIOLOGY- DEFINING SPECIES AND CONSERVATION UNITS

1.2.1 SPECIES

In biology, species is the central and fundamental unit that is used for comparison and measure of biodiversity (O'Brien, 1994; Stearns and Hoekstra, 2000). Formalized taxonomy is critical as it provides a basis of recognition by ranking biological forms according to a hierarchical classification into species, genus, family and order (Van Valen, 1976; Bradley and Baker, 2001; Baker and Bradley, 2006).

1.2.2 SPECIES CONCEPTS

There are a variety of approaches and methods used in different fields to define species (Agapow *et al*., 2004). Some of the species concepts used are; the Morphological Species Concept (Lehman, 1967; Ruse, 1969; Mallet, 1995; Andersson, 1990; Mayr, 2000a), the Biological Species Concept (Mayr, 1942; Van Valen, 1976), the Recognition Species Concept (Paterson, 1980; Coyne *et al.*, 1988; Sluys and Hazevoet, 1999; Mayr, 2000b), the Ecological Species Concept (Van Valen, 1976),

the Phylogenetic Species Concept (Cracraft, 1983; Nixon and Wheeler, 1990), the Evolutionary Species Concept (Simpson, 1961; Wiley, 1978), the Cohesion Species Concept (Templeton, 1989) and the Genetic Species Concept (Mayden, 1997; Baker and Bradley, 2006).

Early work was based on the visual recognition of an entity as being distinct. This gave rise to the Morphological Species Concept (Lehman, 1967; Mayr, 2000a), which has been criticized as it is subjective and unable to distinguish sibling and cryptic species as well as sexual dimorphism and polymorphism (Andersson, 1990; Ruse, 1969; Mallet, 1995; Mayr, 2000a). Nevertheless, it formed the basis of many concepts that were subsequently established. Current classifications of the genus *Chaerephon* are based on morphological studies. This genetic study is designed to complement the morphological characterization of *Chaerephon leucogaster* by Ratrimomanarivo *et al.* (in press).

According to the Biological Species Concept (BSC) (Mayr, 1942; Van Valen, 1976), species are "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such populations". Species are therefore reproductively isolated and represent separate evolutionary lineages. This concept is not universal as it is inapplicable to asexual organisms. Difficulties are also encountered when hybridisation occurs (Agapow *et al*., 2004).

The Phylogenetic Species Concept (PSC) had gained attention due to the advancement of molecular phylogenetics. Cracraft (1983) and Nixon and Wheeler (1990) described a species as a group of organisms that share at least one uniquely-derived character. This species concept has been widely accepted as it applies to asexual as well as allopatric populations. The PSC is valuable as it emphasizes the importance of recognizing species as evolutionary lineages and is compatible with the large amounts of sequence data currently available. However, classification under the PSC has lead to an apparent rise in the number of endangered species due to 'taxonomic inflation' caused by raising subspecies to species level (Baum, 1992; Agapow *et al*., 2004; Freeland, 2005).

Mayden (1997) defined genetic species in terms of "measurement of genetic differences that is used to infer reproductive isolation and evolutionary independence". The application of the Genetic Species Concept (GSC) uses genetic data from both plastid and nuclear genomes in order to identify species and species boundaries (Baker and Bradley, 2006). The GSC overlaps with the BSC, but

where reproductive isolation was the focus of the BSC, genetic isolation is the primary focus in the GSC.

Baker and Bradley (2006) suggest that genetic isolation is a result of the divergence of two genomes which are genetically distinct and share a common evolutionary history. The use of genetic data contributes to the identification of monophyly, sister taxa and hybrid individuals. It can also provide a perspective on types of divergence. Genetic drift is recognized as contributing to uniformity found within populations as well as variation among separate populations (Templeton, 1989). Genetic isolation in mammalian species can be explained using the Bateson-Dobzhansky-Muller (BDM) model (Baker and Bradley, 2006). This model proposes that the accumulation of genetic changes in two isolated populations results in the formation of two separate species. The implementation of the GSC, which is useful for the identification of cryptic species, has resulted in an increase in the number of recognised species. As this is a study of mitochondrial DNA sequences, the GSC will be used to interpret levels of genetic isolation within and among populations and species.

1.2.3 EVOLUTIONARY SIGNIFICANT UNITS

"Molecular data are critical to the shaping of management strategies and has been applied to the identification of units of conservation" (King and Burke, 1999). Conservation units within species are fundamentally important to prioritise and conduct management at a regional or local scale. The Evolutionary Significant Unit (ESU) and the Management Unit (MU) aim to prioritise units of protection below the taxonomic level of the species.

Ryder coined the term 'Evolutionary Significant Unit' in 1986 and defined it as "a subset of the more inclusive entity, species, which possesses genetic attributes significant for the present and future generations of the species in question" (Fraser and Bernatchez, 2001). Moritz (1994) defined an ESU on the basis of historical isolation, which is known to produce a unique and inimitable combination of genotypes (Crandall *et al.*, 2000). This ESU definition allows conservation biologists to apply molecular genetics and also to avoid determining how much genetic variation is needed for a population to be allowed protection (Fraser and Bernatchez, 2001). Moritz (1994) proposed the 'Management Unit' as a conservation unit below that of the ESU which is not afforded the mandated protection that accompanies ESUs (King and Burke, 1999). MUs are involved in short-term management, whilst ESUs are significant in long-term management.

Management and conservation strategies should aim to preserve adaptive diversity and evolutionary processes across the geographic range of a species. Crandall *et al.* (2000) emphasized that in order to conserve evolutionary processes; the goal should be to preserve the network of genetic connections between the populations rather than just distinct populations. Such concepts will become important in the context of this study should *C. leucogaster*, which is currently assessed as 'data deficient' (Chiroptera Specialist Group, 2000), be shown to contain genetically distinct lineages occurring in geographical isolation.

1.2.4 THREATS TO BATS AND CONSERVATION IMPLICATIONS

A variety of factors contribute to either the increase or reduction in size of bat populations worldwide. Hutson *et al.* (2001) and Mickleburgh *et al.* (2002) reviewed the factors that affect bat populations either directly or indirectly. According to Hill and Smith (1984), Fenton (1997), Hutson *et al.* (2001) and Mickleburgh (2002), modification or destruction of bat habitats is one of the most important factors that affect bat species. Habitat selection studies have revealed the importance of several habitat classes for bat survival. These include day- and night-roosting habitat, feeding habitat, and areas connecting roosting and feeding habitats.

In some areas caves, mines, crevices and artificial structures such as houses, churches, attic spaces and chimneys provide ideal roost conditions for hibernation and nursing of bats, including *C. leucogaster*. Depending on the species, bats may use only one roost type or vary the roost type seasonally (Hutson *et al.*, 2001). Structures that are used as night roosts function in conservation of energy, predator protection, social contact/interaction with other bats and breeding (Kunz, 1982). Fenton (1997) highlights the importance of management strategies for protection of these habitats.

In addition to roosts, foraging habitat is also an important factor in bat survival. Carmel and Safriel (1998) assessed habitat use by seven bat species in Israel. Results identified two habitat types, scrub and riparian vegetation, that were foraged by a number of endangered species. Previous studies conducted by Walsh and Harris (1996) found that verspertilionid bats exhibited preferences for broad-leaved woodlands and water bodies. Management programs were implemented to increase the area of riparian vegetation, as it constitutes the major food source for at least three endangered species. Agricultural growth has resulted in changes to the landscape inhabited by certain bat species (Russ and Montgomery, 2002). Walsh and Harris (1996) determined that deciduous

woodland, water and linear landscapes were significant foraging areas for bats. Differences in dietary requirements may be the cause of the difference in foraging habitats (Barlow, 1997). It has been suggested that, for conservation purposes, semi-natural deciduous woodlands with coniferous plantations should be increased. The preservation and improvement of water bodies should also be considered. A study on habitat selection of *Barbastella barbastellus*, which is classed as Vulnerable (Hutson *et al.,* 2001) and Endangered in Italy (Bulgarini *et al.,* 1998) revealed a preference for unmanaged woodlands over open woodland and pasture. Roosts of this species were often in dead or tall trees or beneath loose bark. Russo *et al.* (2004) suggested that preservation and protection of unmanaged woodlands, which provide roosting and feeding opportunities, is essential for the survival of this species. Human activities, such as clearing of these woodlands, decrease roosting and feeding opportunities for these bats. Felling of trees should be avoided, or if unavoidable the trees should be examined for the presence of bats.

The exploitation of land for agricultural purposes causes habitat reduction for many bat species. Limpens and Kapteyn (1991), Jones *et al.* (1995) and Verboom (1998) highlighted the importance of landscape elements such as treelines, hedgerows and canals that act as important connections between roost sites and feeding areas. They recommended that the replacement of tree lines/hedges, one of the most common foraging habitats of bats be discouraged, and that management strategies be focused on the improvement and enhancement of highly-inhabited areas and connecting linear habitats.

There has been major concern that anthropogenic factors threaten bats, particularly because of habitat destruction associated with increasing human populations. Exploitation of land for agricultural purposes plays a role in habitat reduction. Domestic livestock impacts woodlands, grasslands and shrublands. The uncontrolled use of herbicides and pesticides is another threat (Amr *et al.,* 2006). Bio-accumulating organic pesticides kill both the pest and other insects (Hill and Smith, 1984). A decrease in insect population size may result in a corresponding decline in population size of insectivorous bats. The accumulation of certain toxins in adult bats can be the cause of death to the nursing young.

Human activities can however, impact bat populations positively. Some bat species roost exclusively in houses (Hutson *et al.,* 2001). In southern Africa, molossid bats are frequently found roosting in houses, as is the case in this study of *C. leucogaster* from Madagascar

(Rakotonandrasana and Goodman, 2007; Ratrimomanarivo *et al.*, in press). The availability of roost sites in buildings can lead to an increase in bat population size (Brosset, 1966; Kunz, 1982). However, the use of houses as roosts may result in conflict with humans, who dislike bats (Hutson *et al.*, 2001). Fumigation is one destructive method that is used to eradicate bats. Education about bats can result in protection of house-dwelling bats.

In 2002, Mickleburgh *et al.* reported the existence of 1,001 bat species globally. There are a number of international treaties that protect fauna and flora worldwide, some which protect bats directly or indirectly. Seven of these, which include the protection of bats, are highlighted by Hutson *et al.* (2001). There are also a number of treaties that are specifically designed to protect, manage and conserve bat populations across the world. These include: The Agreement on the Conservation of Bats in Europe (EUROBAT) under the Bonn Convention (1994) and the Programme for the Conservation of Migratory bats of Mexico and the United States (PCMM) (1994) (Hutson *et al.*, 2001). The EUROBAT Convention aims to implement strategies for the monitoring of bat populations, the study of migratory species, the implementation of trans-boundary protection programmes and the effective management of woodlands and underground habitats. The PCMM Convention aims to develop programmes to research behavioral characteristics, migratory routes and the economic value of bat populations, as well as to provide education to rural areas that are found close to major roosting caves, and to develop formal legislation for cave conservation.

The Chiroptera Specialist Group of the IUCN's species survival commission has two action plans to examine conservation issues (Mickleburgh *et al.*, 2002). This commission aims to conserve the most threatened species and their habitats. It targets policy makers, organizations and individuals that play a key role in promoting the conservation of bat species (Mickleburgh *et al.*, 2002).

Madagascar is the fourth largest island in the world, situated 400 km east of Mozambique. It was formed during the breakup of the Gondwana landmasses approximately 160 million years (separation from Africa) and 90 million years (separation of Madagascar) ago (de Wit, 2003; Yoder and Nowak, 2006; Ali and Aitchison, 2008). It is considered to be a biodiversity 'hotspot' (Myers *et al*., 2000; Ganzhorn *et al*., 2001; Goodman and Benstead, 2005). It is an area that has a high level of endemism coupled with severe threats to many species of flora and fauna. It is imperative that conservation and management strategies are implemented in such areas (Ganzhorn *et al.*, 2001; Goodman and Benstead, 2005). Madagascar is regarded as a high priority area because endemism

extends to high taxonomic levels among plants and vertebrates; it is therefore regarded as a 'critical component of the global biological heritage' (Myers *et al.*, 2000; Goodman and Benstead, 2005).

Madagascar is one of the world's poorest countries (Dostie *et al.*, 2002; Minten and Barrett, 2008). A large portion of the human population lives in rural settings with incomes below the poverty line (Hutson *et al.*, 2001). Increases in the human population cause extra demands for land, food and other essential resources. This has led to ongoing habitat destruction.

According to Gautier and Goodman (2003), Baron (1889-90) divided the island into eastern, central and western regions. The main vegetation type in the eastern region is dense humid forest. Littoral forest occurs along the eastern coastal strip. A substantial amount of the original vegetation of the eastern region was destroyed, but a significant amount still remains in protected areas (Gautier and Goodman, 2003). Sub-humid forest and dry forest are found to the north of this region, whilst to the south the vegetation undergoes a transition to spiny thicket. The central region covers approximately 40 % of the island (Gautier and Goodman, 2003) and comprises land above 1000 m. The central highlands are defined by moist montane forest. There are a number of major mountains, such as Tsaratanana, Marojejy, Anjanaharibe-Sud, Ankaratra and Andringitra, which are characterized by the presence of forest moss and lichens. Forest habitat in this region exists in a few isolated and highly-fragmented areas. The western region is made up of dry deciduous forest; spiny bush is the principal vegetation type in the southwest. Important forested areas are still present in this region.

The major factors that threaten and contribute to the threat and destruction of the flora and fauna of Madagascar are deforestation, agricultural fires, conversion of natural habitats into pasture and over-exploitation of living resources (Hutson *et al.*, 2001).

Deforestation can be attributed to three main factors:

(a) Slash and Burn (vernacular term: *Tavy*). This predominant land-use practice is the major cause of degradation and deforestation, particularly in eastern Madagascar (Oxby and Boerboom, 1985; Gade, 1996; Hutson *et al.*, 2001; Marcus, 2001; Goodman, 2006; Styger *et al.*, 2007). It is used for the conversion of tropical rainforest to agricultural lands (rice fields) and for wood exploitation (Gautier and Goodman, 2003). Fires kill native and regenerating tree species (Styger *et al.*, 2007). According to Green and Sussman (1990), the

extent of the eastern rainforest was reduced from 68 % to 63 % between 1950 and 1985. *Tavy* also causes endemic biodiversity loss in Madagascar (Styger *et al.*, 2007). Many bat species, including *C. leucogaster*, that use tree crevices as roost sites are threatened or killed by this practice.

(b) Charcoal/Fuelwood production. The spiny forests of Madagascar are being destroyed for the production of charcoal, which is an important source of income in the southwestern portion of Madagascar.

(c) Logging for Timber. Illegal logging poses a significant problem in some protected areas. The current rate of deforestation in the western region is comparable to that in the east (Gautier and Goodman, 2003).

The conservation status of bat species, such as *C. leucogaster*, which are found primarily in western Madagascar, should be carefully assessed as their combined viability may be affected by the deforestation described above. There is also over-exploitation of Madagascar's fauna. In the southwestern portions of Madagascar cave-dwelling bats are consumed during periods of famine (Goodman, 2006). Lemurs and tenrecs are hunted, whilst reptiles and amphibians are collected for international trade (Stiles, 1998; Schlaepfer *et al.*, 2005; Andreone *et al.*, 2008).

Madagascar has a protected area system which is classified into three different reserve types (Randrianandianina *et al.*, 2003). The Strict Nature Reserve protects fauna and flora within a specified boundary, the National Park protects and preserves natural history and is used for educational purposes, whilst the Special Reserve is involved in the protection of a specific ecosystem. This network of protected areas was managed by a government organization known as ANGAP (National Association for the Management of Protected Areas in Madagascar) (Randrianandianina *et al.*, 2003). A new park management system known as SAPM (the System of Protected Areas of Madagascar) replaced ANGAP in 2006. One of the aims of this management system is to simplify the process of creating protected areas and to contribute to sustainable development and poverty reduction.

Madagascar became a member of other international agreements and treaties. The following include the protection of bats:

(a) Convention on Biological Diversity (CBD - Rio Conference).

(b) Convention on protection of Wetlands (Ramsar).

(c) African Convention on the Conservation of Nature's Natural Resources.
(d) Protocol concerning Protected Areas and Wild Fauna and Flora in the Eastern African Region.

Sixty percent of Malagasy bat species are endemic (Goodman and Benstead, 2005). This can be attributed to the geographic isolation of this island (Eger and Mitchell, 2003; Goodman and Benstead, 2005). According to Eger and Mitchell (2003) and Goodman *et al.* (2005), the western region of Madagascar has the greatest species richness and the highest density of bat species. Vegetation type, food supply and roosting sites are suggested to be the factors which determine bat distribution.

According to the 2008 IUCN (The World Conservation Union) Red List of threatened species (Chiroptera Specialist Group, 2000), *C. leucogaster* is classified as data deficient (DD*). This applies to species with uncertain species status (newly described or insufficient material available). One of the aims of this study is to provide data on the genetic diversity of this species for use in determining its conservation status.

1.3 METHODS OF STUDYING GENETIC DIVERSITY

1.3.1 RATIONALE FOR THIS STUDY

Systematic data from morphological and behavioural studies have been traditionally used as indicators of the diversity that exists between populations and species (Savage, 1995; Onarici and Sumer, 2003). DNA analysis has increasingly been used to generate systematic data and provide insight into diversity within ecosystems (Hillis and Davis, 1988). The Polymerase Chain Reaction (PCR) and DNA sequencing are molecular methods that are commonly used to study phylogenetic relationships (Innis *et al.,* 1988; Kocher *et al.,* 1989; Erlich *et al.,* 1991; Onarici and Sumer, 2003). The mitochondrial genome has been a marker of choice in animals owing to its variability. Coding (cytochrome *b*) and non-coding (D-loop) mitochondrial DNA sequences were used in this study to determine phylogenetic and phylogeographic patterns for *C. leucogaster*.

1.3.2 CHOICE OF REGIONS TO SEQUENCE

Mitochondrial DNA sequences are an important tool and have been used to provide insight into the evolutionary histories of animals and in population genetic and phylogeographic studies (Moritz *et al*., 1987). The mitochondrial genome is a small, double-stranded covalently-closed molecule, which ranges in size from 16 - 18 kb (Boore, 1999; Onarici and Sumer, 2001; Freeland 2005) and contains 37 genes. It is maternally-inherited and is therefore an apt indicator of female-mediated gene flow (Moritz *et al.*, 1987; Kondo *et al.*, 1990; Gyllestein *et al.*, 1991). Mitochondrial DNA contains no introns, repetitive DNA or pseudogenes (Avise *et al*., 1987; Moritz *et al*., 1987). A unique characteristic is the lack of recombination, which allows for transmission of a single haplotype from mother to offspring (Clayton, 1982, 1992; Hayashi *et al.*, 1985). Mitochondrial DNA is present in multiple copies in each cell (Pereira, 2000; Michaels *et al*., 1992; Robin and Wong, 1998). The mitochondrial genome has a higher mutation rate than the nuclear genome and may evolve 5 - 10 times faster than single copy nuclear DNA (Brown *et al*., 1979). Transitions outnumber transversions and base substitutions are found to be more common than insertions or deletions (Avise *et al.*, 1987). Although, on average, the mutation rate of the mitochondrial genome is faster than that of the nuclear genome, the gene arrangement of the mitochondrial genome is constant over long periods (Boore, 1999).

Mitochondrial DNA sequences have been used successfully in numerous studies to address systematic questions at the level of both genus and species (Van Den Bussche *et al.* 1998; Avise and Walker 1999). The phylogenetic utility of this gene for resolving species-level relationships in bat genera has been well documented (Sudman *et al.* 1994; Wilkinson *et al.* 1997; Cooper *et al.* 1998; Wright *et al.* 1999; Bastian *et al*. 2001; Lewis-Oritt *et al.* 2001; Kawai *et al.* 2003; Stadelmann *et al*. 2004; Hoofer *et al.* 2006; Li *et al.* 2006; Pulvers & Colgan 2007; Russell *et al.* 2007; Stadelmann *et al*. 2007). Furthermore, the use of this mitochondrial region permits the comparison of my results with those from published data.

There are many disadvantages associated with the use of mitochondrial sequences. Systematic studies based solely on maternally inherited genes such as cytochrome *b* have been criticized because they are susceptible to introgressive hybridization (i.e. the retention of ancestral polymorphisms) and do not reflect male-mediated dispersal. They are also susceptible to independent lineage sorting which can result in increased homoplasy (Avise 1994). Thus, phylogenies incorporating for example cytochrome *b* and nuclear DNA could provide valuable insight into the evolutionary relationships. The nuclear genome is inherited biparentally (Onarici and Sumer, 2001) and may be used to examine male-mediated gene flow (Ruedi and McCracken, 2006). Although mitochondrial DNA has been the marker of choice for the investigation of bats, it is important to note that nuclear DNA has recently been used increasingly with mitochondrial DNA to study phylogeographic patterns of ancient origin (Ruedi and McCracken, 2006).

The cytochrome *b* gene is a coding region and contains slowly- and rapidly-evolving codon positions as well as conservative and variable domains. Overall this region is conserved and has a relatively slow mutation rate. It is usually used for phylogenetic analyses at the species level and above (Pereira, 2000). The complete mitochondrial cytochrome *b* gene is 1,140 bp in bats (Bradley and Baker, 2001) and was amplified as either a single fragment (primers L23 and H15) or as two overlapping fragments (L23 and H53, L46 and H15) (Irwin *et al.*, 1991).

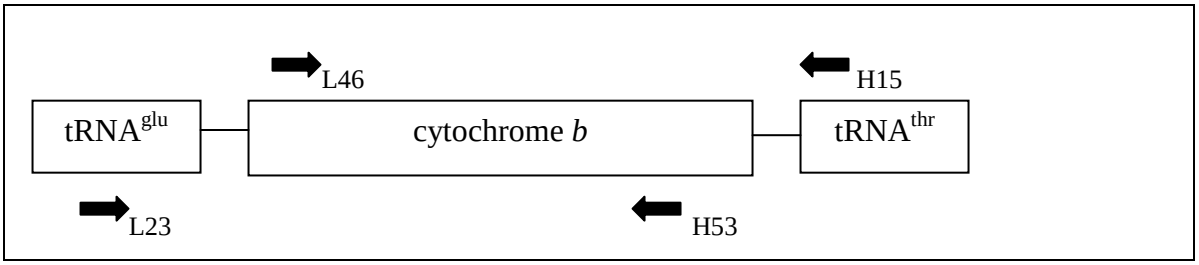

Figure 1.1: Approach used for the amplification and sequencing of the cytochrome *b* gene, which is flanked by $tRNA^{glu}$ and $tRNA^{thr}$ genes. The binding sites of primers used in PCR amplification of the cyt *b* gene are indicated with arrows.

The displacement loop (D-loop), also known as the control region of the mitochondrial genome, is non-coding and is involved in control of replication and transcription of mitochondrial DNA (Loew,

2000). It is estimated to be approximately 0.8 - 1.0 kb in size (Fernandez-Silva *et al*., 2003). Hypervariable mitochondrial DNA alternates with conserved sequence blocks within this region. Sequences can thus be compared within and between species and populations, depending on the variability of the region used. In this study the 5' hypervariable region of the D-loop was sequenced using primers P (located in the tRNA for proline) and F (located in conserved sequence block F) (Wilkinson and Chapman, 1991).

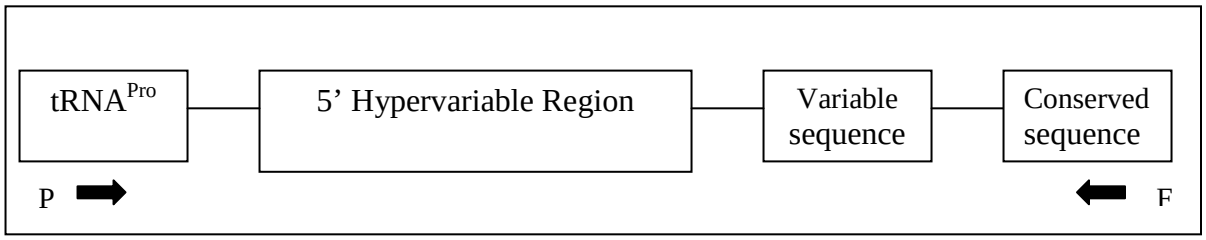

Figure 1.2: Approach used for the amplification and sequencing of the mitochondrial D-loop region. Binding sites of primers used in PCR amplification of the D-loop are indicated with arrows.

1.3.3 GENETIC STUDIES ON BATS

"Studies of genetic variation can contribute substantially to the understanding and management of species" (Worthington-Wilmer *et al*., 1994). The application of molecular techniques allows the study of genetic differentiation at inter- and intra-species level. Mitochondrial DNA analyses have played an integral role in analyses where phylogenetic and phylogeographic relationships are inferred.

Phylogenetics and Phylogeography

Cytochrome *b* has been shown to be useful in elucidating relationships within and between closely related bat genera (Porter and Baker, 2004). Cytochrome *b* gene sequences have been used at an intra-generic level to define species in a number of bat families. Examples include the genera *Phyllostomus* (Van de Bussche and Baker, 1993), *Glossophaga* (Hoffman and Baker, 2001), *Pipistrellus* (Hulva *et al*., 2004), *Myotis* (Rodriguez and Ammerman, 2004), *Miniopterus* (Tian *et al*., 2004) and *Vampyressa* (Porter and Baker, 2004).

A number of authors have adopted the use of mitochondrial DNA alone or in combination with other data to resolve relationships and classifications. Recent studies have utilized cytochrome *b* sequences in combination with ND2, ND3 - ND4, D-loop, *RAG*2 and 16S rDNA sequences. Petit

and Mayer (2000) used mitochondrial D-loop sequences to differentiate between populations of *Nyctalus noctula* (Vespertilionidae); Cooper *et al.* (2001) analysed the cytochrome *b* and ND2 regions from populations of *Myotis* to reveal a single species of *Myotis* (large-footed) that occurs in Australia (*M. macropus*) that is genetically distinct from the Indonesian *M. adversus*; Kiefer and Veith (2001) revealed a new species of *Plecotus* (Vespertilionidae) using the D-loop and 16S regions; Lim *et al.* (2003) used the ND3 - ND4 and cytochrome *b* regions to show that the genus *Ectophylla* is closely related to *Mesophylla*; Hoffmann *et al.* (2003) compared cytochrome *b* sequence variation with chromosomal and geographic variation in *Uroderma bilobatum* of the family Phyllostomidae; Stadelmann *et al.* (2004) used cytochrome *b* sequences to determine the phylogenetic relationships of eight *Myotis* species from Ethiopia relative to other *Myotis* samples from across the globe in an attempt to resolve the contentious position of *M. vivesi*; Davalos (2005) used cytochrome *b* and nuclear *Rag*2 genes to define eight species in the genus *Natalus* (Natalidae); Piaggio and Perkins (2005) used cytochrome *b* and D-loop sequences to reveal the existence of three distinct species in the genus *Corynorhinus* and Goodman *et al*. (2006) used morphological and cytochrome *b* sequence data to describe a new species of bat of the genus *Emballonura* (Emballonuridae) from Madagascar.

Phylogeography involves characterization of the distribution of phylogenetic lineages across the geographical landscape. Ditchfield (2000) revealed low cytochrome *b* divergence (less than 4 %) between bat species with little phylogeographic structure. Hoffmann and Baker (2003) suggested that the uplift of the Andes and Panamanian contributed significantly to biodiversity in the Neotropical genus *Carollia* (Phyllostomidae). Lloyd (2003) used cytochrome *b* sequences to determine the intraspecific phylogeny of *Mystacina tuberculata* (Mystacinidae), an endemic to New Zealand. Lineages of this bat are geographically structured, reinforcing the presence of a barrier to inward gene flow on the South Island. Worthington-Wilmer *et al*. (1994) showed geographic structuring of genes in *Macroderma gigas*, of the family Megadermatidae (Ghost bats), as a result of long-term isolation of populations and female philopatry.

Cryptic species

Genetic data has become a powerful tool in the detection of cryptic species (Ruedi and McCracken, 2006), which may be morphologically similar but genetically distinct or morphologically distinct but genetically similar (Pfenninger and Schwenk, 2007). It is therefore wise to use both morphological and genetic data to identify species. Jacobs *et al.* (2004) analysed mitochondrial

cytochrome *b* sequences and proposed that two forms of *C. pumilus* (light- and dark-winged), which showed a 0.9 % average sequence divergence were a single species. In 2006, Jacobs *et al*. conducted a study on the bat species *Scotophilus dinganii*, which comprises two types that are similar in size and share a yellow venter but differ in echolocation frequency. The two types were found to be reciprocally-monophyletic and suggested to be sibling species. These studies indicate the complexity of identifying species genetically, as a wide range of cytochrome *b* divergences are found between morphologically-defined species (Baker and Bradley, 2006).

Barriers to dispersal on islands

Like other mammals, bats exhibit different responses to geographic barriers. Barriers that are narrow can result in restriction of dispersal, while large bodies of water may not be a barrier to dispersal (Ruedi and McCracken, 2006). Pestano *et al*. (2003) used mitochondrial 16S rRNA and cytochrome *b* sequences to investigate the genetic differentiation and evolutionary relationships of *P. teneriffae* from Tenerife, La Palma and El Hierro islands in the Canarys. Levels of divergence of the cytochrome *b* gene between populations on Tenerife and those on El Hierro and La Palma were 1.1 % and 1.5 % respectively. Haplotype analysis indicated that gene flow had occurred from Tenerife to the other two islands. These authors also reported that *Barbastellus barbastellus* on the Canary Islands shows a 4.0 % divergence from populations of the same species found on mainland Spain.

A study on the bat *Myotis myotis* by Castella *et al*. (2000) examined whether the Gibraltar Strait was a geographical barrier that lead to genetic discontinuities in bat populations on either side. They found using cytochrome *b* and nuclear data that the two populations on either side of this strait were genetically distinct. A similar study by Juste *et al*. (2004) showed a 20 % sequence divergence between *Plecotus auristus* and *P. austriacus*. The Strait of Gibraltar was proposed as a geographic barrier which led to the separation of these lineages.

Carstens *et al.* (2004) used cytochrome *b* sequence analysis to explore the colonization history of the phyllostomid bats (*Ardops nichollsi*, *Brachyphylla cavernarum* and *Artibeus jamaicensis*) in the Lesser Antilles. *Ardops nichollsi* and *B. cavernarum* each comprised a single well-supported clade which exhibited little genetic variation within species across islands, whilst *Artibeus jamaicensis* comprised two highly-divergent clades. It was suggested that the two divergent haplotypes of *Artibeus jamaicensis* were a result of inter-island migration and vicariance (Carstens *et al.*, 2004).

Tadarida brasiliensis (Brazilian free-tailed bat) of the family Molossidae occurs in Central and South America as well as North America. Russell *et al*. (2005) analysed D-loop sequences of *T.b mexicana* from four groups of migratory and non-migratory bat populations. Sequence divergence values within and among groups ranged from 0.2 - 7.1 %. Bayesian inference showed no structuring of mitochondrial lineages according to migratory behaviour. These authors found that high haplotype diversity in *T. b mexicana* may be the result of female gene flow, as well as the retention of ancestral polymorphisms.

Russell *et al.* (2006), Emerson (2002); Raxworthy *et al.* (2002) and Evans *et al*. (2003) highlight the need to incorporate biogeographical studies with phylogenetic studies. This approach was used to determine the biogeographical history of *Triaenops* from Madagascar (Russell *et al.,* 2006). Bayesian analysis of cytochrome *b* sequence variation in *Triaenops* species from Madagascar and Africa indicates that there were possibly two independent, unidirectional dispersal events from Africa to Madagascar.

1.4 DATA ANALYSIS

1.4.1 INFERENCE OF PHYLOGENETIC TREES

The use of morphological characteristics enabled biologists to examine and estimate relationships between and among taxa. Molecular data are now also widely used to investigate evolutionary relationships of organisms (Irwin *et al.,* 1991; Whelan *et al.,* 2001; Vandamme, 2003). The inference of a phylogeny, based on molecular data, is achieved by the comparison of homologous residues (Vandamme, 2003).

There are a number of methods and criteria for phylogenetic tree construction (Jin *et al.,* 2007). Methods using molecular data can use discrete character states (cladistic approach) or a distance matrix of pairwise similarities (phenetic approach) to infer phylogenetic trees. The phenetic approach does not consider historical relationships of organisms. It is used to measure the genetic distances between species and thereby construct a tree by a hierarchical clustering method. Cladistic methods differ from phenetic methods in that they can provide information on ancestral relationships.

1.4.2 NUCLEOTIDE SUBSTITUTION MODELS

Sequences that are derived from a common ancestor eventually diverge from each other due to accumulation of nucleotide substitutions. As sequence divergence increases, so does the likelihood of multiple substitutions at a single site. Observed genetic distances may be an underestimate of the true distance where there have been multiple substitutions at the same site. According to Page and Holmes (1998), statistical models may be implemented to estimate the corrected distance from the observed difference. Some of the more commonly-used models are outlined below.

The Jukes-Cantor model was proposed in 1969 [JC69]. It is the simplest model of sequence evolution and assumes that the four bases have equal frequencies and that the frequency of transitions is equal to that of transversions (Strimmer and von Haesler, 2003). The disadvantage of this model is that it is seldom applicable to real sequence data sets.
The Kimura-2-parameter [K2P] model differs from the JC69 model in that it allows for variation in the rate of transitions and transversions although it still assumes that the four bases occur with equal frequency. The Felsenstein model [F81] was proposed in 1981. This allows the four base frequencies to vary and assumes that transitions and transversions occur at equal rates (Page and Holmes, 1998; Strimmer and von Haesler, 2003).

Hasegawa *et al.* (1985) proposed the HKY85 model, which is a combination of the K2P and F81 models (Strimmer and von Haesler, 2003). It takes into account both transitional/transversional and GC content biases.

The most comprehensive model is the General Time Reversible Model [GTR]. It considers two independent rate parameters i.e. the ratio of transitions and transversions and the ratio of two types of transitions. Each possible substitution has its own probability (Page and Holmes, 1998).

The models described above are among the most well known and frequently-used in phylogenetic studies. The gamma distribution (G) of rate variation across sites and the proportion of invariable sites (I) in a sequence data set may also be taken into account.

1.4.3 MODELTEST

Modeltest (Posada and Crandall, 1998) is a program that determines the nucleotide substitution model which best fits a sequence data set. It is used in conjunction with the programme Phylogenetic Analysis Using Parsimony (PAUP) (Swofford, 2002) and allows for comparisons to be drawn between 56 models of substitution. Mr Modeltest (Nylander, 2004) is a simplified version of Modeltest which fits the data to a smaller set (24) of substitution models.

The Akaike Information Criterion (AIC) selects a chosen model that has a good fit. Akaike weights are used in model averaging and selection as described by Burnham and Anderson (2002).

1.4.4 DISTANCE MATRIX METHODS

Genetic distances reflect the dissimilarity between sequences (Strimmer and von Haeseler, 2003). Distance matrix methods examine the dissimilarity of pairs of Operational Taxonomic Units (OTUs) and produce a pairwise distance matrix from which a tree may be generated (Vandamme, 2003). The most commonly-used methods to infer distance-based trees are the Unweighted Pair Group Method with Arithmetic Means (UPGMA) and the Neighbour-Joining (NJ) method, which will be used in this study.

The Neighbour-Joining method was developed by Saitou and Nei (1987). It constructs a tree by finding pairs of taxa that are connected by a single interior node (Vandamme, 2003). This algorithm requires that all internal branches be minimized, a condition which would ultimately lead to the shortest tree length (Nei and Kumar, 2000; Vandamme, 2003). The advantages of this method are that it is not computer-intensive and produces a tree quickly. The disadvantage lies in the loss of information about the ancestral nodes, as distance methods do not consider the character state of taxa (van de Peer, 2003). The NJ method is most commonly used to infer distance-based trees.

Minimum evolution is also a distance based method that uses the minimum sum of branch lengths and is based on the assumption that the topology with the least total branch length score best represents the evolutionary relationship. I did explore minimum evolution however it produced many trees of equal score.

1.4.5 CHARACTER STATE METHODS

These are cladistic methods that are based on shared derived characters. Examples of character state methods include maximum parsimony, maximum likelihood and Bayesian analysis

Parsimony is used extensively in phylogenetic studies. Taxa are grouped if they share a common characteristic which is different from that of a common ancestor. The Maximum Parsimony method chooses the tree that requires the fewest evolutionary changes (Page and Holmes, 1998). This algorithm evaluates each tree possible at every informative site (Nei and Kumar, 2000; Vandamme, 2003; Kolaczlowski and Thornton, 2004). A disadvantage of this method is that it is time-consuming and does not allow for the use of models to correct for multiple substitutions at the same site (Page and Holmes, 1998).

Bayesian inference is based on posterior probabilities. Bayesian analysis uses the Markov Chain Monte Carlo (MCMC) process to sample trees from a distribution of posterior probabilities (Huelsenbeck *et al.,* 2001). Trees are sampled at fixed intervals and the posterior probability of a tree is expressed by the proportion of time spent there by the chains. Bayesian Analysis is similar to Maximum Likelihood in that it requires an approximate model of evolution. A major advantage of this method is that it is more efficient due to improvements in hardware and speed than the as well as the lack of calibration in common hiearchial settings in Maximum Likelihood method (Karol *et al*., 2001), and, for this reason, is used in this study in preference to Maximum Likelihood (Browne and Draper, 2006).

1.4.6 ESTIMATING RELIABILITY OF PHYLOGENETIC TREES

Bootstrap Analysis

This method was first applied by Felsenstein in 1985. Bootstrapping is described by Efron *et al.* (1996) as a computer-based technique that is used to evaluate the measure of confidence of clades within a phylogenetic tree. It involves resampling from the original data set (Lanyon, 1987). The resampled data are termed pseudoreplicates and these matrices are identical in size to the original matrices (Alfaro *et al.,* 2003). Statistics are calculated from each pseudoreplicate. This is a measure of the sampling error. The process may be repeated 100 to 1000 times, thus resulting in the generation of bootstrapped trees (Lanyon, 1987; Page and Holmes, 1998). An advantage of using

bootstrap analysis is that it is an effective technique and it can be utilized in most tree-construction methods. A disadvantage of this method is that it is time-consuming. According to Hillis and Bull (1993), branches/taxa that are supported with a greater than 70 % bootstrap support are reliable whilst a value less than 70 % should be treated with care.

Posterior Probabilities

Inference of phylogeny using Bayesian analysis is based on posterior probabilities (Huelsenbeck *et al.,* 2001). The Markov Chain Monte Carlo algorithm is used to estimate the posterior probabilities of nodes in phylogenetic trees (Erixon *et al*., 2003). These probabilistic measures of support are used to determine how likely it is that a certain clade is accurate.

1.4.7 PHYLOGEOGRAPHY

The concept of phylogeography was first introduced by Avise *et al.* in 1987. It provides an insight into events that may have occurred over a few million years (Hewitt, 2004). Phylogeographic analysis can identify historically and evolutionary independent geographical regions. Nested Clade Analysis (NCA) is a statistical method that uses haplotype trees to define a nested series of clades which allow the evolutionary nested analysis of the geographic distribution of the genetic variation in a dataset at intra- and inter-species level (Templeton, 1998). Although publications criticizes the use of this method (Knowles, 2008), the rebuttal by Templeton (2009) discusses the validation for the use of this method.

The programme TCS (Clement *et al.,* 2000) is used to construct a haplotype network from the sequence data. A nested cladogram is created from the haplotype network. Geodis (Posada *et al.*, 2000) is used to carry out permutational contingency analysis to test for statistically significant associations of haplotypes with geographical location. The results are interpreted according to Templeton's key (Templeton *et al.,* 1995). NCA is implemented using the automated method of Panchal (2007). NCA examines various other issues such as gene flow, bottlenecks and population expansion.

1.4.8 METHODS USED IN THIS STUDY

This study contains both phenetic and phylogenetic analyses. Appropriate genetic distance models were estimated with either Modeltest (Posada and Crandall, 1998) or Mr Modeltest (Nylander, 2004) (for Bayesian analysis). Distance-based trees were constructed using the neighbour-joining method. The reliability of nodes in both neighbour-joining and maximum parsimony trees was estimated by bootstrapping. The reliability of nodes in Bayesian trees was indicated as posterior probabilities. Population demography was estimated using neutrality statistics and mismatch analysis. Nested Clade analysis of the geographical distribution of genetic haplotypes was carried out using the automated method of Panchal (2007). MaxEnt Ecological Niche Modelling (Phillips *et al.,* 2006) was used for predicted distribution.

1.5 AIMS AND OBJECTIVES OF THIS STUDY

The aim of this project was to investigate the genetic diversity of *Chaerephon leucogaster* populations from Madagascar and the islands of Mayotte and Pemba in the western Indian Ocean. A further aim was to investigate the relationship of *C. leucogaster* to *C. pumilus* from Madagascar and to draw conclusions on the implications of my study for conservation. The scope of this study is limited by the non-availability of African samples, so the conspecificity of the small west African forms is not addressed.

The objectives of this project were:

(i) To assess genetic diversity by sequencing the mitochondrial cytochrome *b* and D-loop regions from representative samples.

(ii) To analyse sequence variation using phenetic (Neighbour-Joining) and cladistic (Bayesian Analysis and Maximum Parsimony) methods.

(iii) To establish the number of haplotypes present in the sample, to characterize them, and to search for the presence of statistically-significant phylogeographic structure among haplotypes.

(iv) To carry out a study of the population demography of *C. leucogaster* using diversity and neutrality statistics and mismatch distribution analysis of D-loop sequence data.

(v) To create a predicted distribution for *C. leucogaster* in Madagascar using MaxEnt Ecological Niche Modelling (Phillips *et al.*, 2006).

(vi) To make inferences about the number of species, Evolutionary Significant Units (ESUs) and/or Management Units (MUs) present in the sample.

(vii) To make recommendations about the conservation and management of genetic diversity of *Chaerephon leucogaster* in Madagascar and the neighbouring islands of Mayotte and Pemba.

2. MATERIALS AND METHODS

2.1 SAMPLE COLLECTION AND STORAGE

Samples of *Chaerephon leucogaster* tissue from Madagascar, Mayotte (Comoros) and Pemba (Tanzania) were provided by Steven M. Goodman, Fanja Ratrimomanarivo and William Stanley. Samples were stored in 70 % ethanol and, after being transferred to the laboratory, were stored at -20º C until required for use (Table 2.1 – Appendix 1). The outgroups used in this study comprised samples of other *Chaerephon* species (*pumilus*) from eastern Madagascar, and two Malagasy molossids from a different genus – *Mops leucostigma* and *M. midas* (Table 2.2 – Appendix 1).

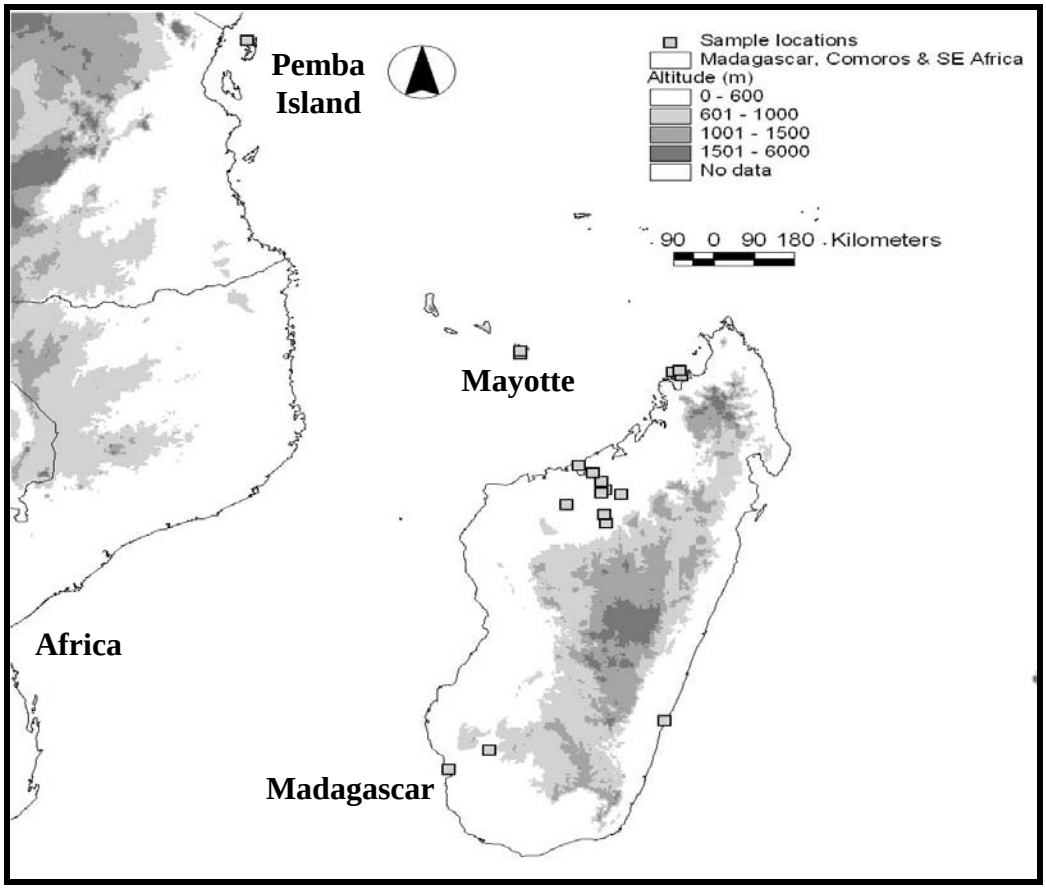

Figure 2: Map showing the distribution of *Chaerephon leucogaster* study samples. These were taken from the western region of Madagascar, a locality on the east coast of Madagascar (Manakara), the north-west Malagasy offshore islands (Nosy Be and Nosy Komba), Mayotte (Comoros Archipelago) and the offshore Tanzanian island of Pemba.

2.2 DNA ISOLATION

DNA was extracted using the Qiagen® DNeasy Extraction Kit (250) according to the manufacturer's instructions. Sample stocks in buffer AE (Qiagen) were stored at -20º C. Working stocks were stored at 4º C.

2.3 DNA CONCENTRATION AND MOLECULAR WEIGHT ASSESSMENT

2.3.1 FLUOROMETRY

The isolated DNA was quantified using a Hoefer ™ DyNA Quant® 200 Fluorometer. The blank solution consisted of 9 ml of water mixed with 1 ml 10X TNE and 1 µL H33258 dye stock. 2 ml of the blank solution was added to a cuvette and the fluorometer was zeroed. Two microlitres (2 µl) of the DNA standard (calf thymus – 1 mg ml^{-1}) was then added to 2 ml of blank solution and the fluorometer was calibrated to 100 ng μl^{-1}. The machine was zeroed, after which 2 µl DNA solution was added and the concentration measured.

2.3.2 SPECTROPHOTOMETRY

Spectrophotometry was also used to quantify DNA samples. One microlitre (1 µl) of double-deionised water was used to zero the Nanodrop spectrophotometer. One microlitre (1 µl) of blank solution (AE buffer from Qiagen) was then added to calibrate the instrument, after which 1 µl DNA solution was added and the concentration measured.

2.3.3 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was conducted to ensure that the isolated DNA was not degraded. A 1 % (w/v) gel of agarose in 0.5X TBE buffer was cast. One hundred microlitres (100 µl) ethidium bromide (0.05 mg ml^{-1}) was added to the gel to allow visualization of the DNA bands. Five micolitres (5 µl) of each DNA sample was mixed with 3 µL loading dye prior to loading into the well. Five microlitres (5 µl) molecular weight marker III (Roche, Germany) or O'Gene Ladder (100 bp) (Fermentas) were co-electrophoresed on each gel as a molecular weight marker.

Samples were electrophoresed at 100 V for one and half hours in 0.5X TBE running buffer. A Uvitec UV transilluminator was used to visualize the DNA and the image was captured using a Uvitec digital camera.

2.4 DNA SEQUENCING USING THE POLYMERASE CHAIN REACTION (PCR)

2.4.1 PCR AMPLIFICATION

The polymerase chain reaction was used in this study to amplify the mitochondrial cytochrome *b* gene and D-loop region. 25 μl PCR reaction mixes contained 30 ng template DNA, $MgCl_2$ (4 mM), Buffer (1x), forward primer (1 μM), reverse primer (1 μM), dNTPs (2 mM) and *Taq* DNA polymerase (1 u). Reagents were kept on ice at all times. PCR amplification reactions were conducted in a Perkin Elmer GeneAmp® PCR system 2400 thermal cycler according to a preset thermal cycling procedure. Cytochrome *b* is a large gene (1140 bp) and was therefore amplified as two overlapping fragments using two sets of primers ([L14723 and H15553], [L15146 and H15915]) (Table 2.3). The cycling parameters for cytochrome *b* amplification are described in Table 2.4.

Table 2.3: Sequences of primers used in PCR amplification of the mitochondrial cytochrome *b* gene and D-loop regions.

Region Amplified	**Primer name**	**Primer sequences**
Cytochrome *b*	L14723	5'ACCAATGCAATGAAAAATCATCGTT3'
	H1553	5'TAGGCAAATAGGAAATATCATTCT3'
	L15146	5'CATGAGGACAAATATCATTCTGAG3'
	H15915	5'TCTCCATTTCTGGTTTACAAGAC3'
Mitochondrial D-loop	P	5'TCCTACCATCAGCACCCAAAGC3'
	F	5'GTTGCTGCTTTCACGGAGGTAG3'

Table 2.4: Thermal cycling parameters for amplification of the cytochrome *b* gene.

1 cycle	**36 cycles**			**1 cycle**	**Hold**
94°C	94°C	50°C	72°C	72°C	15°C
4 mins	1min	1.30 mins	2 mins	10 mins	∞

The mitochondrial D-loop was amplified as a single PCR product using primers P and F (Table 2.3). The thermal cycling parameters for D-loop amplification are described in Table 2.5.

Table 2.5: Thermal cycling parameters used for the amplification of the mitochondrial D-loop region.

1 cycle	**40 cycles**			**1 cycle**	**Hold**
95°C	95°C	55°C	72°C	72°C	15°C
2 mins	1min	1.30 mins	2 mins	7 mins	∞

Amplification was done in triplicates to show reproducibility and accuracy. A negative control was also run under the same amplification conditions. The reaction contained all reagents with the exception of the DNA.

The PCR products were separated by electrophoresis in 1.5 % agarose gels prepared and run as described in section 2.3.3. Samples were electrophoresed overnight at 15 V. The amplified bands were selected by their position relative to the molecular weight marker and excised from the gel for later DNA extraction. Excised bands were stored in a 1.5 ml microfuge tube at -20° C.

2.4.2 PURIFICATION OF PCR PRODUCTS

DNA was extracted from gel slices using a QIAquick® Gel Extraction Kit (using a micro-centrifuge) according to manufacturer's instructions. Samples were electrophoresed in 1 % (w/v) agarose gels to confirm the lack of undesired co-amplification products and to provide a visual estimate of the concentration and integrity of the DNA in comparison with a known concentration of Molecular Weight Marker III (Roche, Germany). 20 µl DNA (10 ng μl^{-1}) was sent for sequencing.

2.4.3 DNA SEQUENCING

Sequencing of PCR products were conducted at Inqaba Biotechnical Industries Pty. Ltd. Samples were sequenced in the forward and reverse directions using the primers used in the initial amplifications. Electropherograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia).

2.4.4 DATA ANALYSES

2.4.4.1 Construction of consensus sequences

Consensus sequences were constructed by comparing forward and reverse sequences using the Bioedit Sequence Alignment Editor, version 5.0.9 for windows 95/98 NT (Hall, 1999). The Clustal W function of Bioedit (version 5.0.9) (Hall, 1999) was used to perform a multiple alignment which was then further edited by visual inspection. Sequences were trimmed to a uniform length. Aligned and trimmed files were imported into Clustal X, version 1.81 (Thompson *et al.*, 1997), and re-saved as nexus (.nxs) files.

2.4.4.2 Data Saturation

The programme DAMBE (Xia and Xie, 2001) was used to check for saturation of the data by plotting transitions and transversions versus genetic distance. Xia's test of substitution saturation (Xia *et al.*, 2003) was conducted to provide insight into the level of saturation of the data.

2.4.4.3 Molecular Model

Modeltest (Posada and Crandall, 1998) using the AIC criterion was applied to determine the model of substitution which best fits the sequence data. The model selected for cytochrome *b* was the HKY+I+G model and the model selected for the D-loop dataset was the GTR model.

The models were selected using AIC. This approach has several advantages as highlighted by Posada and Buckley (2004) they are able to simultaneously comparemultiple nested or nonnested models account for model selection uncertainty, and allow for model-averaged inference**.**

2.4.4.4 Bayesian analysis

Bayesian analysis (Mr Bayes, version 3.0B4, Ronquist and Huelsenbeck, 2001) utilized four Markov chains, which were run for five million generations each, with sampling every 1000 generations, resulting in a sample of 50 000 trees and using default priors. We assessed convergence by measuring the standard deviation of the split frequency among parallel chains. Chains were considered to have converged once the average split frequency was lower than 0.01. We summarized results by discarding the first 5000 trees and doing a majority-rule consensus from the remaining 45 000 trees. The burn-in value was estimated by plotting likelihood values against number of generations to determine where the likelihood plateaued.

Parallel analyses were conducted however we did not present the results as they were congruent.

2.4.4.5 Parsimony and Genetic Distance Analyses

PAUP4.0b10 for Macintosh (Swofford, 2002) was used to create neighbour joining and maximum parsimony trees. For parsimony analysis, the random additions sequence option (n = 100) for discrete, unordered characters was used. The heuristic search option was used to search for the shortest tree using the tree bisection-reconnection (TBR) branch swapping option. Bootstrap support was calculated by using bootstrap re-sampling analysis (1000 iterations) (Felsenstein, 1985; Felsenstein and Kishino, 1993; Hillis and Bull, 1993).

2.4.4.6 Analysis of Molecular Variance

Arlequin 3.01 (Excoffier *et al.,* 2005) was used to test for significant molecular variance between the groups defined by phylogeographic analysis. Individuals were separated into three groups i.e. the 13º S latitude band, the 15-17º S latitude band and the 22-23º S latitude band. Fixation indices were calculated 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.

2.4.4.7 Haplotype and population genetic analyses

DnaSP (DNA Sequence Polymorphism) version 4.10.9 (Rozas *et al.,* 2003) was used to determine the number of haplotypes in each data set. A statistical parsimony haplotype network was created to depict the relationships between haplotypes using the program TCS version 1.13 (Clement *et al.*, 2000). Population genetic analyses of the D-loop dataset were carried out in DnaSP to determine haplotype (*h*) and nucleotide (π) diversity values, neutrality test statistics (Fu's [1997] F*s* and Fu and Li's [1993] D* and F*) and a mismatch distribution. Time since population expansion was calculated using the formula of Rogers and Harpending (1992) - $\tau = 2ut$. Tau (τ) was calculated using DnaSP version 4.10.9, *u* was the product of the mutation rate (mutations per site per generation) and sequence length and t is time in generations since expansion.

2.4.4.8 Phylogeographic Analysis

Inferences of phylogeographic concordance within Malagasy samples, based on the D-loop dataset, were calculated using Nested Clade Phylogeographic Analysis implemented with the automated method of Panchal (2007). TCS (Clement *et al.*, 2000) was used for estimating gene genealogies and Geodis (Posada *et al.*, 2000) was used for cladistic nested analysis of the geographical distribution of the haplotypes. The criteria of Crandall and Templeton (1993) were used to resolve uncertainties in the results, which were interpreted using Templeton's key (Templeton *et al.*, 1995).

2.4.4.9 Ecological Niche Modelling Analysis

The Maximum Entropy (MaxEnt) method (Phillips *et al.*, 2006) was used to estimate the predicted distribution of *C. leucogaster*. Ten continuous environmental variables (altitude and nine bioclimatic variables) were used (WORLDCLIM version 1.4., www.biogeo.berkeley.edu; Hijmans *et al.*, 2005) and showed means, extremes and seasonal variation of temperature and precipitation. These included Bio1 (mean annual temperature), Bio4 (temperature seasonality), Bio5 (maximum temperature of warmest month), Bio6 (minimum temperature of coldest month), Bio7 (annual range of temperature), Bio12 (annual precipitation), Bio13 (precipitation of wettest month), Bio14 (precipitation of driest month) and Bio15 (precipitation seasonality). The MaxEnt model was run with all distribution records (100 % training), the regularization multiplier was set at 1.0 and the maximum number of iterations was 1000. The model performance was assessed with sensitivity, specificity and discrimination ability (area under the curve [AUC] of a receiver operating

characteristic [ROC] plot of sensitivity versus 1-specificity). The continuous model output was transformed to a map representing probabilities. The jack-knife procedure that is implemented in MaxEnt was used to evaluate the contribution of each explanatory variable.

3. RESULTS

3.1 DNA PURITY, CONCENTRATION AND MOLECULAR WEIGHT

High molecular weight DNA was required as the substrate for PCR amplification. Agarose gel electrophoresis indicated the presence of high molecular weight bands (Fig 3.1). 200 µl DNA was extracted for each sample, and ranged in concentration from 30 to 90 ng μl^{-1}. There were no extensive smears produced, indicating that there was little degradation.

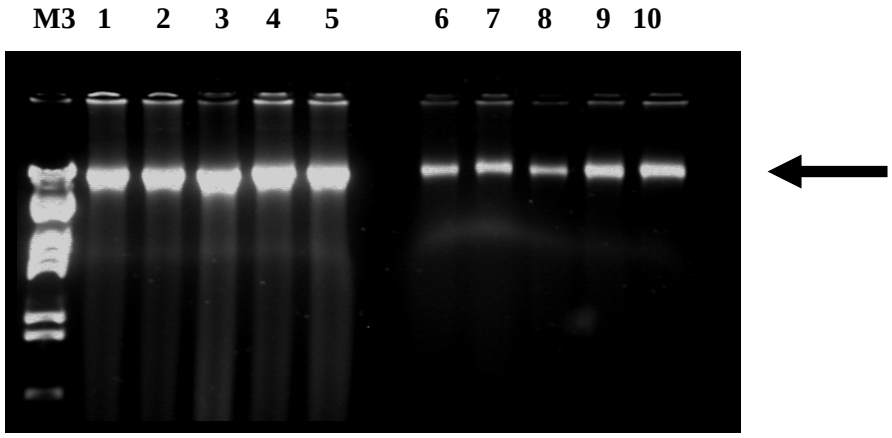

Figure 3.1: Electropherogram of DNA isolated from *Chaerephon leucogaster* and separated by electrophoresis in a 1 % agarose gel stained with ethidium bromide. Lane M3 contains molecular marker III (Roche). Lanes 1-5 show DNA isolated from Mahajanga samples and Lanes 6-10 show DNA isolated from Andranofasika samples. High molecular weight DNA is marked with an arrow.

3.2 PCR AMPLIFICATION

PCR amplification of the cytochrome *b* gene using the primer combinations L23 with H53 and L46 with H15 was successful (Figure 3.2). The PCR product produced bright bands representing the

desired amplified regions. Products were identified by their molecular weight relative to that of the molecular weight marker. Only one clear band was produced in each amplification.

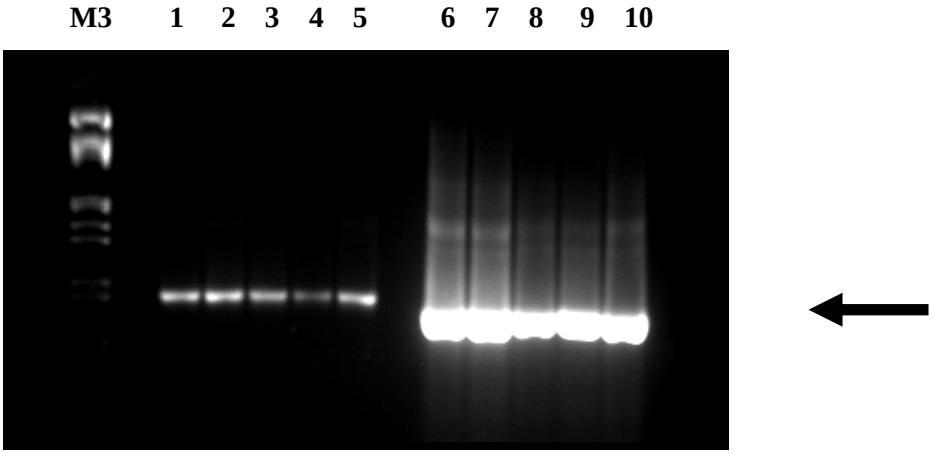

Figure 3.2: PCR amplification of the cytochrome *b* region of *Chaerephon leucogaster* samples from Andranofasika. Samples were separated by electrophoresis in 2 % agarose gels. Lane M3 represents the molecular marker XVII (Roche). Lanes 1-5 indicate the 5' fragment (primers H53 and L23), and Lanes 6-10 indicates the 3' fragment (primers H15 and L46).

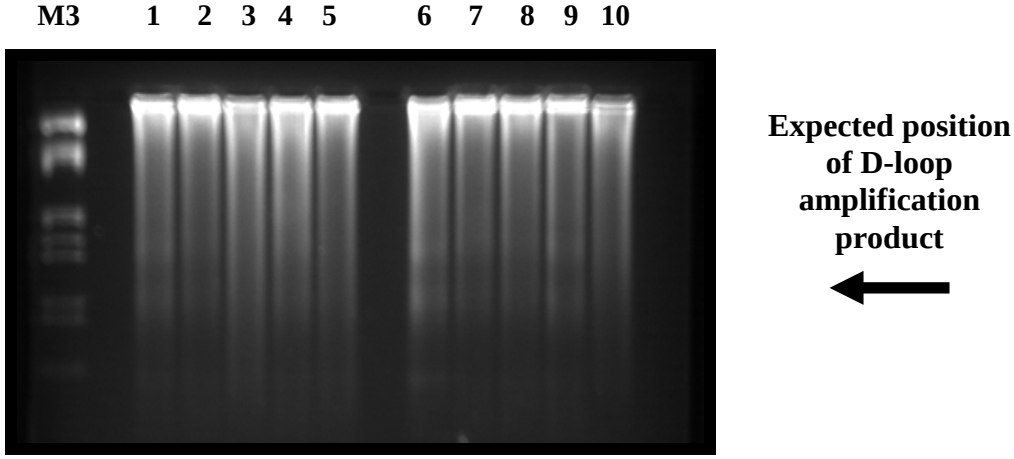

Figure 3.3: Electropherogram of initial attempt at PCR amplification of the mitochondrial D-loop. No amplification product was present. Lanes 1 - 5 show samples from Andranofasika and Lanes 6 - 10 show samples from Ankazomborona.

The PCR amplification of the mitochondrial D-loop region using the P and F primers presented some problems. Initial attempts showed streaking and no distinct amplification (Figure 3.3). The presence of high molecular weight bands near the wells indicated that the template DNA concentration might have been too high. A 1:6 dilution of the DNA resulted in successful PCR showing strong D-loop amplification (Figure 3.4).

The bands were selected based on previous literature and the expected position as described by Irwin *et al*. (1991) and Wilkinson and Chapman (1991). After sequencing the product was then checked and verified by making comparisons to other amplified cyt *b* and D-loop genes on Genbank.

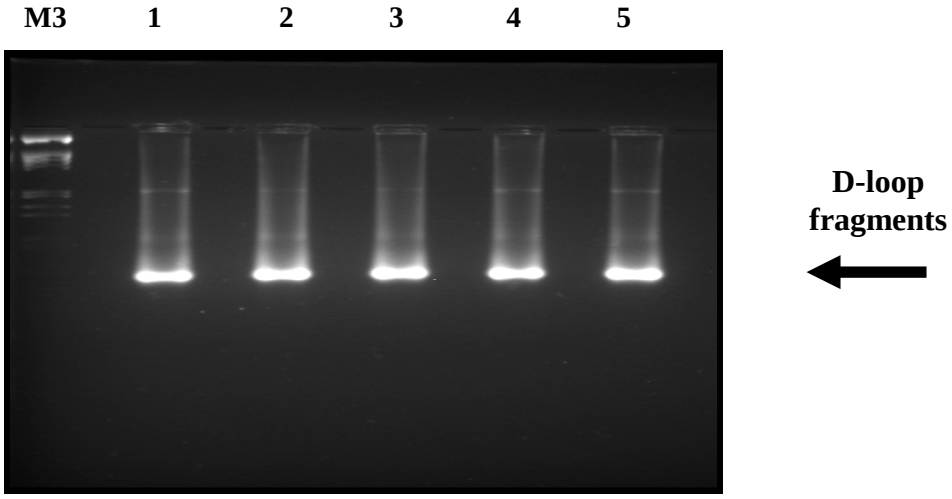

Figure 3.4: PCR amplification of the mitochondrial D-loop. Lane M3 contains molecular marker III (Roche). Lanes 1 - 5 contain samples from Mahajanga. The desired D-loop fragment is indicated by the arrow.

3.3 ASSESSMENT OF THE PURITY OF THE PCR-AMPLIFIED REGIONS

Amplified bands were excised from the gel and the DNA extracted as described previously in section 2.4.2. Samples were electrophoresed in 1 % agarose gels; a single band indicated the

absence of contaminating co-amplification products (Figure 3.5). The concentration of the purified DNA ranged from 5 - 50 ng μl^{-1}.

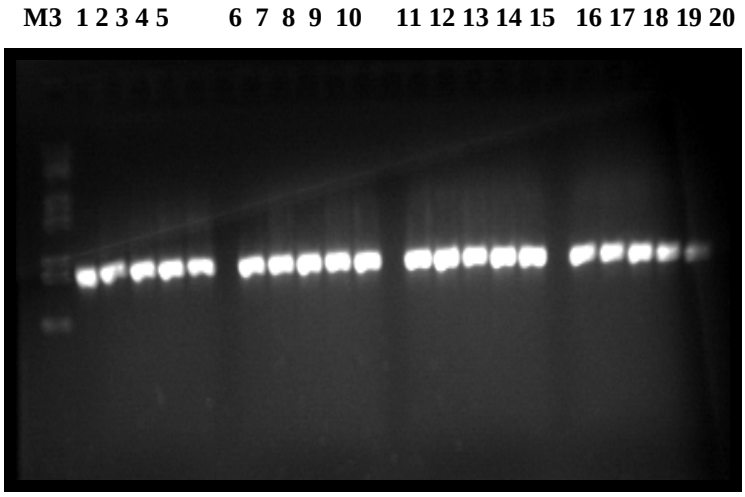

Figure 3.5: 1 % agarose gel electrophoretic separation of DNA fragments purified from gel slices. Lane M3 contains molecular marker III (Roche). Lanes 1 – 20 contain purified mitochondrial D-loop fragments. Lanes 1 - 5 and 11 - 15 contain samples from Mahajanga. Lanes 6 - 10 and 16 - 20 contain samples from Andranofasika.

3.4 SATURATION DATA

The program DAMBE (Xia and Xie, 2001) was used to show the relationship between transitions/transversions and genetic distance and to assess the level of saturation in the sequence data set (Figures 3.6 and 3.7). Saturation analyses demonstrated that the data set showed little/no substitution saturation for cytochrome *b* and mitochondrial D-loop sequences as it appeared to fit a straight line model for the ingroup data.

Xia's test of substitution saturation (Xia *et al.*, 2003) was conducted on the cytochrome *b* dataset. The index of substitution saturation (Iss - 0.0913) was less than the critical value (Iss.c - 0.7728) ($p < 0.0001$), demonstrating that there was little saturation in the data.

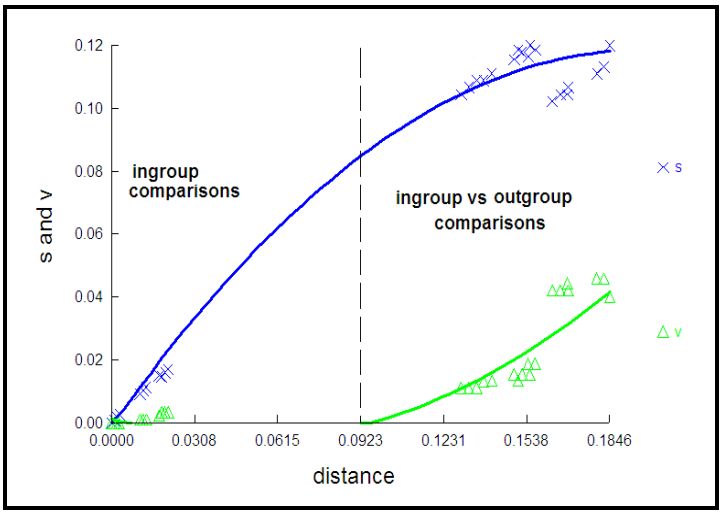

Figure 3.6: Transversions (Δv)/ transitions (xs) versus genetic distance for mitochondrial cytochrome *b* sequences. Solid lines represent the least squares best-fit.

Xia's test of substitution saturation (Xia *et al.*, 2003) was conducted on the D-loop dataset. The index of substitution saturation (Iss - 0.1780) was lower than the critical value (Iss.c - 0.6920) ($p < 0.0001$) demonstrating that the ingroup sequence data had little saturation.

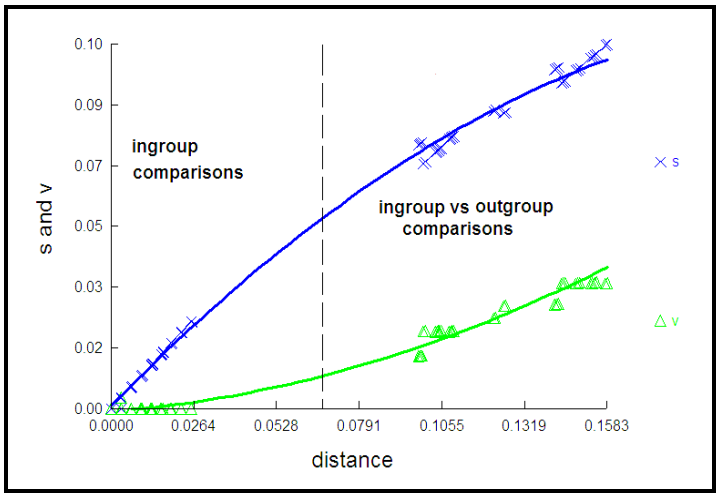

Figure 3.7: Transversions (Δv)/transitions (xs) versus genetic distance of mitochondrial D-loop sequences. Solid lines represent the least squares best-fit.

3.5 SEQUENCE DATA ANALYSIS

3.5.1 CYTOCHROME *b*

3.5.1.1 Phylogeographic analyses

Haplotypes

DnaSP version 4.0.9 (Rozas *et al*., 2003) was used to determine the number of haplotypes present in the cytochrome *b* dataset.

Haplotype analysis of 39 *C. leucogaster* samples based on 863 nucleotides of the cytochrome *b* gene yielded six haplotypes based on four variable sites (Table 3.1). The haplotype diversity (*h*) was 0.718 (variance 0.00214) and the nucleotide diversity (π) was 0.00111 (variance < 0.00001). The average number of nucleotide differences between *C. leucogaster* samples was 0.95.

Table 3.1: Cytochrome *b* haplotype data.

Taxon	Hap	N	Sample codes
Chaerephon leucogaster	1	19	FMNH 192886, 184259, 184954, 184955, 184956, 184957, 184958, 184959, 185030, 188496, 184923, 184922, 184239, 184240, 184238, 194028, 192889, 194019, 185228
	2	2	FMNH 184923, 184924
	3	5	FMNH 184925, 184926, 184950, 184951, 184953
	4	4	FMNH 184975, 184976, 184977, 184978
	5	1	FMNH 184979
	6	8	FMNH 187754, 187755, 188498, 187750, 188643, 188642, 188640, 188644
Chaerephon pumilus	8	4	FMNH 185230, 185259, 185286, 185314
	9	1	FMNH 187816
	10	1	FMNH 185322
	11	1	FMNH 184678
Mops leucostigma	12	1	FMNH 194508
Mops midas	13	1	FMNH 184306

Hap – Haplotype number. N – number of samples.

Haplotype Network

A statistical parsimony network of the cytochrome *b* data showing mutational relationships between haplotypes when set at a 95 % confidence limit is presented in Figure 3.8. There are six haplotypes in this network; adjacent haplotypes are separated by one mutational step. *Chaerephon leucogaster* from Manakara, a locality on the eastern side of Madagascar shares haplotype 1 (Table 3.1) with *C.*

leucogaster from the western side of the island. *C. pumilus* (Madagascar) is separated from the closest *C. leucogaster* haplotypes by 15 mutational steps. This connection was associated with less than 95 % confidence.

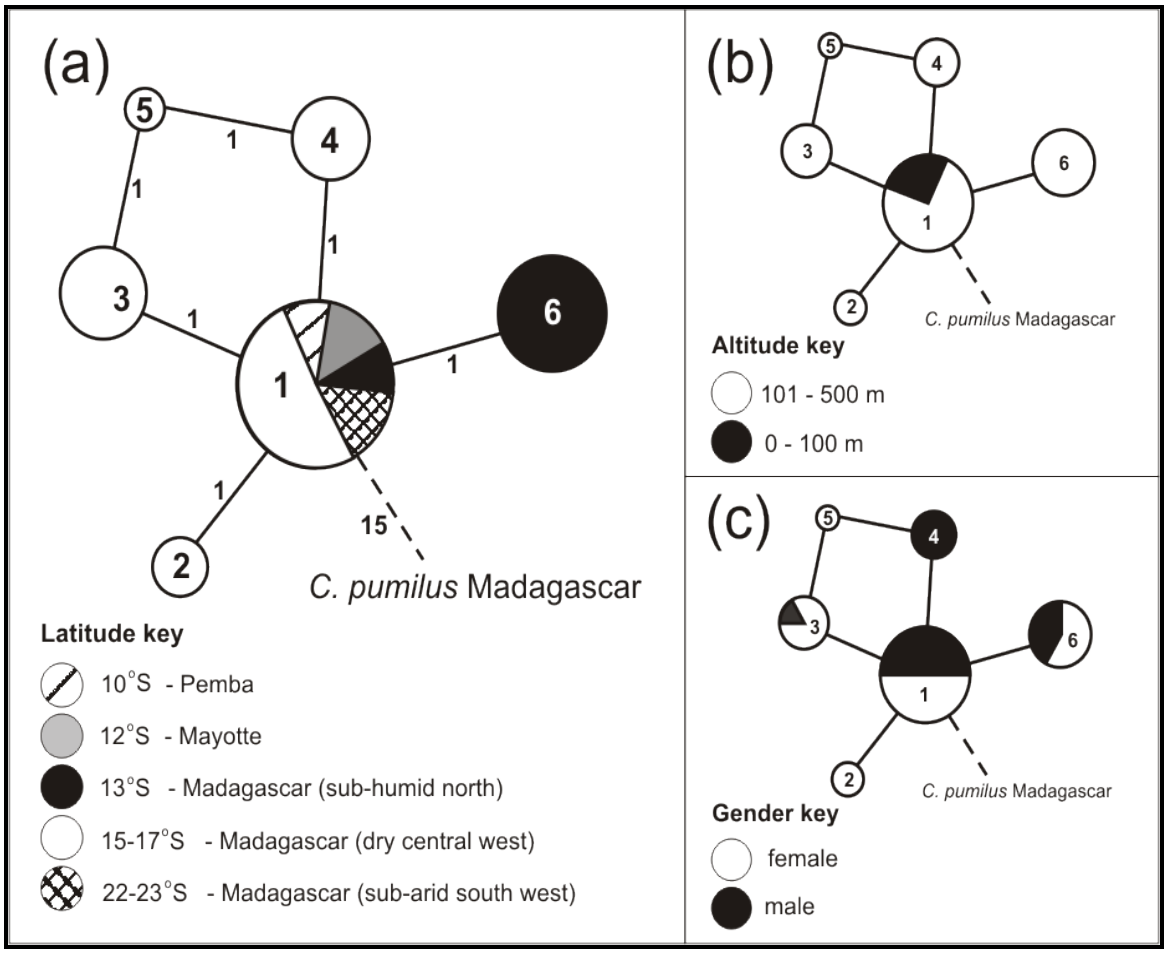

Figure 3.8: Networks showing the mutational relationships between six mitochondrial cytochrome *b* haplotypes of *Chaerephon leucogaster* with reference to the outgroup, *C. pumilus* (Madagascar): (a) overlay of latitude, (b) overlay of altitudes and (c) overlay of gender. Numbers within circles are haplotype numbers. Numbers adjacent to the connecting lines are mutational steps.
_____95 % confidence, --------- < 95 % confidence.

Relationship between haplotypic variation and latitude, altitude and gender

Sample latitude, altitude and gender were superimposed on the haplotype network to illustrate relationships (or lack thereof).

(a) Latitudinal Variation: There are five location-specific *C. leucogaster* haplotypes (2, 3, 4, 5 and 6) and one shared haplotype (1) found at all locations. Haplotype 6 comprises samples from the 13º S latitude band, namely the offshore islands of Nosy Be and Nosy Komba, and adjacent regions of mainland Madagascar. Haplotypes 2, 3, 4 and 5 are from the middle latitude band (15º S - 17º S), which is characterized as the dry central west. Haplotype 1 is the most common haplotype and is shared by samples from the central, north and extreme south (Sakaraha) of Madagascar, as well as Pemba (Tanzania) and Mayotte (Comoros).

(b) Altitude Variation: Most *C. leucogaster* samples were obtained from altitudes greater than 100 m (101 - 500 m). Haplotype 1 comprises samples from altitudes above and below 100 m. There is no apparent association of haplotypes with altitude.

(c) Gender: There is no apparent association of haplotypes with gender. The major haplotypes (1, 3 and 6) were shared by both genders, whilst the minor haplotypes were either exclusively female (2 and 5) or exclusively male (4).

3.5.1.2 Phylogenetic and Phenetic Analyses

The analyses for the cytochrome *b* gene were performed using the HKY+I+G substitution model (section 2.4.4) in PAUP 4.0b10 (Swofford, 2002).

Genetic Distances

Individual pairwise cytochrome *b* genetic distances (HKY+I+G) were calculated for the six *C. leucogaster* cytochrome *b* haplotypes, four *C. pumilus* haplotypes, *M. leucostigma* and *M. midas* (Table 3.2). Genetic distances between *C. leucogaster* samples from Madagascar ranged from 0.12 % to 0.35 %. Genetic distances of 0.35 % occurred between haplotypes 2 and 5 as well as 5 and 6. *Chaerephon leucogaster* separated from *C. pumilus* (Madagascar) with a mean genetic distance of 2.16 % (range, 1.77 % - 2.62 %). The *Mops* outgroups (*M. midas* and *M. leucostigma*) are separated from the *C. leucogaster* ingroups by mean distances of 12.0 % (range 11.78 % – 12.07 %), and 11.9 % (range 11.75 % – 12.07 %), respectively.

Table 3.2: HKY+I+G genetic distance ($x10^2$) based on 863 nt of the mitochondrial cytochrome *b* gene, between six *Chaerephon leucogaster* haplotypes, and the outgroups (*C. pumilus*, *Mops leucostigma* and *M. midas*).

	hap	1	2	3	4	5	6	7	8	9	10	11
C. leucogaster	1	-										
C. leucogaster	2	0.116	-									
C. leucogaster	3	0.116	0.232	-								
C. leucogaster	4	0.116	0.232	0.232	-							
C. leucogaster	5	0.232	0.349	0.116	0.116	-						
C. leucogaster	6	0.116	0.232	0.232	0.232	0.349	-					
C. pumilus	7	2.375	2.497	2.497	2.497	2.619	2.497	-				
C. pumilus	8	2.132	2.253	2.253	2.253	2.375	2.253	0.232	-			
C. pumilus	9	1.769	1.890	1.890	1.890	2.011	1.890	0.583	0.349	-		
C. pumilus	10	1.890	2.011	2.011	2.011	2.132	2.011	0.701	0.466	0.116	-	
M. leucostigma	11	11.914	11.917	12.071	11.751	11.907	12.071	11.896	11.577	11.258	11.259	-
M. midas	12	11.918	12.065	12.065	11.771	11.917	12.065	12.495	12.198	11.757	11.903	12.682

Bayesian, maximum parsimony and neighbour-joining trees were congruent in structure (Figure 3.9). *Chaerephon* haplotypes formed an exclusive cluster (bootstrap 100 %, pp 1.00) with respect to the outgroups (*M. leucostigma* and *M. midas*). *Chaerephon leucogaster* haplotypes from Madagascar, Mayotte and Pemba formed a strongly supported cluster (bootstrap 99 - 100 %, pp 1.00) which was distinct from the sister species *Chaerephon pumilus* from Madagascar, which also formed a strongly supported group (bootstrap 100 %, pp 1.00). The *Chaerephon leucogaster* group contains a subcluster which comprises haplotypes 3 and 5, but which is essentially unsupported (bootstrap < 50 %, pp 0.68).

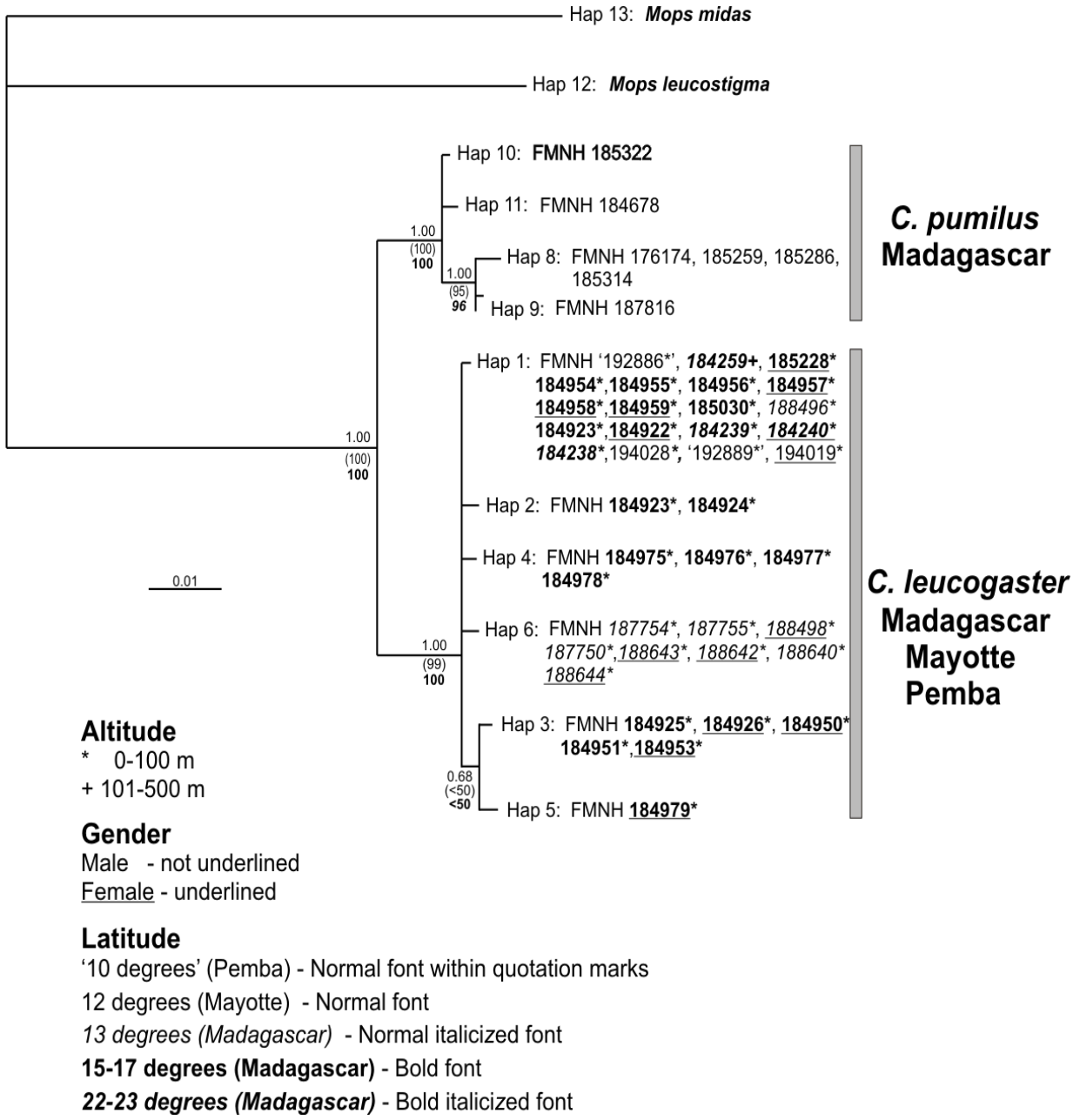

Figure 3.9: Dendrogram (Bayesian, maximum parsimony and neighbour-joining) based on 863 nt of the mt cytochrome *b* gene, showing relationships between 39 samples of *Chaerephon leucogaster* with reference to the outgroups, *C. pumilus* (Madagascar), *Mops leucostigma* and *M. midas*. Nodal support is shown as Bayesian posterior probabilities (normal font - top), and bootstrap values for maximum parsimony (brackets - middle) and neighbour joining (bold - bottom).

3.5.1.3 Analysis of Molecular Variance (AMOVA)

27.98 % of the variance occurred among the three geographically defined groups (13^{o} S, 15 – 17^{o} S and 22 – 23^{o} S) (*P* (random value >= observed value) = 0.01760) (Table 3.3). Variance among populations within groups was 40.12 % with *P* (random value >= observed value) < 0.0002. Differences within populations accounted for 31.90 % of the variance, with *P* (random value >= observed value) < 0.0002.

Table 3.3 Analysis of Molecular Variance (AMOVA) of cytochrome *b* data.

Source of Variation	df	Sum of squares	Variance components	% of variation
Among groups	**2**	**3.806**	**0.12757 Va**	**27.98**
Among populations within groups	**10**	**6.108**	**0.18295 Vb**	**40.12**
Within populations	**22**	**3.200**	**0.14545 Vc**	**31.90**
Total	**34**	**13.114**	**0.45597**	

Fixation Indices	
$\mathbf{F_{SC}}$	**0.55709**
$\mathbf{F_{ST}}$	**0.68100**
$\mathbf{F_{CT}}$	**0.27977**

Significance tests (1023 permutations)

Vc and FST : P(random value < observed value) < 0.0001
P(random value = observed value) < 0.0001
P(random value <= observed value) < 0.0002+-0.0000

Vb and FSC : P(random value > observed value) < 0.0001
P(random value = observed value) < 0.0001
P(random value >= observed value) < 0.0002+-0.0000

Va and FCT : P(random value > observed value) = 0.01564
P(random value = observed value) = 0.00196
P(random value >= observed value) = 0.01760+-0.00392

3.5.2 MITOCHONDRIAL D-LOOP

3.5.2.1 Phylogeographic Analyses

Haplotypes

Analysis of 71 *C. leucogaster* sequences revealed nine variable sites (of 338 nucleotides) and eleven haplotypes (Table 3.4). The haplotype diversity was 0.870 (variance 0.00052), whilst the nucleotide diversity per site was 0.00737 (variance 0.00002). The average number of nucleotide differences between samples was 2.00.

Table 3.4: D-loop haplotype data for taxa used in the phylogeographic analysis.

Taxon	Hap	N	Sample codes
Chaerephon leucogaster	1	2	FMNH 184263, 184264
	2	18	FMNH 187750, 187751, 187752, 187753, 187754, 187755, 187756, 188495, 188497, 188498, 188499, 188500, 188640, 188641, 188642, 188643, 188644, 185228.
	3	4	FMNH 184237, 184238, 184239, 184240
	4	6	FMNH 188496, 184902, 184922, 184923, 184955, 185028
	5	5	FMNH 184604, 184605, 184606, 184607, 184608
	6	10	FMNH 184896, 184897, 184898, 184899, 184900, 184901,184915, 184917, 184919, 184920
	7	6	FMNH 184916, 184924, 184954, 184957, 184958, 184959
	8	6	FMNH 184925, 184926, 184950, 184951, 184952, 184953
	9	10	FMNH 184975, 184977, 184979, 185020, 185021, 185022,185027, 185029, 185030, 184956
	10	2	FMNH 184973, 184974
	11	2	FMNH 192889, 194028
Chaerephon pumilus	12	2	FMNH 188088, 188089
	13	2	FMNH 187834, 187835
	14	3	FMNH 185260, 185286, 185315
	15	2	FMNH 187797, 187799
Mops leucostigma	16	1	FMNH 185098
Mops midas	17	1	FMNH 184306

Haplotype Network

Statistical parsimony analysis of D-loop data (using TCS version 1.2.1) was used to show mutational relationships between haplotypes when set at a 95 % connection limit. In the haplotype network (Figure 3.10), all nearest-neighbours are separated by one mutational step with the exception of haplotype 11, comprising samples from Mayotte and Pemba, which is separated by six mutational steps from its closest neighbors in the network (haplotypes 1, 2 and 8). *Chaerephon pumilus* (Madagascar) is 25 mutational steps different from any of the Malagasy *C. leucogaster*

haplotypes. As in the cytochrome *b* network, *C. leucogaster* (from Manakara in eastern Madagascar), shares a haplotype (haplotype 2 - Table 3.3) with the rest of the *C. leucogaster* samples from the western portion of Madagascar.

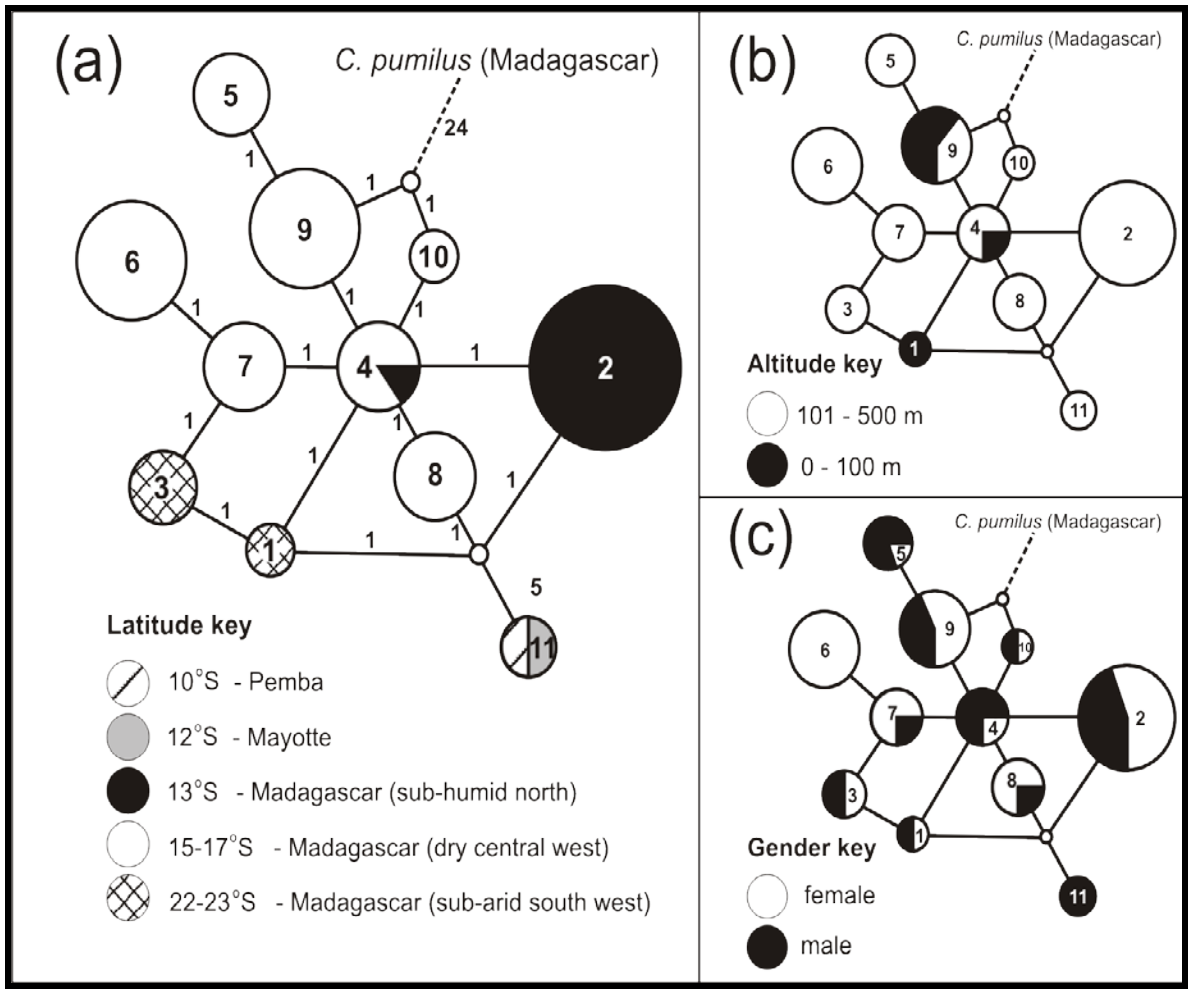

Figure 3.10: Networks showing the mutational relationships between eleven mitochondrial D-loop haplotypes of *Chaerephon leucogaster* with reference to the outgroup, *C. pumilus* (Madagascar): (a) overlay of latitude, (b) overlay of altitude and (c) overlay of gender. Numbers within circles are haplotype numbers. Numbers adjacent to the connecting lines are mutational steps.

_____ 95 % confidence, --------- < 95 % confidence.

Relationship between haplotypes and latitude, altitude and gender

Latitude, altitude and gender of samples were superimposed on the haplotype network to give a visual indication of relationships, or lack thereof, between the variables.

(a) Latitudinal Variation: Of the 11 *Chaerephon leucogaster* haplotypes, only two (4 and 11) were found at more than one location. Haplotype 2 consisted exclusively of samples from 13º S latitude, (the northwest offshore islands of Nosy Be and Nosy Komba, and adjacent regions of the Malagasy mainland) (Figure 3.11). Samples showing haplotypes 1 and 3 occurred exclusively in the extreme south regions and are separated from each other and the rest of the haplotype network by one mutational step. Haplotypes 5 - 10 comprise samples from the dry central west latitude band of 15 - 17º S. Haplotype 4 comprised samples from the 13º S and 15 - 17º S bands.

(b) Altitude Variation: Most of the samples were collected from altitudes above 100 m. Figure 3.10 shows no apparent association of haplotypes with altitude.

(c) Gender: Figure 3.10 shows no apparent association of the haplotypes with gender, although haplotype 6 consists exclusively of females.

3.5.2.2 Phylogenetic and Phenetic Analyses

Neighbour-joining analysis of the D-loop data was not possible owing to a large number of undefined distances generated in PAUP. This may be associated with gaps (indels) within the dataset. Maximum parsimony and Bayesian analyses of haplotypes produced congruent trees (Figure 3.11), which were also congruent with the dendrogram produced by the analysis of the cytochrome *b* data (Figure 3.9). *Chaerephon* (*leucogaster* plus *pumilus*) forms a monophyletic clade with respect to the *Mops* outgroups. This is well-supported (100 % bootstrap) in the maximum parsimony analysis, but poorly-supported in the Bayesian analysis (pp 0.60). *Chaerephon leucogaster* forms a well-supported clade (bootstrap value 98 %, pp 0.95), in which haplotype 11, comprising samples from Mayotte and Pemba, appears basal to a moderately-supported (bootstrap value 76 %, pp 0.95) Madagascar-only clade. There is no well-supported structure within this clade. *Chaerephon pumilus* (Madagascar) forms a sister-clade to *C. leucogaster*; this is not strongly supported (bootstrap value 82 %, pp 0.65).

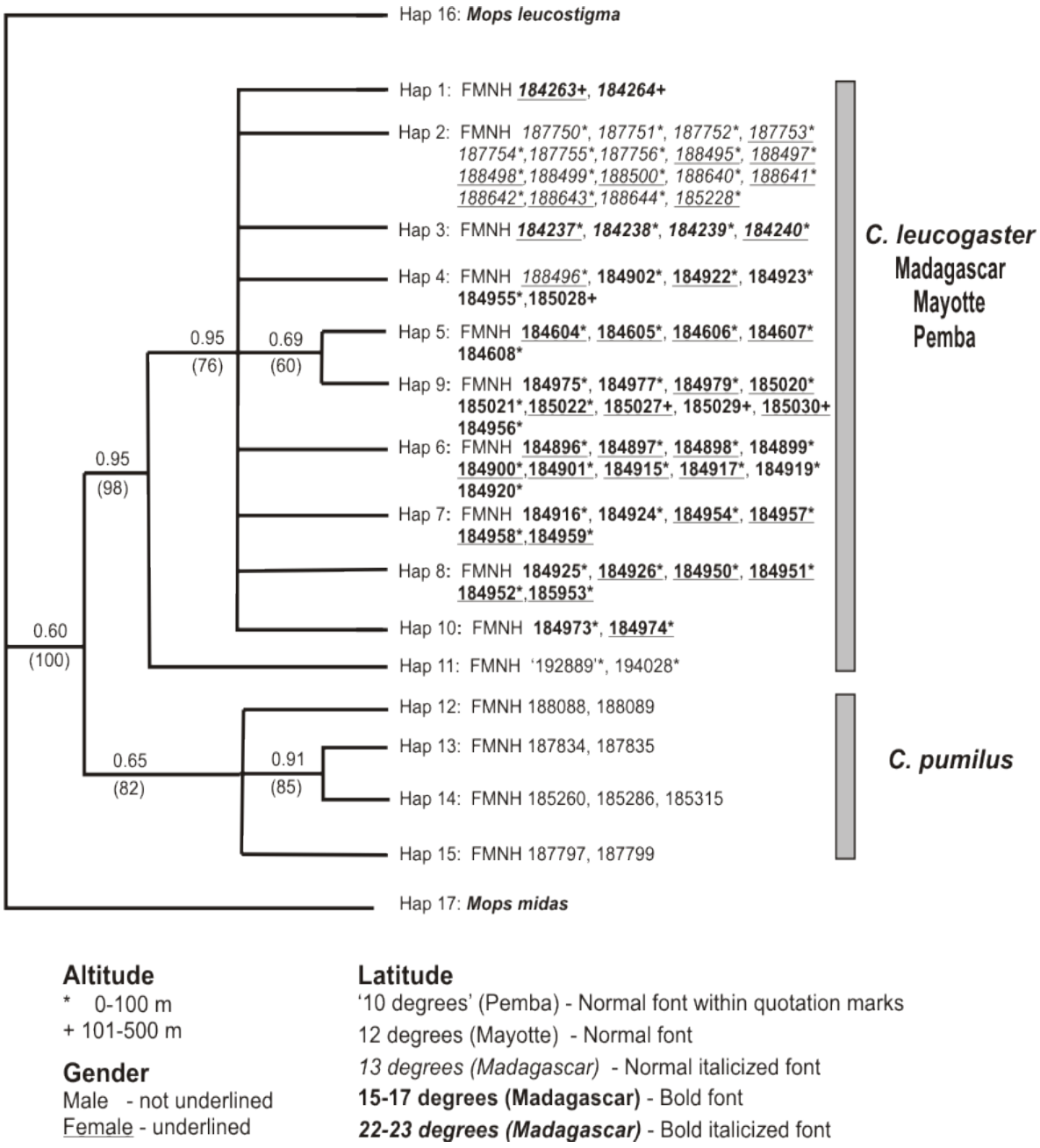

Figure 3.11: Dendrogram (Bayesian and maximum parsimony) based on 338 nt of the mitochondrial D-loop showing relationships between 71 samples of *Chaerephon leucogaster* with reference to the outgroups, *C. pumilus* (Madagascar), *Mops leucostigma* and *M. midas*. Nodal support is indicated as Bayesian posterior probabilities (normal font - top) and bootstrap values for maximum parsimony (brackets - bottom).

3.5.2.3 Analysis of Molecular Variance (AMOVA)

40.44 % of the variance occurred among the three geographically defined groups (13^{o} S, 15 – 17^{o} S and 22 – 23^{o} S) (*P* (random value >= observed value) = 0.00098) (Table 3.5). Variance among populations within groups was 42.27 % with *P* (random value >= observed value) < 0.0002. Differences within populations accounted for 17.29 % of the variance, with *P* (random value >= observed value) < 0.0002.

Table 3.5: Analysis of Molecular Variance (AMOVA) of D-loop.

Source of Variation	df	Sum of squares	Variance components	% of variation
Among groups	**2**	**28.920**	**0.64186 Va**	**40.44**
Among populations within groups	**14**	**43.134**	**0.67090 Vb**	**42.27**
Within populations	**54**	**14.819**	**0.27443 Vc**	**17.29**
Total	**70**	**86.873**	**1.58719**	

Fixation Indices	
F_{SC}	**0.70970**
F_{ST}	**0.82710**
F_{CT}	**0.40440**

Vc and FST: P(random value < observed value) < 0.0001
P(random value = observed value) < 0.0001
P(random value <= observed value) < 0.0002+-0.0000

Vb and FSC: P(random value > observed value) < 0.0001
P(random value = observed value) < 0.0001
P(random value >= observed value) < 0.0002+-0.0000

Va and FCT: P(random value > observed value) = 0.00098
P(random value = observed value) < 0.0001
P(random value >= observed value) = 0.00099+-0.00098

3.5.2.4 Demographic Analysis

Diversity tests, neutrality tests and mismatch distribution analysis were conducted on D-loop data to examine the demographic history (Russell *et al.,* 2005) of *C. leucogaster*. Results are presented in Table 3.6 and Figure 3.12. Two different methods had been conducted to show support that the

population was in equilibrium. Although some studies have indicated which tests are more suitable, studies have also used both of these analyses (Lamb *et al.*, 2012).

Analysis of 370 nucleotides of the mitochondrial D-loop revealed a high haplotype diversity (h = 0.891) and a low nucleotide diversity (π = 0.00934) (Table 3.6). Neutrality tests showed Fu and Li's D*test statistic (1.47561) (P>0.10), Fu and Li's F*test statistic (1.15366) (P >0.10) and Fu's (1997) F*s* statistic (-1.730) (P >0.05) to be non-significant. The expansion co-efficient (S/d) was high (4.80), which confirmed an expanding population. The overall mismatch distribution for the D-loop data was unimodal (Figure 3.12) with a non-significant raggedness value (r =0.0768) (P >0.05) and fitted the model for an expanding population (Rogers and Harpending, 1992).

Chaerephon leucogaster may be considered an expanding population as it meets the criteria set by Hull and Girman (2005). Madagascarien populations have been expanding for between 5,842 years (based on a 33 % divergence rate) and 11, 143 years BP (based on a 17 % divergence rate) (according to Rogers and Harpending, 1992).

Table 3.6: Diversity and neutrality statistics based on 370 nucleotides of the mitochondrial D-loop.

	D-loop	Expectation#
Nucleotide diversity (π)	0.00934	Low
Haplotype diversity (h)	0.891	High
Expansion coefficient (S/d)	4.800	High
Fu and Li's (1993) F *	1.15366	Not significant
Fu and Li's (1993) D *	1.47561	Not significant
Fu's (1997) Fs	-1.730	Significant
Raggedness (rg)	0.0768	
Mismatch distribution	Unimodal	Unimodal
Tau (τ)	1.30322	
Time since expansion (yr BP)	5,842 – 11,143 yr	

\# Expected trends for a model of demographic population expansion (Hull and Girman, 2005)

$\perp$ Value obtained from formula $\tau = 2ut$, following Rogers and Harpending (1992). u was the product of mutation rate (μ) per generation and sequence length (370 bp). 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 $\mu = 3.3 \times 10^{-7}$. t was the time (in generations) since expansion (generation time taken as 2 years).

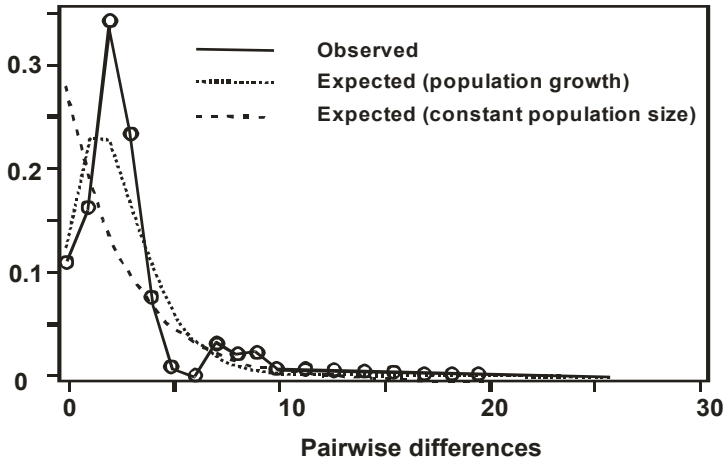

Figure 3.12: Distribution of pairwise nucleotide differences for 370 nucleotides of the mitochondrial D-loop of 71 *Chaerephon leucogaster* samples from Madagascar, Pemba and Mayotte.

3.5.2.5 Nested Clade Analysis

Phylogeographic Nested Clade Analysis of D-loop data using the programmes TCS (Clement *et al.*, 2000) and Geodis (Posada *et al.,* 2000) generated step-wise clustering of *C. leucogaster* haplotypes and is presented as a nested cladogram (Figure 3.13) and a table (3.7) indicating which of the clades showed significance in the permutational contingency test, the chain of inference for those clades and the interpretation according to Templeton's key (Templeton *et al.,* 1995).

Results from the permutational contingency test showed significance for the following clades: **1.2** ($P < 0.001$), **1.3** ($P = 0.0021$), **2.1** ($P = 0.0005$), **2.2** ($P = 0.0150$) and the total cladogram ($P = 0.0001$). This analysis shows statistically distinct northern and southern haplotypes of *C. leucogaster*. Significant phylogeographic association within clades **1.3** and **2.2** supports the uniqueness of haplotypes from the southern latitude band. Similarly, significant phylogeographic association within clades **1.2** and **2.1** supports the uniqueness of haplotypes from the northern latitude band. The total cladogram (**2.1** vs. **2.2**) further illustrates the significant phylogeographic association of haplotypes according to latitude.

Table 3.7: Table showing inferences from Nested Clade Analysis of *Chaerephon leucogaster* D-loop haplotypes.

Clade	**Chain of inference**	**Interpretation**
1.2	1-2-3-5-6-13-14 NO	Long-distance colonization and/or past fragmentation.
1.3	1-2-3-5-6-13 YES	Long-distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion.
2.1	1-2-3-5-6*-7-8 YES	Restricted gene flow/dispersal but with some long-distance dispersal over intermediate areas not occupied by the species; or past gene flow followed by extinction of intermediate populations.
2.2	1-2-11-12-13-14 NO	Long-distance colonization and/or past fragmentation (not necessarily mutually exclusive).
Total Cladogram	1-19 NO	Allopatric fragmentation.

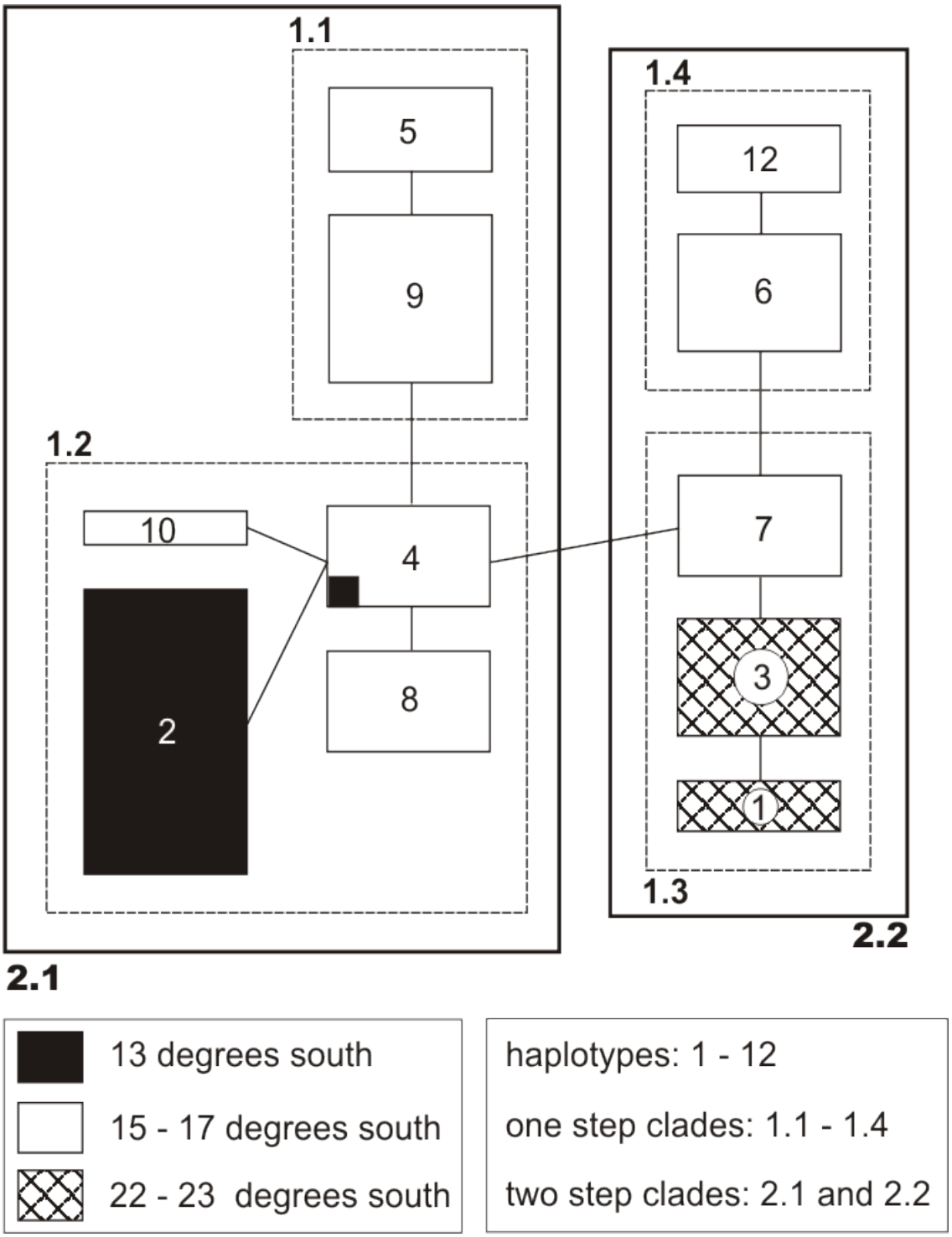

Figure 3.13: Nested cladogram based on the network showing relationships between haplotypes derived from 69 D-loop sequences of *Chaerephon leucogaster* from Madagascar generated using the program TCS (Clement *et al.*, 2000).

3.6 Ecological Niche Modelling

The MaxEnt method (Phillips *et al.,* 2006) was used to estimate the predicted distribution of *C. leucogaster* (Figure 3.14). The MaxEnt algorithm converged after 1,200 iterations with a regularized training gain of 1.833. Model performance as assessed by the area under the curve (AUC) was very high (0.95), indicating efficient classification of suitable versus unsuitable habitats.

. Temperature seasonality (bio_4) was the environmental variable that exhibited the highest explanatory power. Annual mean temperature (bio_1), when removed, was shown to decrease the overall gain of the model. Results from the model revealed clear occurrence, between *C. leucogaster* populations from the northwest, central west and southwest, of regions of unsuitable habitat.

Suitable habitat was sparsely distributed in the northwest (sub-humid forest), east (humid forests) and central west (deciduous forest), whilst it was more widely distributed in the south (semi-arid forest). According to the MaxEnt model, *C. leucogaster* recorded on the southeastern side of Madagascar at Manakara occupies unsuitable habitat.

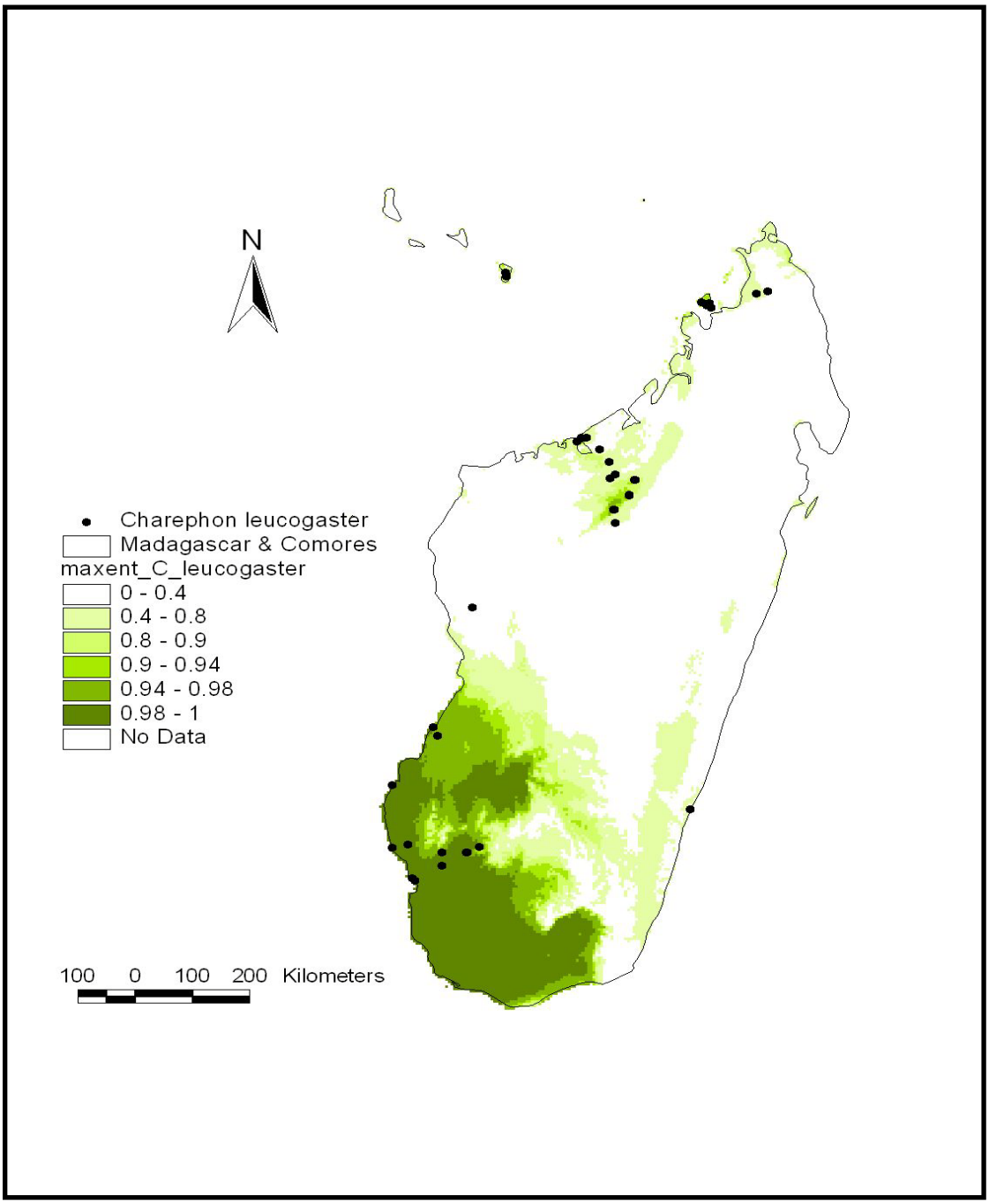

Figure 3.14: MaxEnt model of *Chaerephon leucogaster* distribution based on 103 known records of occurrence from Madagascar and Mayotte. Localities derived from museum collection records are shown as dots. Shading represents habitat suitability as indicated in Table 3.7.

4. DISCUSSION

4.1 TAXONOMY, PHYLOGENY AND PHYLOGEOGRAPHY OF *CHAEREPHON LEUCOGASTER*

Phylogenetic and phenetic analysis of cytochrome *b* sequences shows strong support for the monophyly of *Chaerephon leucogaster* from the western Indian Ocean region (Madagascar, Mayotte and Pemba). The topologies of neighbour-joining, maximum parsimony and Bayesian cytochrome *b* gene trees (Figure 3.9) and maximum parsimony and Bayesian D-loop gene trees (Figure 3.11), which support this monophyly, are congruent.

The low genetic variability of *C. leucogaster* cytochrome *b* sequences was also apparent in the haplotype network (Figure 3.8), which reflects six haplotypes for 39 samples, with adjacent haplotypes separated by only a single mutation. The low genetic variability is also reflected in the sharing of haplotypes between individuals from Madagascar, Pemba and Mayotte, despite the separation of these islands by distances of up to 1300 km.

The mean cytochrome *b* genetic distance between *C. leucogaster* samples from Madagascar, Mayotte and Pemba was low (0.20 %). According to Baker and Bradley (2006), the mean inter-population cytochrome *b* distance for bats is 1.7 % (range 1.4 % to 1.9 %, based on 2 studies), whilst the mean intraspecific distance is 1.6 % (range 0.6 % to 2.3 %, 10 studies) and the mean inter-species difference is 8.3 % (range 3.3 % to 14.7 %, 10 studies). As the genetic distance between *C. leucogaster* samples is less than both the inter-population and intra-specific distances reported by Baker and Bradley (2006), this suggests that *C. leucogaster* from Madagascar, Mayotte and Pemba maybe a single species group and that the different islands may contain different populations, or, but less likely, one panmictic population. Although *C. leucogaster* is one of the smallest Molossidae, evidence suggests that all Molossids are capable of long-distance flight, and that *C. leucogaster* is capable of crossing the Mozambique Channel. It should be noted that the cytochrome *b* distances in this study (HKY+I+G) are not strictly comparable to those reported in Baker and Bradley (2006), as their report was compiled from a number of studies and did not include any Molossidae. Additionally, their inter-population estimate was based on only two studies, and might change considerably should further studies (for example this one) be included in the dataset.

Analysis of both cytochrome *b* and D-loop sequence data shows low variation within *C. leucogaster* relative to some other molossid bats. Haplotype diversity for cytochrome *b* and the D-loop (0.718, 0.870) is low compared to that of populations of *Otomops madagascariensis* (0.945, 0.968), *O. martiensseni* (0.876, 0.952) (Lamb *et al.,* 2008) and *Tadarida brasiliensis* (0.987, 0.998) (Russell *et al.,* 2005), although higher than that calculated for *Mops midas* (0.608, 0.468) (Ratrimomanarivo *et al.,* 2007) and *M. leucostigma* (0.367, 0.758) (Ratrimomanarivo *et al*., 2008).

Phylogenetic (Figure 3.8) analyses indicate that *C. leucogaster* and *C. pumilus* from the eastern side of Madagascar are reciprocally-monophyletic sister taxa. The congruency of cytochrome *b* and D-loop analyses (Figures 3.9 and 3.11) lends support to this conclusion. The genetic distances between *C. leucogaster* and *C. pumilus* range from 1.8 % to 2.7 % (Table 3.2). Baker and Bradley (2006) reported mean inter-species distances for ten bat species of 1.6 % (range 0.6 % - 2.3 %) and for ten sister species pairs of 8.3 %, (range 3.3 % -14.7 %). The *leucogaster/pumilus* (Madagascar) distance values of 1.8 % - 2.7 % are not within the sister species range reported by Baker and Bradley (2006) for ten studies on (non – Molossid) bats.

Examples of groups that have been recognized as species even though separated by relatively low genetic distances include *Mops leucostigma* and *M. condylurus*, sister molossid taxa separated by a 2.5 % genetic distance (Ratrimomanarivo *et al*., 2008). Lower levels of sequence divergence could separate valid species which may have recently diverged and still manifest incomplete lineage sorting and a low level of ongoing gene flow (Ditchfield 2000; Mayer and von Helversen 2001; Hoffman and Baker 2003; Juste *et al.,* 2003; Russell *et al.,* 2005). The genetic distance means and ranges reported by Baker and Bradley (2006) are based on a defined number of comparisons of non-Molossid data. The ranges are great and there is overlap between the inter-population and intra-specific data. Nabholz *et al.* (2008) reported a wide variation in mitochondrial DNA substitution rates in mammals, and that bats, which, themselves exhibit wide variation; evolve slower than many non-flying mammals. Data should be interpreted holistically, particularly where a point falls at the lower end of or just below a reported range, in combination with phylogenetic patterns (reciprocal monophyly) and morphological data.

The well-supported reciprocal monophyly of both cytochrome *b* and D-loop sequences of *C. leucogaster* and *C. pumilus* on Madagascar coupled with the morphological distinction (Ratrimomanarivo *et al.,* in press) between them appear to support their status as separate species.

The *Chaerephon leucogaster* animal collected from Manakara, a locality on the east coast of Madagascar and outside the acknowledged range of *C. leucogaster,* exhibits no genetic or morphological variation from other *C. leucogaster* specimens (Ratrimomanarivo *et al.,* in press) from the west coast. It may indicate a recent colonization of the east of Madagascar by *C. leucogaster*. This population on the east coast occurs in sympatry with *C. pumilus* in its acknowledged range, which lends further support to the notion that these are distinct sister species between which gene exchanges does not seem to occur.

4.1.1 GENETIC VARIATION OF *CHAEREPHON LEUCOGASTER* SAMPLES FROM MAYOTTE AND PEMBA

Analysis of cytochrome *b* sequences reveals no genetic separation between *Chaerephon leucogaster* samples from Mayotte and Pemba even though Pemba and Mayotte are 1300 km and 320 km (respectively) from the closest coast of Madagascar. These results can be attributed to the relatively low variability of the *C. leucogaster* cytochrome *b* region and are consistent with the finding that large bodies of water may not be a barrier to dispersal of bats (Ruedi and McCracken, 2006).

D-loop data, however, shows some genetic differentiation of samples from mainland Madagascar and the islands of Mayotte and Pemba (1.4 % - a minimum of six mutational steps). This difference is not unexpected as the D-loop is known to evolve around three times faster than the cytochrome *b* gene and the latter is therefore more likely to reveal low levels of genetic differentiation.

This relative lack of genetic variability between *C. leucogaster* populations from Madagascar, Mayotte and Pemba contrasts with the results of many other studies on bats separated by large bodies of water. Castella *et al.* (2000) found that the Straits of Gibraltar formed a geographical barrier which separated two genetically distinct populations of *Myotis myotis* which occur on either side of it. In 2003, Pestano *et al.* reported an insignificant amount of gene flow between populations of *Plecotus teneriffae* on Canary Island and those from the western portion of this archipelago (Tenerife, La Palma and El Hierro), and also reported a 4 % cytochrome *b* divergence between *Barbastella barbastellus* populations on Canary island and mainland Spain.

The genetic similarity of *C. leucogaster* on Madagascar, Mayotte and Pemba may be attributed to inter-island dispersal. Genetic structure has been noted in bats with low dispersal capabilities and not within more wide-ranging species (Worthington-Wilmer *et al.,* 1994; Webb and Tidemann,

1996; Burland *et al.,* 1999; Russell *et al.,* 2005). The flight capability of molossid bats suggests that *C. leucogaster* is capable of long distance dispersal (Goodman and Cardiff, 2004).

Morphologically, *C. leucogaster* from Pemba was found to be larger than *C. leucogaster* found in Madagascar and Mayotte, and to have darker pelage coloration and a more extensive white coloration of the ventral region (Ratrimomanarivo *et al.*, in press). These population-level differences are not reflected in the cytochrome *b* and D-loop sequences of this study; this, insofar as variation in these sequences is a measure of general genetic isolation, indicates that these morphological differences may not be genetically based. The distinctive size and coloration of these bats could be due to environmental factors such as differences in the ecological niche these bats inhabit. The lack of concordance between morphological and genetic variation of *C. leucogaster* from Madagascar, Mayotte and Pemba parallels the results of a study conducted by Ratrimomanarivo *et al.* (in press). *Mops leucostigma* exhibits morphological but not genetic variation between the eastern and western slopes of Madagascar, which exhibit different bioclimatic conditions. Therefore, as with *C. leucogaster*, these morphological differences may be attributed to dietary, climatic or other factors.

4.1.2 GENETIC VARIATION WITHIN MALAGASY *CHAEREPHON LEUCOGASTER* SAMPLES

Genetic distinctiveness of animals from the 13º S latitude band

This region of sub-humid forest includes the northwest offshore islands of Nosy Be and Nosy Komba, which are situated 14 km from the Madagascan mainland. It is separated from the 15º - 17º S latitude band of dry deciduous forest by a region of habitat predicted, based on the current sample distribution, to be unsuitable for *C. leucog*aster by MaxEnt ecological niche modelling.

Analysis of cytochrome *b* sequences indicates that eight out of nine sample animals from the 13º S zone show a unique haplotype (Figure 3.8). This is supported by the D-loop data, where 17 out of 18 samples reflect a distinct haplotype (Figure 3.10). Only one animal from the northwest offshore islands shared a haplotype with Malagasy samples from more southerly regions. Thus samples from the 13º S latitude band show strong phylogeographic structure. These results are supported by Nested Clade Phylogeographic Analysis (Table 3.7, Figure 3.13), which displays similar

associations and provides statistical support for the phylogeographic distinctness of samples from the 13º S latitude band.

One possible interpretation, according to Templeton's key (Templeton *et al.,* 1995) is restricted gene flow, but with some long distance dispersal over intermediate areas not occupied by the species. The region separating the 13º S zone from 15º - 17º S zone is unoccupied by *C. leucogaster* and comprises unsuitable habitat (MaxEnt modeling). *C. leucogaster*, which feeds on insects (unknown species), is capable of flying between these regions. Its feeding ecology is, however, largely unstudied. It is likely that it would restrict its feeding to a narrower range around the area of its day roosts. If this is the case, animals living in the 13º S zone would be likely to feed on insects found within that zone, possibly in the sub-humid forests where its night roosts may be located, although, again, this is not known. Unless food is unavailable with the 13º S zone, it would seem unlikely (energetically unfavorable) that a bat would forage several hundred kilometers south across a region of habitat that is predicted as unsuitable. Thus the effective range of *C. leucogaster* might be much more restricted that its flight capability suggests, which may account for the development of genetically-isolated populations in this region. As the vegetation in this 13º S zone, sub-humid forest, differs from that in the more southerly zones (dry deciduous forest and spiny bush), the type of insects available as food may differ, leading to a degree of specialization and thus regional isolation. Studies on the feeding ecology of *C. leucogaster* are necessary to provide further insight. Such studies are difficult, however, as these high-flying bats are seldom trapped in flight.

Other explanations of the phylogeographic structure observed in this region include past gene flow followed by extinction of intermediate populations, long-distance colonization or past fragmentation. Without detailed specific information on past climate and vegetation change in these regions of Madagascar, it is difficult to comment on whether there were intermediate populations, which perhaps foraged and roosted in the regions which are currently predicted to comprise unsuitable habitat, and are now extinct. There is no evidence in the fossil record to support this.

Genetic distinctiveness of animals from the 15º S to 17º S latitude band

Both cytochrome *b* and D-loop data show haplotypes exclusive to this central region of dry deciduous forest, which is separated from the habitat zones in the north (13º S) and south (22º S - 23º S) by unoccupied regions of unsuitable habitat predicted by MaxEnt ecological niche modelling.

Genetic distinctiveness of animals from the 22º S to 23º S latitude band

Animals sampled from the southwest region of Madagascar came from Toliara and Sakaraha. This region is separated from the 15º S to 17º S latitude band by an area of unsuitable habitat (predicted by MaxEnt ecological niche modelling). Analysis of cytochrome *b* sequences reveals no genetic differentiation of samples from this region relative to areas from further north. Mitochondrial D-loop data however, is more variable and reveals the existence of two exclusive haplotypes within this area (Figure 3.10). These haplotypes are separated by one mutational step from each other and the closest haplotype is separated by one mutational step from the rest of the haplotype network. This genetic distinctiveness is supported by Nested Clade Phylogeographic Analysis (Figure 3.13, Table 3.7) which reflects significant phylogeographic associations in clades 1.3, 2.2 and the total cladogram.

Long distance colonization, possibly coupled with past fragmentation, were the processes inferred to explain these geographical associations of haplotypes (Table 3.7). Much of the discussion above, relating to the distinctiveness of animals from the 13º S latitude zone applies here. Distinctiveness of *C. leucogaster* from the 22º S to 23º S latitude band may relate to vegetation and feeding ecology. *Chaerephon leucogaster* may have specialized by feeding on insects that are found in the spiny bush characteristic of this region, which is separated from the 15º S to 17º S habitat zone by more than 500 km. The lack of suitable vegetation (for night roosts) and food in this region of predicted unsuitable habitat may have resulted in the confinement and specialization of *C. leucogaster* populations to the southern zone. Overall, it appears that the confinement of exclusive haplotypes to specific latitude and vegetation zones is consistent with allopatric fragmentation and that habitat vicariance may be involved in the current evolution of significant phylogeographically-structured groups of *C. leucogaster*. Although the regions between the three latitude bands are predicted to contain unsuitable habitat by MaxEnt ecological niche modeling, it is possible that more intensive sampling in the gap areas might reveal the existence there of populations of *C. leucogaster*. These samples, if included in the niche modeling might change the areas of predicted suitable habitat. This could account for the apparent genetic and ecological gaps. If this is the case, sampling in these largely unsampled areas might provide links which would result in genetic and ecological clines which fit a model of isolation by distance.

Morphological analysis of *C. leucogaster* reveals variation in size of animals from the different latitude (and vegetation) bands (Ratrimomanarivo *et al.*, in press) as discussed in the section on genetic analysis. Animals from the extreme south, southwest and northern regions are larger in certain morphological characters than those from the central western zone (Ratrimomanarivo *et al.*, in press). Nested Clade analysis of D-loop data shows that animals from the northern and southern latitude bands are genetically distinct. AMOVA analysis further supports this conclusion (Table 3.3 and Table 3.5). It is not clear, though, that there is any causative relationship between this genetic and morphological distinctness.

The explanations for current patterns of genetic diversity may lie in historical, climatic and habitat variation. Frequent changes in climate led to the ice ages of the Quaternary (2.4 Myr to the present) (Hewitt, 2000), which may have influenced speciation and the geographic distribution of species, including *C. leucogaster* or its ancestors. The expansion and contraction of species' ranges reflected the change in suitable habitats of species. Expansion would have allowed individuals to broaden their range and this would have involved selection and adaptation to different environmental conditions (Hewitt, 2000). Wilme *et al.* (2006) examined the effects of paleoclimatic changes on patterns of dispersal and vicariance at intra-island level. In Madagascar, it was proposed that the riverine habitats acted as refugia for retreat to higher altitude zones. This led to fragmentation of ancestral populations, which in turn led to allopatric differentiation or extinction of populations, and later recolonization (Hewitt, 2000; Burney *et al.*, 2004; Wilme *et al.*, 2006). During glacial minima, species used the riverine corridors to expand their geographical ranges. Burney *et al.* (2004) suggested that the lowland forests were restricted to refugia along the east coast and in the northwest of the island. This could explain the origin (during an earlier Pleistocene glacial maximum) of *C. pumilus* (adapted to the east cost conditions) and *C. leucogaster* (adapted to the west coast conditions), as well as the latitudinal genetic variation shown in the D-loop data for *C. leucogaster*. During the last glacial maximum, 18,000 years ago, *C. leucogaster* populations may have retreated into the northwestern refugium and subsequently extended their geographical range southwards along the western portion of Madagascar.

Origin of *C. leucogaster* populations on Madagascar

Current fossil records for mammals do not extend much earlier than the late Cretaceous/early Paleocene (Alroy 1999; Foote *et al.*, 1999). For most bat species the fossil record is insufficient; this poses a problem in determining the origin and dispersal patterns of bat species (Jones *et al.*, 2005).

Determining the population structure of bats at macrogeographic level aids in resolving patterns of dispersal and colonization (Burland and Worthington-Wilmer, 2001; Abbott and Double, 2003).

According to Russell *et al.* (2006) the theories that relate to the origin of Malagasy fauna involve biogeographical scenarios of dispersal because the isolation of Madagascar from the African mainland (165 Mya) occurred before the evolution of Chiropterans (71 - 58 Mya). Some species may have arrived via aerial dispersal from the neighboring landmasses (Steven and Heesy, 2006). According to Goodman and Cardiff (2004), "members of the genus *Chaerephon* are capable of dispersal over water and subsequent colonization and speciation on distant oceanic islands". Island hopping was suggested to have occurred across the Mozambique Channel during the uplift along the Davie Ridge in the Channel (Forster, 1975; Houle, 1998; Wells, 2003).

The results from this study of cytochrome *b* and D-loop data indicate that *C. leucogaster* from Madagascar, Mayotte and Pemba form a single, genetically rather uniform group, which is consistent with suggestions that large bodies of water such as the Mozambique Channel (minimum 300 km wide) are not a significant barrier to dispersal and hence gene flow of *C. leucogaster* between Madagascar, the Comoros, Pemba, and, it would seem, mainland Africa, approximately 20 km distant from Pemba. Although molossid bats appear capable of crossing large expanses of water, the frequency with which this happens is not clear.

Most species that are found on Madagascar and other islands in the western Indian Ocean are suggested to have African origins (Yoder *et al.,* 2004; Vences *et al*., 2004; Rabinowitz and Woods, 2006; Ratrimomanarivo *et al.,* 2008; Lamb *et al.,* 2008; Ratrimomanarivo *et al.,* in press). The Malagasy population of *Mops leucostigma* is suggested to have been derived from African stock of *M. condylurus,* and to have been sufficiently isolated for speciation to have occurred (Ratrimomanarivo *et al.,* 2008). A study done by Russell *et al.* (2006) found that phylogenies of the genus *Triaenops* are paraphyletic with respect to the African species. They suggested that there were two dispersal events between Africa and Madagascar.

Certain bat species show evidence of relatively recent migration from founder populations in Africa. The Malagasy and African populations of *Mops midas* show little morphological or genetic variation, which suggests that there is some recent or regular gene flow between them (Ratrimomanarivo *et al.,* 2007). A study conducted by Taylor *et al.* (in press), shows paraphyly in

C. pumilus from southern Africa and *C. leucogaster*, thus also supporting the findings of possible and regular gene flow between Africa and Madagascar. Although the current sample does not include animals from mainland Africa, owing to lack of availability, an African origin of *C. leucogaster* appears likely.

4.2 POPULATION EXPANSION

Haplotype diversity and expansion coefficients values, and a unimodal mismatch distribution indicate that populations of *Chaerephon leucogaster* on Madagascar may have been expanding over the last 5 842 - 11 143 years (Table 3.6). Neutrality tests of the D-loop region also show support for expanding *C. leucogaster* populations, as Fu's (1997) F*s* is significant and Fu and Li's (1993) D* and F* are not significant (Hingston *et al*., 2005; Russell *et al.*, 2005).

According to Burney *et al.* (2004), human colonization of Madagascar dated back to about 2300 BP. The expansion of *C. leucogaster* is therefore likely to have begun before this colonization and the construction of buildings which have been used by *C. leucogaster* for day roosting. This implies that the roost sites used during the first several thousand years of the expansion were not synanthropic, as they are today, and raises the question of what roost sites were used during the early expansion period, although it is likely that they were natural. Burney (1997, 1999) highlighted the change in climate and vegetation since the late Pleistocene and Holocene in Madagascar. It was reported that the most noticeable vegetation changes occurred in the western portion of Madagascar, where *C. leucogaster* is currently found. It is clear that, before the expansion of *C. leucogaster*, these bats inhabited natural day roost sites (Goodman and Cardiff, 2004). The change in roosting habitat from natural to synanthropic sites within the past hundred years may have contributed further to the expansion of the effective population size of *C. leucogaster*, as well as to the possible expansion of its range.

Although the current use of synanthropic sites as roosts by *C. leucogaster* may have led to an increase in population sizes of this bat, it still is puzzling why this species abandoned the original day roosts. In this regard it may be important that anthropogenic factors have reduced forest cover in the lowland region of Madagascar (Green and Sussman, 1990; Smith, 1997; Harper *et al.*, 2007). If *Chaerephon leucogaster* used trees of the native forests to roost, their disappearance may have

influenced the population structure of this species and contributed to its migration to anthropogenic day roost sites.

4.3 CONSERVATION IMPLICATIONS AND MANAGEMENT

Islands are considered to be biodiversity 'hotspots' and are important in the conservation of our global biological heritage. Madagascar is particularly rich in biodiversity and endemic taxa (Myers *et al*., 2000; Ganzhorn *et al*., 2001; Goodman and Benstead, 2005), many of which are not well studied. According to the current classification by the IUCN (Chiroptera Specialist Group, 2000), *C. leucogaster* is 'Data Deficient' (DD*). This category is applied to species that are newly described or to those for which there is insufficient research material available (Hutson *et al.*, 2001). Based on this study, it contributes to other body of work to alleviate the DD* status of *C. leucogaster*. This study provides information on the genetic diversity of *C. leucogaster* populations on Madagascar, Mayotte and Pemba, which supports morphological analysis conducted by Ratrimomanarivo *et al.* (in press). It also provides evidence that *C. leucogaster* populations are expanding, are geographically-structured into three different latitudinal groups on Madagascar, and that a further genetically distinct group occurs on the islands of Pemba and Mayotte.

It is recommended that the status of this species be re-assessed today with other significant data points. Results from genetic analyses can contribute for *C. leucogaster* to be considered under the Lower Risk category. This study highlights that *C. leucogaster* is an expanding population and that no rare genotypes exists that require special attention. *Chaerephon leucogaster* is commonly-found within the range from which it has been collected (except at Manakara) and, in some cases, is regarded as a 'pest species'. Although there are genetically-distinct 'northern', 'middle' and 'southern' forms these are not rare genotypes which warrant special preservation. However, Pullin (2002) indicates that all genetic lineages require preservation as together they represent the future evolutionary potential of a species. The sample from Manakara, although morphologically similar to western samples of *C. leucogaster*, and genetically identical to the most common 'western' haplotype, may warrant further attention owing to the unusual niche which it occupies. Samples from Pemba and Mayotte are genetically distinct at D-loop but not cytochrome *b* levels, and are also not rare.

The haplotypes that are found in the three latitude bands (13º S, 15º S to 17º S and 22º S to 23º S) are possibly separated by unsuitable habitat (depending on future studies which need to sample the gap areas extensively). Management actions should focus on the preservation of the different classes of vegetation in these habitat zones, as phylogeographically distinct groups of *C. leucogaster* may be dependent on different sets of insect species supported by different habitats (see Crandall *et al.,* 2000). The southwest portion of Madagascar has been shown by MaxEnt Modelling to contain the most suitable habitat for *C. leucogaster*. Destruction of vegetation by slash and burn, logging and charcoal production in such areas may negatively influence the population size and dispersal of these bats as it may affect the availability of foods. Although *C. leucogaster* is insectivorous, it is not known which insect species it feeds on, or what factors affect the prevalence of these species. This points to the importance of initiating studies of the feeding ecology of this species. These may establish the insect species on which these bats preferentially feed, and the habitats in which these species are found. Depending on status, these areas may then be targets for conservation.

Management and conservation strategies should aim to preserve adaptive diversity and evolutionary processes across the geographic range of a species (Crandall *et al.,* 2000). The genetic diversity that does exist within these populations should be maintained by continuous assessment and monitoring, particularly at established key roost sites. The availability of day roosts is not a constraint for this species. Human colonization has resulted in the construction of buildings which are used by *C. leucogaster* as day roosts and has thus allowed the expansion of this species. Bats living in synanthropic settings can however, be affected by negative practices and perceptions on the part of humans (Hutson *et al.,* 2001; Mickleburgh *et al.,* 2002; Knight, 2008). Public awareness is therefore vital to protect synanthropic bats from destruction by practices such as fumigation, which is used to eradicate synanthropic bats from buildings (Hutson *et al.,* 2001). The presence of bats may also trigger fears about the possible risk from diseases such as rabies. Other concerns from residents include noise, smell and the accumulation of bat droppings. It is therefore, however, important to recognize the key day roosts for this species and legislate their protection into local, regional and national policies to prevent decline in numbers of *C. leucogaster* (Hutson *et al.,* 2001).

Threats to bats are related to ignorance of the roles of these mammals in ecosystems (Hutson *et al.,* 2001; Mickleburgh *et al.,* 2002). Educational campaigns need to be developed and implemented and should ideally target the general public as well as interest groups that may influence or have an

impact on bat colonies. Appropriate systems need to be developed to resolve the conflicts that could arise between humans and bats. Bat interest groups should be established to promote the understanding and diversity of these mammals.

Madagascar is a member of a number of international agreements and treaties that protect bat species. These conventions would be required to protect *C. leucogaster* through legislation. Government organizations (i.e. SAPM) need to focus management strategies on conservation of haplotypes of this species in Madagascar and the western Indian Ocean islands of Mayotte and Pemba, as these identified haplotypes of *C. leucogaster* represent its genetic evolutionary potential.

4.4 FUTURE RESEARCH

Genetic variation is an important component of biodiversity. The conservation of biodiversity requires knowledge of the origins, distribution, dispersal and feeding behavior of mammals. This study examined the genetic diversity of *C. leucogaster* from Madagascar, Mayotte and Pemba. Results suggested that *C. leucogaster* can be considered a separate species from *C. pumilus* (east Madagascar) and that populations on Madagascar are phylogeographically structured on the basis of latitude. This structuring may reflect the occurrence on Madagascar of climatically- and vegetationally-suitable regions which harbour the insects upon which *C. leucogaster* feeds. These regions are likely to be separated by regions of unsuitable habitat. Further studies are essential to understand the ecology and feeding behavior of *C. leucogaster* populations.

This study has focused on *C. leucogaster* from Madagascar (from where the type specimen originates), Mayotte and Pemba islands. It is important that future studies include *C. leucogaster* collected from the rest of its range, which extends into west-central Africa. *Chaerephon leucogaster* from the study region is suggested to be a sister species to *C. pumilus* from Madagascar. This scenario is likely to be more complex if *C. pumilus* and *C. leucogaster* from mainland Africa are included in the analysis.

Nuclear DNA is able to provide substantial systematic and phylogenetic information. It is inherited bi-parentally and so would provide more information on male-mediated gene flow and mating systems (Ruedi and McCraken, 2006). If DNA sequencing of nuclear genes is to provide phylogenetic and phylogeographic information relevant to this study, a region which evolves at

least as fast as the mitochondrial D-loop would need to be utilized (Porter *et al.*, 2003; Hoofer *et al.*, 2003). Simple sequence repeats (microsatellites) are variable enough to give fine-scale information on population and colony structure, and would provide information or male-mediated gene flow and the potential contribution of male versus female philopatry to population and colony structure (Kerth *et al.*, 2002).

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APPENDICES

APPENDIX 1

Table 2.1: Sample, locality and collection details of *Chaerephon leucogaster* used in this project (FMNH – Field Museum of Natural History)

Location	Geographic coordinates		FMNH number	Genbank # Cyt *b*	Genbank # D-loop	Sex	Collector(s)
	Latitude	Longitude					
Ambalanjankomby	16°42.062'	46°04.304'	184922	EU716039	EU727502	F	F.H. Ratrimomanarivo, S.M. Goodman
			184923	EU716006	EU727503	M	
			184924	EU716007	EU727504	M	
			184925	EU716008	EU727505	M	
			184926	EU716009	EU727506	F	
Andranofasika	16°20.229'	46°50.794'	184955	EU716015	EU727512	M	F.H.Ratrimomanarivo, C. Andriarileva
			184956	EU716016	EU727513	M	
			184957	EU716017	EU727514	F	
			184958	EU716018	EU727515	F	
			184959	EU716019	EU727516	F	
Ambondramamy	16º26.173'	47º09.329'	184950	EU716010	EU727507	F	F.H. Ratrimomanarivo, C. Andriarileva
			184951	EU716012	EU727508	F	
			184952	EU716011	EU727509	F	
			184953	EU716013	EU727510	F	
			184954	EU716014	EU727511	F	
Antanimbary	17º11.104'	46º51.306'	184896	-	EU727489	F	F.H.Ratrimomanarivo, A.E. Randrianarivo
			184897	-	EU727490	F	
			184898	-	EU727491	F	
			184899	-	EU727492	M	
			184900	-	EU727493	F	
			184901	-	EU727494	F	
			184902	-	EU727495	M	
Maevatanana	16°57.452'	46°49.433'	184915	-	EU727496	F	F.H.Ratrimomanarivo, C. Andriarileva
			184916	-	EU727497	M	
			184917	-	EU727498	F	
			184918	-	EU727499	F	
			184919	-	EU727500	M	
			184920	-	EU727519	M	
Ankazomborona	16º06.961'	46º45.400'	184975	EU716020	EU727521	M	F.H.Ratrimomanarivo, C. Andriarileva
			184976	EU716021	EU727520	M	
			184977	EU716022	EU727522	M	
			184978	EU716023	EU727523	M	
			184979	EU716024	EU727484	F	
			184604	-	EU727485	F	

Mahajanga	15°42.778'	46°18.752'	184605	-	EU727486	F	F.H.Ratrimomanarivo, S.M. Goodman
			184606	-	EU727487	F	
			184607		EU727488	F	
			184608	-	EU727517	M	
Ankijibe	16°24.807'	46°45.876'	184973	-	EU727518	M	F.H. Ratrimomanarivo, C. Andriarileva
			184974	-	EU727524	F	
Berivotra	15°54.245'	46°35.873'	185020	-	EU727525	F	F.H.Ratrimomanarivo, S.M. Goodman
			185021	-	EU727526	M	
			185022	-	EU727527	F	
			185027	-	EU727528	F	
			185028	-	EU727529	M	
			185029	-	EU727530	M	
			185030	EU716025	EU727532	F	
Sakaraha	22°54.546'	44°31.574'	184259	EU716005	EU727531	M	F.H. Ratrimomanarivo, J. Rakotomavo
			184263	-	EU727461	F	
			184264	-	EU727470	M	
Toliara	23°23.704'	43°43.219'	184237	-	EU727462	F	F.H.Ratrimomanarivo, S.M. Goodman, J. Rakotomavo
			184238	EU716038	EU727471	M	
			184239	EU716036	EU727483	M	
			184240	EU716037	EU727474	F	
Nosy Be (Dzamandzar)	13°21.095'	48°11.307'	188497	-	EU727475	F	S.M. Goodman
			188498	EU716028	EU727476	F	
			188499	-	EU727477	M	
			188500	-	EU727463	F	
Nosy Be (near Hell-ville)	13°24.308'	48°18.201'	187750	EU716030	EU727464	M	E. Rakotonandrasana
			187751		EU727478	M	
Nosy Komba	13°26.562'	48°20.874'	188640	EU716032	EU727479	M	S.M. Goodman, E. Rakotonandrasana
			188641	-	EU727480	F	
			188642	EU716033	EU727481	F	
			188643	EU716034	EU727482	F	
			188644	EU716035	EU727472	F	
Nosy Be (Hell-ville)	13°24.254'	48°16.425'	188495	-	EU727473	F	S.M. Goodman
			188496	EU716029	EU727465	F	
Nosy Be (Ambatozazavy)	13°22.012'	48°18.927'	187752	-	EU727466	M	E. Rakotonandrasana
			187753	-	EU727467	F	
			187754	EU716026	EU727468	M	
			187755	EU716027	EU727469	M	
			187756	-	EU727501	M	
Manakara	22°09.418'	48°01.009'	185228	EU716031	EU727535	F	F.H. Ratrimomanarivo
Mayotte	12°53.609'	45°08.550'	194019	EU716041	EU727536	F	S.M. Goodman
	12°49.923'	45°08.215'	194028	EU716040	EU727533	M	
Pemba	4.96487°	39.71456°	192886	EU716003	EU727534	M	W.T. Stanley
			192889	EU716004	EU727502	M	

Table 2.2: Origin and catalogue numbers of outgroups used in this study.

Outgroups	Country	Catalogue Number
Mops leucostigma	Madagascar	FMNH 185098
Mops midas	Madagascar	FMNH 184306
Chaerephon pumilus	Madagascar	FMNH 188088, 188089, 187834, 187835, 185260, 185286, 185315, 187797, 187799

APPENDIX 2: GEL ELECTROPHORESIS SOLUTIONS

10 X TBE stock solution

53.89 g Tris-HCl

24.96 g Boric acid powder

1.86 g EDTA

Make up to 500 ml with distilled water, adjust pH to 8.3. Autoclave before use.

0.5 X TBE solution (1:19 dilution)

5 ml (10 X) TBE

95 ml deionised/distilled water

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 (10 mg/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 3: FLUOROMETRY SOLUTIONS

10 X TNE buffer stock solution

12.11 g Tris (100 mM)

3.72 g EDTA Na2.2H2O (10 mM)

116.89 g NaCl (2 M)

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.

Hoechst 33258 stock dye solution (1 mg/ml)

10 mg Hoechst 33258 dye

10 ml sterile H_2O

Stored in a dark bottle for a maximum of 6 months

Low Range Assay Solution

1 µl H 33258 stock solution (Hoeschst dye)

1 ml 10 X TNE

9 ml filtered distilled water

The assay solution was 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

CHAPTER TWO

Stable Pleistocene-era populations of *Chaerephon pumilus* (Chiroptera: Molossidae) in south eastern Africa do not use different echolocation calls

ABSTRACT

Our extension of the phylogenetic study of Taylor *et al*. (2009) on a larger, more geographically representative sample confirmed their finding of genetically distinct sympatric lineages of bats currently referred to as *Chaerephon pumilus* sensu lato (s.l.) (Family Molossidae) in south eastern Africa. *Chaerephon pumilus* s.l. comprised two cytochrome *b* lineages separated by a mean genetic distance of 0.7% (0.1% - 1.4%), consistent with intraspecific variability. The *C. pumilus* s.l. clade was paraphyletic, containing a nested *C. leucogaster* (Madagascar) clade. As well as the expected four mitochondrial control region lineages, we identified a new strongly-supported clade from the Durban area. Indices of diversity and neutrality, combined with a ragged multimodal mismatch distribution, are inconsistent with demographic expansion of a single *C. pumilus* s.l. population in south eastern Africa, and suggest that the control region lineages are stable units at demographic equilibrium. Dating analyses suggest that these lineages were established during the late Pleistocene, between 60 000 and 13 000 years ago. We found no evidence to support our hypothesis that the sympatric genetic lineages of *C. pumilus* s.l. are associated with distinct sonotypes, as Discriminant Function Analysis based on four

echolocation parameters could not discriminate between the four clades. We hypothesise that the different genetic lineages may be distinguished by differences in social communication and behaviour.

Keywords: *Chaerephon pumilus*, mitochondrial DNA, differentiation, cryptic species, echolocation

INTRODUCTION

Molecular phylogenetic techniques have proven to be a powerful tool in revealing cryptic lineages in different biological groups (Pfenninger & Schwenk 2007) including large mammals (Birungi & Arctander 2000; Ravaoarimanana *et al*. 2004; Brown *et al*. 2007), small mammals (Peppers & Bradley 2000; Olson *et al*. 2004; Yoder *et al*. 2005), amphibians (Vences & Glaw 2005) and birds (Omland *et al*. 2000; Brambilla *et al*. 2008; Towes & Irwin 2008). Cryptic species may go undetected because diagnostic features of individuals usually involve sensory modalities very different from our own. For example, bats navigate, forage and communicate in an acoustic environment that is largely beyond human hearing (Kingston *et al*. 2001). Advances in technology to record and analyse echolocation calls have unmasked many cryptic lineages based on differences in their calls. In southern Africa, Taylor *et al.* (2012) described four new species in the *Rhinolophus hildebrandtii* species-complex of horseshoe bats, whose evolution has entailed adaptive shifts in species-specific peak frequencies representing the allometric effect of adaptive divergence in skull size. Stoffberg *et al*. (2012) found five geographic groups in *Rhinolophus clivosus* sensu lato supported by genetic data, echolocation characteristics and wing morphology, and suggested diversification may have been facilitated by glaciation events in the Plio-Pleistocene. Bioacoustic information combined with genetic data have provided insight into speciation and cladogenesis amongst many families of insectivorous bats (Russo & Jones 2000; Rydell *et al*. 2002; Kingston & Rossiter 2004; Thabah *et al*. 2006; Ramasindrazana *et al*. 2011), except the Molossidae.

As classically defined, the little free-tailed bat, *Chaerephon pumilus* (Cretzchmar 1830-31), is a synanthropic molossid that is widely distributed across sub Saharan Africa, extending to Arabia (Peterson *et al*. 1995; Bouchard 1998; Simmons 2005). Accompanying the wide geographic distribution is variation in morphology, including wing and pelage colour, size, degree of development of the male aural crest and the extent of palatal emargination (Bouchard 1998; Taylor

et al. 1999a). This has contributed to a number of differing taxonomic designations, for *C. pumilus* (Simmons 2005), which currently includes nine synonyms.

Five forms of *C. pumilus* have been named from the eastern and southern portions of the African continent, namely *C. limbata* (Peters, 1852), *C. naivashae* (Hollister, 1916), *C. hindei* (Thomas, 1904), *C. elphicki* (Roberts, 1926) and *C. langi* (Roberts, 1932). A further four forms have been named from western Indian Ocean islands. *Chaerephon* individuals from western Madagascar were assigned to *C. leucogaster* (Ratrimomanarivo *et al*. 2009). Two forms of *C. pumilus* were found by Goodman *et al*. (2010) to be genetically distinct from samples of this species originating from the type locality (Massawa, Eritrea); the eastern Malagasy form was named as a new species, *Chaerephon atsinanana*, whereas *C. pumilus* from the western Seychelles and the Comoros archipelago was referred to *C. pusillus*. A morphologically distinct Malagasy form was found to warrant species status and named *C. jobimena*, (Goodman & Cardiff 2004), although more recent molecular studies have shown this form to be too divergent to be included in *Chaerephon* (Lamb *et al*. 2011). As our unpublished results show that *C. pumilus* from the southern portion of the African continent is genetically distinct from individuals obtained from the type locality of this species (Massawa, Eritrea) (Goodman *et al*. 2010), we refer to these specimens from here on as *C. pumilus* sensu lato (s.l.).

There have been several studies of genetic divergence in forms of *Chaerephon* from mainland Africa. Jacobs *et al*. (2004) found < 0.9% genetic divergence in mitochondrial cytochrome *b* sequences between light-winged *C. pumilus* s.l. from Zambia and Tanzania and dark-winged forms from South Africa, suggesting that these two forms are not distinct species. Taylor *et al*. (2009) found genetically distinct lineages within animals currently referred to as *C. pumilus* s.l. in eastern South Africa and neighbouring Swaziland. Analyses of the mitochondrial cytochrome *b* and control regions show at least four clades from the greater Durban area, separated by low inter-clade genetic distances (0.6–0.9%, cytochrome *b*). Two of the clades appeared to have undergone

Pleisocene-era population expansions 60000 – 14700 and 13000 – 3300 years before present, lending support to the idea that these clades are independently-evolving genetic units. Interestingly, Malagasy *C. leucogaster*, although morphologically distinct (Ratrimomanarivo *et al.* 2009), is nested within *C. pumilus* s.l. from the Durban area and is separated from two clades by cytochrome *b* genetic distances of 0.7% and 0.6% respectively. Taylor *et al*. (2009) hypothesised that the *C. pumilus* s.l. clades may represent cryptic species with distinct echolocation characteristics.

The bioacoustic characteristics of *C. pumilus* s.l. from eastern and southern Africa are varied. Aspetsberger *et al*. (2003) reported ultrasonic frequencies of 19–23 kHz, with a peak echolocation frequency (PF) of 21.0 kHz in a population in Tanzania. Taylor (2005) recorded similar PFs (~25 kHz) in populations from Kenya and South Africa. Echolocation frequencies are also varied in the Durban (KwaZulu-Natal) region. Taylor (1999b) and Fenton *et al*. (2004) both reported two distinct sonotypes ascribed to *C. pumilus* s.l. (PFs 23.9 and 16.3 kHz), and Schoeman & Waddington (2011) recorded PFs of ~28.2 kHz at two rivers in Durban.

Our aim in this study was to investigate genetic and sensory patterns of cryptic diversity in south eastern African *C. pumilus*. We examine phylogenetic structure in *C. pumilus* s.l. based on published data (Taylor *et al*. 2009) and new cytochrome *b* and control region sequences, thus more than doubling the previous sample size and expanding the geographic sampling to include a wider area of southern and northern KwaZulu-Natal (KZN). Further, we investigated the possible relationships between the genetic data and recorded echolocation call data to test if genetic lineages can be identified by distinct echolocation characteristics. Finally, we examine the historical demography of *C. pumilus* s.l. in this region in order to determine whether this species behaves as a single population, or several distinct populations.

MATERIALS & METHODS

Study sites

Individuals of *Chaerephon pumilus* s.l. were captured using mist nets placed at their points of entry to and exit from house roofs and in their flight paths over rivers. The captured bats were kept in cotton bags overnight and released the following evening where they were caught. The South African sampling locations were (acronyms for sites and geographical coordinates in parentheses): Chatsworth (CH, 29.930S, 30.925E), Effingham Heights (EF, 29.769S, 31.010E), Pinetown (PNT, 29.828S, 30.866E), Umbilo River at the Umbilo Waste Water Works (UWWW, 29.846S, 30.890E), Paradise Valley (URPV, 29.831S, 30.892E), Queensburgh (QB, 29.857S, 30.899E), and the Phinda Nature Reserve situated in northern KZN (PH, 27.695S, 32.356E) (Appendix). Genetic analyses included 35 individuals utilized by Taylor *et al*. (2009) and an additional 60 individuals captured during the present study. All manipulation of living animals was carried out in accordance with guidelines of the American Society of Mammalogists (Sikes *et al.* 2011) and was approved by the Animal Ethics Committee of the University of Kwa-Zulu Natal.

Tissue sampling

Two biopsies of skin were taken from the membrane of each wing using a 3 mm diameter medical biopsy punch (Wilkinson *et al*. 1997). Biopsies were taken from areas with no visible large blood vessels and placed in 1.5 mL microfuge tubes containing 90% ethanol.

Molecular analysis

Total genomic DNA was isolated from wing biopsies using a DNeasy® Blood and Tissue Kit (QIAGEN Inc.). Polymerase Chain Reaction (PCR) amplifications were performed in 25 µl volumes. Each reaction contained 9 µl DNA (3 ng μl^{-1}), 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 *Taq* polymerase (5 u/µl) (Super-Therm) and 4 µl of each primer (6 µM) (forward and reverse) per reaction.

The cytochrome *b* gene was PCR-amplified as two overlapping double-stranded fragments using 2 primer pairs. The 5' fragment was amplified using primers L14723 (5'-ACCAATGCAATGAAAAATCATCGTT-3') and H15553 (5'-TAGGCAAATAGGAAATATCATTCTGGT -3') whilst the 3' fragment was amplified using L15146 (5'-CATGAGGACAAATATCATTCT GAG-3') and H15915 (5'-TCTCCATTTCTGGT TTACAAGAC-3') (Irwin *et al.* 1991). The thermal cycling parameters used were: 94°C for 4 minutes, 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 minutes. The control region was PCR-amplified as a single fragment using primers P (5'-TCCTACCATCAGCACCCAAAG C –3') and E (5'- CCTGAAGTAGGAACCAGA TG -3') (Wilkinson & Chapman 1991). The thermal cycling parameters used were: 94°C for 4 minutes, followed by 40 cycles of (94°C for 1 minute, 55°C for 1.30 minutes and 72 °C for 2 minutes) and followed by 72 °C for 7 minutes.

Target fragments were purified from excised gel bands using the ZymoClean Gel Extraction Kit (Zymogen Inc.) and sequenced at Inqaba Biotechnical Industries, Hatfield, Pretoria, South Africa.

Sequences were aligned using the CLUSTAL W option (Thompson *et al.* 1994) in BioEdit v 5.0.9 (Hall 1999) and by visual inspection. Aligned sequences were cut to a common length of 306 base pairs (bp) for the control region, 830 base pairs for cytochrome *b* and 1178 base pairs for the concatenated cytochrome *b* – control region dataset. Sequences were deposited in GenBank (Appendix).

Sequence analysis

DnaSP 5.10 (Librado & Rozas 2009) was used to generate haplotype data files from the cytochrome *b* and control regions. The genetic results are presented as haplotype trees for the cytochrome *b* and control region datasets. jModelTest (Posada 2008) was used to select the most appropriate model of nucleotide substitution under the AIC criterion. The model selected for all datasets was the HKY sequence-evolution model (Hasegawa *et al*. 1985) with invariant sites (HKY+I). Maximum parsimony (MP) and neighbour-joining (NJ) trees were generated using PAUP* 4.0 (Swofford 2002). Nodal support of the MP and NJ trees was estimated by bootstrap resampling analysis using 1000 pseudoreplicates.

Bayesian analysis was implemented in MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2001). Four Markov chains were run (three hot and one cold) for 15 million generations, and the first 500 000 trees were discarded as burn-in. The priors for the five active parameters were: 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 branch lengths were unconstrained = exponential (10.0). The phylograms were 50% majority-rule consensus trees with nodal support indicated as posterior probabilities. *Chaerephon atsinanana*, *Mops leucostigma* and *M. midas* were used as outgroups (Appendix).

Population genetic analyses

The control region dataset was used for population genetic analyses as it is more variable than the cytochrome *b* gene. Analyses were carried out in DnaSP to determine haplotype and nucleotide diversity values and neutrality test statistics (Fs and Fu & Li's [1993] D* and F* (Fu 1997)). Mismatch distribution analysis was used to examine the mutational profile of the *C. pumilus* s.l. samples from south eastern Africa, and to determine whether they behave as a single overall population, and if so, whether it is expanding. Past population sizes were also estimated using a

Bayesian skyline plot, implemented in BEAST v.1.5.4 (Drummond *et al*. 2005). The number of grouped intervals (m) was set to five and the prior to 4E-4. The chains were run for 10 000 000 generations, sampling once per 1000 iterations. The Bayesian skyline plot was created using Tracer v.1.5 (Rambaut & Drummond 2009).

Molecular dating analysis

A time-scale for the divergence of *C. pumilus* s.l. clades was calculated using Bayesian inference as implemented in BEAST version 1.5.4 (Drummond & Rambaut 2010) with the 307bp control region alignment. The substitution model was set to HKY with a gamma distribution of rate heterogeneity. The analysis consisted of 50 million generations with a 10% burnin. A relaxed molecular clock (uncorrelated lognormal) was employed and a Yule species prior was used to calibrate the analysis with data for the expansion of Clade 1.1 (13000 – 3300 years before present (BP); Taylor *et al*. 2009). Time estimates were calculated based on an XML file from BEAST in TreeAnnotator version 1.5.4 (Drummond & Rambaut 2010). The dated tree was then viewed and edited in FigTree v.1.3.1. The Bayesian skyline plot was based on a mutation rate of 1.73×10^{-7} mutations per site per generation.

Network analysis

Statistical parsimony haplotype networks were constructed for the cytochrome *b* and control region datasets in TCS (Clement *et al*. 2000). The program implements the estimation of gene genealogies from DNA sequences as described by Templeton *et al*. (1992).

Echolocation and body size

Body mass was measured to the nearest 0.5 g using a Pesola scale. Forearm length was measured using digital callipers (to the nearest 0.1 mm). Echolocation calls of 36 captured bats were recorded directly onto a laptop computer (Hewlett Packard Pavilion 6210 notebook) connected to an

Avisoft Ultrasound 116 bat detector (Avisoft Bioacoustics, Berlin, Germany) as they were hand-released back into open habitat. The sampling rate was set at 500 000 Hz (16 bits, mono) with a threshold of 16. Bats were released at dusk the day after capture and followed for as long as possible after release to ensure that the search-phase calls were recorded (O'Farrell *et al*. 1999).

Peak echolocation frequency (PF in kHz), maximum frequency (Fmax in kHz) and minimum frequency (Fmin in kHz) were measured from the power spectrum (Obrist 1995). Fmax and Fmin were measured at ± 18 dB from the peak frequency on the power spectrum, call duration (Dur in ms) was measured from the time amplitude display (Biscardi *et al*. 2004), and bandwidth (BW in kHz) was the difference between Fmax and Fmin (Fullard *et al*. 2003). To avoid pseudoreplication, a single high quality search-phase call was selected on the basis of a high signal to noise ratio (bat signal at least three times stronger than background noise).

Statistical analyses

SPSS, version 19.0 (SPSS Inc. 2010) was used for statistical analysis. Data were screened for normality using the Kolmogorov-Smirnov test (Dytham 2003; all $P > 0.2$). A t-test was performed to check for sexual dimorphism. A multivariate ANOVA (MANOVA) and post-hoc Tukey's tests were performed to identify differences in bioacoustic variables among the four south eastern African *C. pumilus* s.l. lineages. In order to determine whether these lineages showed distinct echolocation characteristics, Discriminant Function Analysis (DFA) was used on PF, Dur, BW, Fmin and Fmax. The parameters were set to 'all groups equal' within the prior probabilities to circumvent the problem of unequal sample sizes.

RESULTS

Genetic analysis

We present three trees illustrating the phylogenetic structure of *Chaerephon pumilus* s.l. from south eastern Africa. Figures 1 (cytochrome *b*) and 2 (control region), which include present experimental samples and samples from Taylor *et al*. (2009) (n = 95), are included for phylogeny estimation. Figure 3 (concatenated cytochrome *b* and control region) includes 36 recently collected samples for which echolocation data were obtained, and which are included in the Discriminant Function Analysis. In all cases, maximum parsimony (MP), neighbour-joining (NJ) and Bayesian inference (BI) analyses were largely congruent and are presented as single figures (Figs 1, 2 and 3) (Nodal support ≥ 95% (BV) or 0.98 (PP) is strongly supported, ≥ 70% - 94% (BV) or ≥ 0.90 – 0.97 (pp) is well supported, < 70% (BV) or < 0.90 (pp) is weakly supported). In these analyses, the *Chaerephon* haplotypes formed a strongly-supported monophyletic clade with respect to the *Mops* outgroups; (MP - 100% bootstrap value (BV); NJ - 100% BV; BI - 1.00 posterior probability (PP)). *Chaerephon atsinanana* (Madagascar) formed a sister clade to the other *Chaerephon* samples included in this study; this was strongly supported (MP - 100% BV; NJ - 100% BV; BI - 1.00 PP) in the cytochrome *b* (Fig. 1) and control region analyses (Fig. 2). The *C. pumilus* s.l. clade was paraphyletic, containing the nested *C. leucogaster* Clade 2.3 (Fig. 1: MP - 97% BV; NJ - 70% BV; BI - 0.94 (<0.98 PP) (Fig. 2: MP - 98% BV; NJ - 97% BV; BI - 0.94 (<0.98 PP)).

The aligned, trimmed cytochrome *b* dataset (830 bp) comprised 76 individuals and 17 haplotypes. The nucleotide diversity was 0.014 and the haplotype diversity 0.822. The *C. pumilus* s.l. clade comprised well-supported subclades (Clade 2.1 and Clade 2.2; Fig. 1) (≥ 70% (BV) and ≥ 0.90 (pp)). HKY+I genetic distances among *C. pumilus* s.l. haplotypes ranged from 0.1% to 1.4% (Table 1). The mean genetic distance between *C. pumilus* s.l. clades (1, 2.1 and 2.2) was 0.7%

(0.1% - 1.4%). The mean genetic distance between Clade 2.3 (*C. leucogaster*) and the *C. pumilus* s.l. clades was 0.95% (0.1% - 1.3%).

Control region sequences of 100 individuals yielded 25 haplotypes. Sister to the *C. atsinanana* clade was a very strongly supported *C. pumilus* s.l. clade (Fig. 2). Overall, the structure of this clade was similar to its equivalent in the cytochrome *b* tree, although some of the groupings had lower nodal support values; a similar clade naming system has been used in all trees. Within Clade 1 there was moderate - strong support for Clade 1.1 (MP - 94% BV; NJ – 60% BV; BI – 0.97 PP) and moderate - weak support for Clade 1.3 (MP - 70% BV; NJ - 59% BV; BI – 0.86 PP). An additional clade (1.2) comprising samples from the Durban area was moderately supported (MP - 85% BV; NJ - 87% BV; BI – 0.86 PP). Clade 2.1 was moderately-weakly supported (MP - 79%BV; NJ - 53% BV; BI – 0.94 PP), whereas Clade 2.2 (MP - 76 % BV; BI - 1.00 PP) was moderately supported and Clade 2.3 (*C. leucogaster*) was strongly supported.

Table 1. Individual pairwise genetic distances using the HKY+I model (below diagonal) and uncorrected p-distances (above diagonal) between *Chaerephon* haplotypes based on 306 bp of the control region. Abbreviations used: CP - *C. pumilus* s.l.; CL- *C. leucogaster*; outgroups CA - *C. atsinanana*, MM - *Mops midas*, ML – *Mops leucostigma*.

Species	Hap	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9	CP10	CL11	CL12	CL13	CA14	CA15	MM	ML
	1	-	0.001	0.002	0.011	0.010	0.015	0.004	0.011	0.006	0.013	0.006	0.007	0.008	0.025	0.022	0.108	0.108
	2	0.001	-	0.001	0.009	0.008	0.013	0.002	0.010	0.005	0.012	0.005	0.006	0.007	0.024	0.020	0.107	0.107
	3	0.002	0.001	-	0.011	0.010	0.015	0.004	0.011	0.006	0.013	0.006	0.007	0.008	0.025	0.022	0.108	0.108
C. pumilus s.l.	**4**	0.011	0.009	0.011	-	0.001	0.011	0.010	0.012	0.010	0.010	0.007	0.008	0.010	0.024	0.020	0.107	0.107
	5	0.009	0.008	0.009	0.001	-	0.010	0.008	0.011	0.008	0.008	0.006	0.007	0.008	0.022	0.019	0.106	0.106
	6	0.014	0.013	0.014	0.011	0.009	-	0.013	0.006	0.013	0.004	0.011	0.012	0.013	0.024	0.023	0.113	0.109
	7	0.003	0.002	0.004	0.009	0.008	0.014	-	0.012	0.007	0.012	0.007	0.008	0.010	0.026	0.023	0.109	0.107
	8	0.010	0.009	0.010	0.012	0.010	0.006	0.012	-	0.012	0.002	0.010	0.010	0.012	0.025	0.021	0.112	0.110
	9	0.006	0.004	0.006	0.009	0.008	0.014	0.007	0.012	-	0.012	0.004	0.006	0.007	0.022	0.018	0.105	0.102
	10	0.013	0.012	0.013	0.009	0.008	0.003	0.012	0.002	0.012	-	0.009	0.010	0.012	0.025	0.022	0.112	0.108
C. leucogaster	**11**	0.006	0.004	0.006	0.007	0.006	0.010	0.007	0.009	0.004	0.009	-	0.001	0.002	0.022	0.018	0.107	0.107
	12	0.007	0.006	0.007	0.008	0.007	0.012	0.008	0.010	0.006	0.010	0.001	-	0.004	0.023	0.019	0.108	0.108
	13	0.008	0.007	0.001	0.009	0.008	0.013	0.009	0.012	0.007	0.012	0.002	0.003	-	0.024	0.020	0.107	0.107
C. atsinanana	**14**	0.025	0.024	0.025	0.024	0.023	0.024	0.027	0.025	0.022	0.025	0.022	0.023	0.024	-	0.004	0.106	0.109
	15	0.022	0.020	0.022	0.020	0.019	0.023	0.023	0.022	0.018	0.022	0.018	0.019	0.020	0.003	-	0.102	0.106
M. midas	**16**	0.121	0.120	0.121	0.120	0.118	0.127	0.123	0.126	0.117	0.126	0.120	0.121	0.120	0.118	0.113	-	0.112
M. leucostigma	**17**	0.121	0.120	0.121	0.120	0.118	0.122	0.119	0.124	0.113	0.121	0.120	0.121	0.120	0.122	0.118	0.125	-

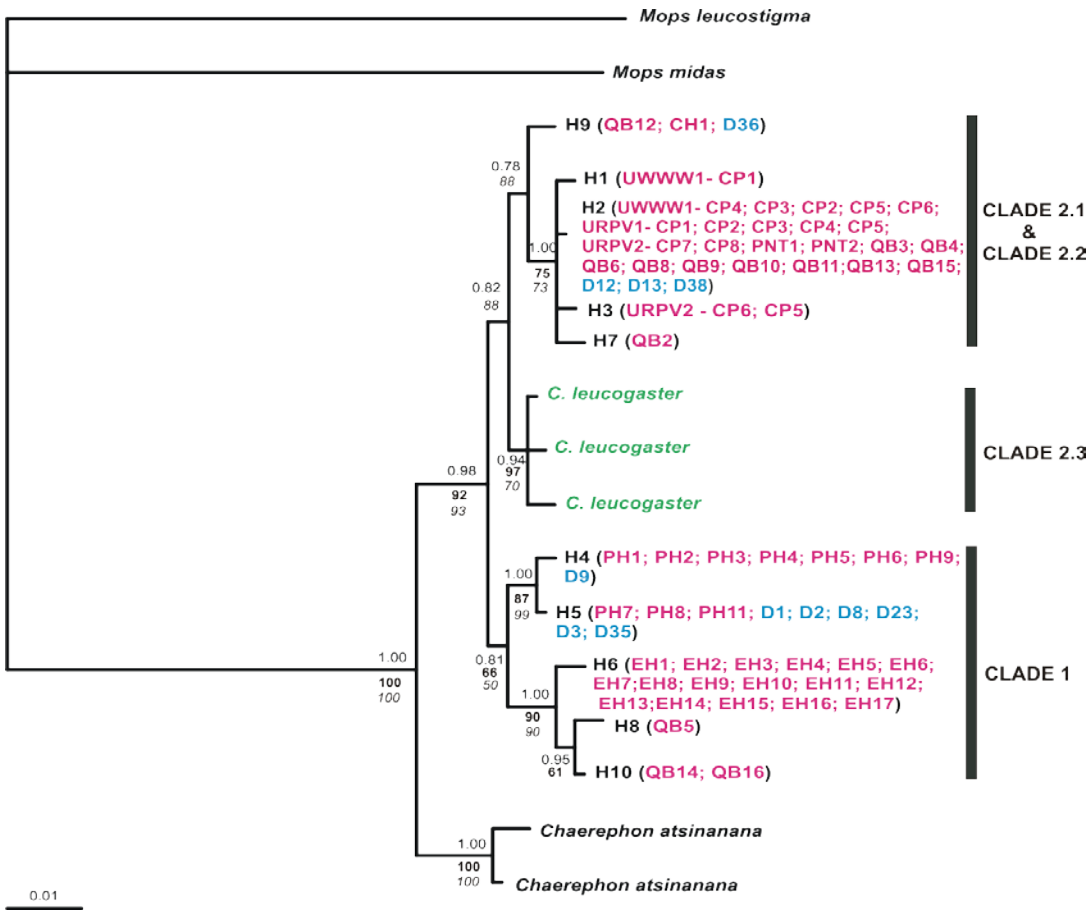

Figure 1. Bayesian phylogram based on analysis of 830 bp of the mitochondrial cytochrome *b* region illustrating the evolutionary relationships between haplotypes of *Chaerephon pumilus* s.l., *C. leucogaster*, *C. atsinanana* and the chosen outgroups, *Mops leucostigma* and *M. midas*. Values at nodes represent bootstrap support for maximum parsimony (bold font) and neighbour-joining (italic font) analyses, and Bayesian posterior probabilities (normal font). Scale bar units are substitutions per site. Font colours: blue - samples used by Taylor *et al*. (2009); pink - new samples; green - *C. leucogaster* from Madagascar. The codes provided for each haplotype - refer to the field numbers provided in the Appendix.

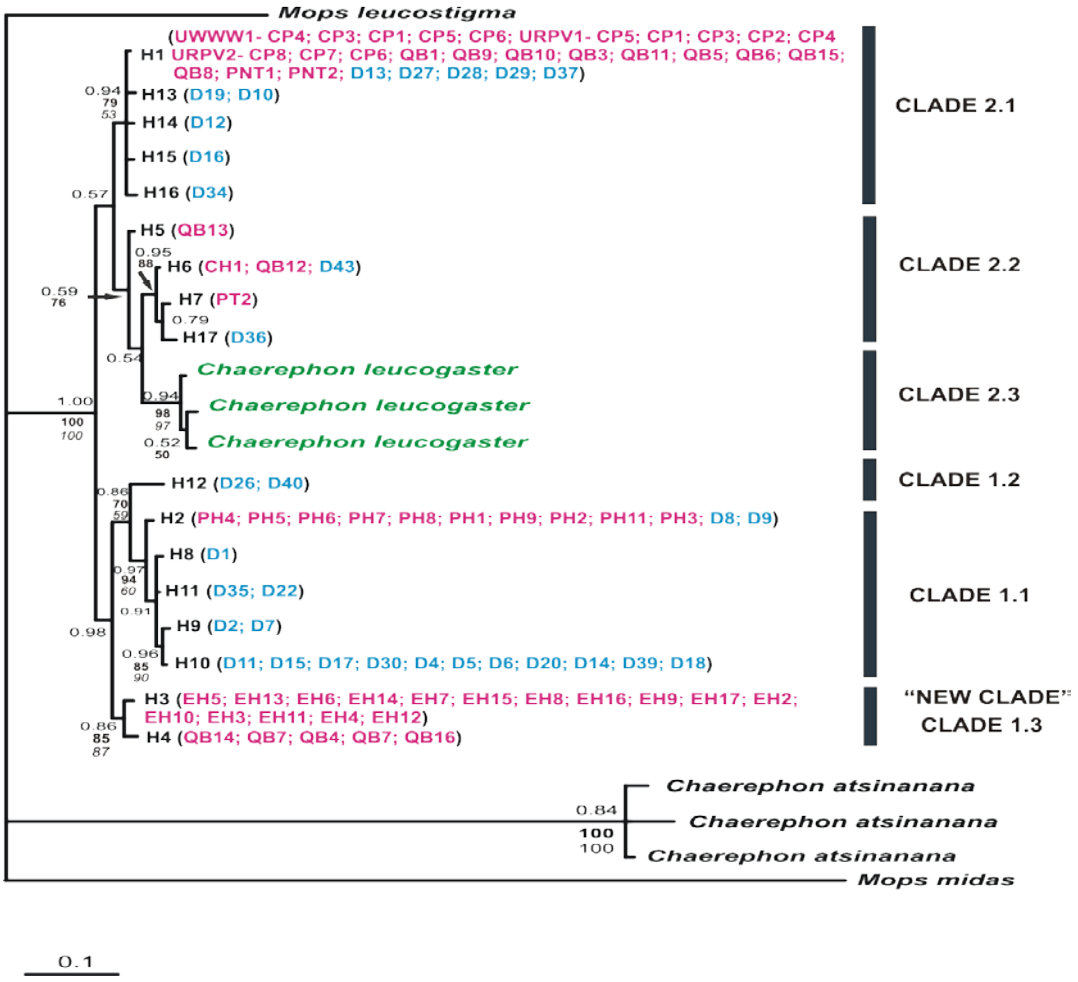

Figure 2. Bayesian phylogram based on analysis of 306 bp of the mitochondrial control region illustrating the evolutionary relationships between *Chaerephon pumilus* s.l. , *C. leucogaster* and *C. atsinanana* haplotypes and outgroups, *Mops midas* and *M. leucostigma*. Values at nodes represent bootstrap support obtained for maximum parsimony (bold) and neighbour-joining (italicised) analyses, and posterior probabilities for Bayesian Inference analysis (normal). Scale bar units are substitutions per site. Font colours: blue - samples used by Taylor *et al*. (2009), pink - samples captured for this study, green - *C. leucogaster* (Madagascar) samples.

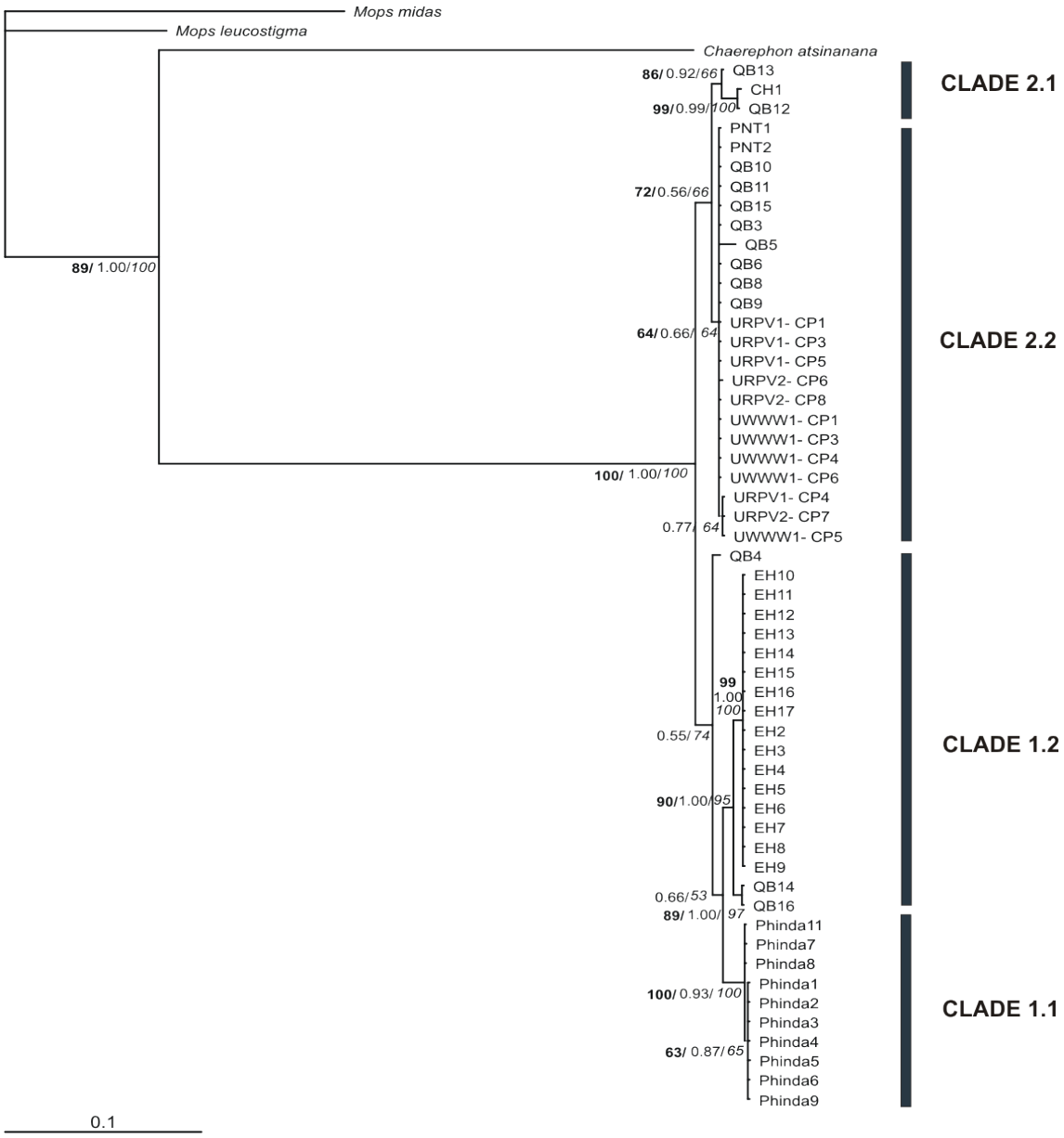

Figure 3. Bayesian phylogram based on analysis of 1178 bp of the concatenated mitochondrial cytochrome *b* and control regions illustrating the evolutionary relationships between *Chaerephon pumilus* s.l. samples and the outgroups *C. atsinanana*, *Mops leucostigma* and *M. midas*. Values at nodes represent bootstrap support for maximum parsimony (bold font) and neighbour-joining (italic font) analyses and posterior probabilities for Bayesian Inference analysis (normal font).

Population analyses

Analysis of 306 bp of the control region of 92 *C. pumilus* s.l. individuals yielded 242 variable sites of which 78 were parsimony informative. The ingroup dataset comprised 17 haplotypes; the haplotype diversity was 0.839 and nucleotide diversity 0.036. Consistent with the expectation of an expanding population (Russell *et al*. 2005), Fu and Li's (1993) F* and D* were not significant. However contrary to this expectation, Fu and Li's (1997) Fs was not significant (Table 2) and the mismatch distribution was ragged. Thus overall the data were inconsistent with the profile of an expanding population. The multimodal *C. pumilus* s.l. mismatch distribution (Fig. 4) was consistent with a sample that had differentiated into stable subdivisions (Rogers & Harpending 1992). The shape of the Bayesian skyline plot indicated a constant population size from ~500 000 - ~40 000 years before present. The apparent decrease in size from ~40 000 years ago to the present has a high degree of uncertainty in the 95% credibility range of the plot and should be viewed with caution (Fig. 5).

Table 2. Diversity and neutrality statistics for *Chaerephon pumilus* s.l. populations based on 306 bp of the mitochondrial control region.

	Control region dataset	**Expectation[#]**
Nucleotide diversity (Pi)	0.03685	Low
Haplotype diversity (h)	0.839	High
Expansion coefficient (S/*k*)	3.918	High
Fu & Li's (1993) F*	1.37	Not significant
Fu & Li's (1993) D*	1.33	Not significant
Fu & Li's (1997) Fs	4.16	Significant
Raggedness (r)	0.0784	Not significant
Mismatch distribution	Multimodal	

#Expected trends for a model of demographic population expansion (Hull & Girman 2005).
*D** and *F** -- departures from neutrality assessed as a deviation between estimates of nucleotide diversity derived from external branches of a phylogeny and from the total number of mutations (*D**) or from the average pairwise diversity (*F**). Fs - an estimate of the probability of observing a random sample with a number of alleles equal to or smaller than the observed value, given the observed level of diversity (Fu 1997).

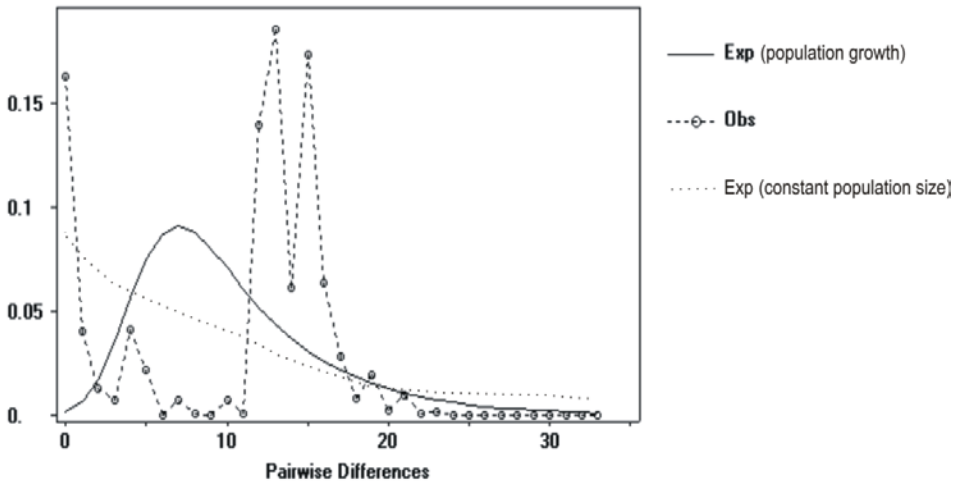

Figure 4. Mismatch distribution for populations of *Chaerephon pumilus* s.l. from south eastern Africa. Dashed line –observed (multimodal) distribution; solid line - expected distribution under a model of population growth/decline; dotted line – expected distribution under a model of constant population size.

Figure 5. Bayesian skyline plot of past *Chaerephon pumilus* s.l. (south eastern Africa) population sizes based on a mutation rate of 1.73×10^{-7} mutations per site per generation. The heavy line represents the median and the solid intervals represent the 95% credibility range.

Network analyses

The cytochrome *b* haplotype network included 10 *C. pumilus* s.l. haplotypes (Fig. 6a) in addition to *C. leucogaster* and *C. atsinanana*. *C. atsinanana* was excluded from the haplotype network when connections were set at a 95% connection limit. *C. leucogaster* occupied a central position within the *C. pumilus* s.l. network, and was separated from H2 and H9 by 7 and 4 mutational steps respectively. Adjacent haplotypes within the *C. leucogaster* network were separated by between 1 and 6 mutational steps. Haplotypes belonging to Clade 1 were separated from those belonging to Clades 2.1 & 2.2 (defined in Fig. 1) by 5 mutational steps. *C. atsinanana* was separated from *C. pumilus* s.l. and *C. leucogaster* by a minimum of 16 and 19 mutational steps respectively.

The control region haplotype network consisted of 17 *C. pumilus* s.l. haplotypes (Fig. 6b). Analyses based on 95% connection limit yielded five *C. pumilus* s.l. networks, in addition to *C. leucogaster* and *C. atsinanana*. Haplotypes within *C. pumilus* s.l. networks were separated by between 1 and 5 mutations, whereas networks were separated by between 4 and 37 mutations. *C. pumilus* s.l. was separated from *C. leucogaster* by a minimum of 12 mutational steps, and from *C. atsinanana* by a minimum of 46 mutational steps.

Dating analysis

Estimates of the divergence times of the major clades were obtained using a relaxed Bayesian clock approach based on an alignment of 307 bp of the mtDNA control region (see Fig. 2). Clades appear to have arisen between 60 and 13 000 years BP (Table 3).

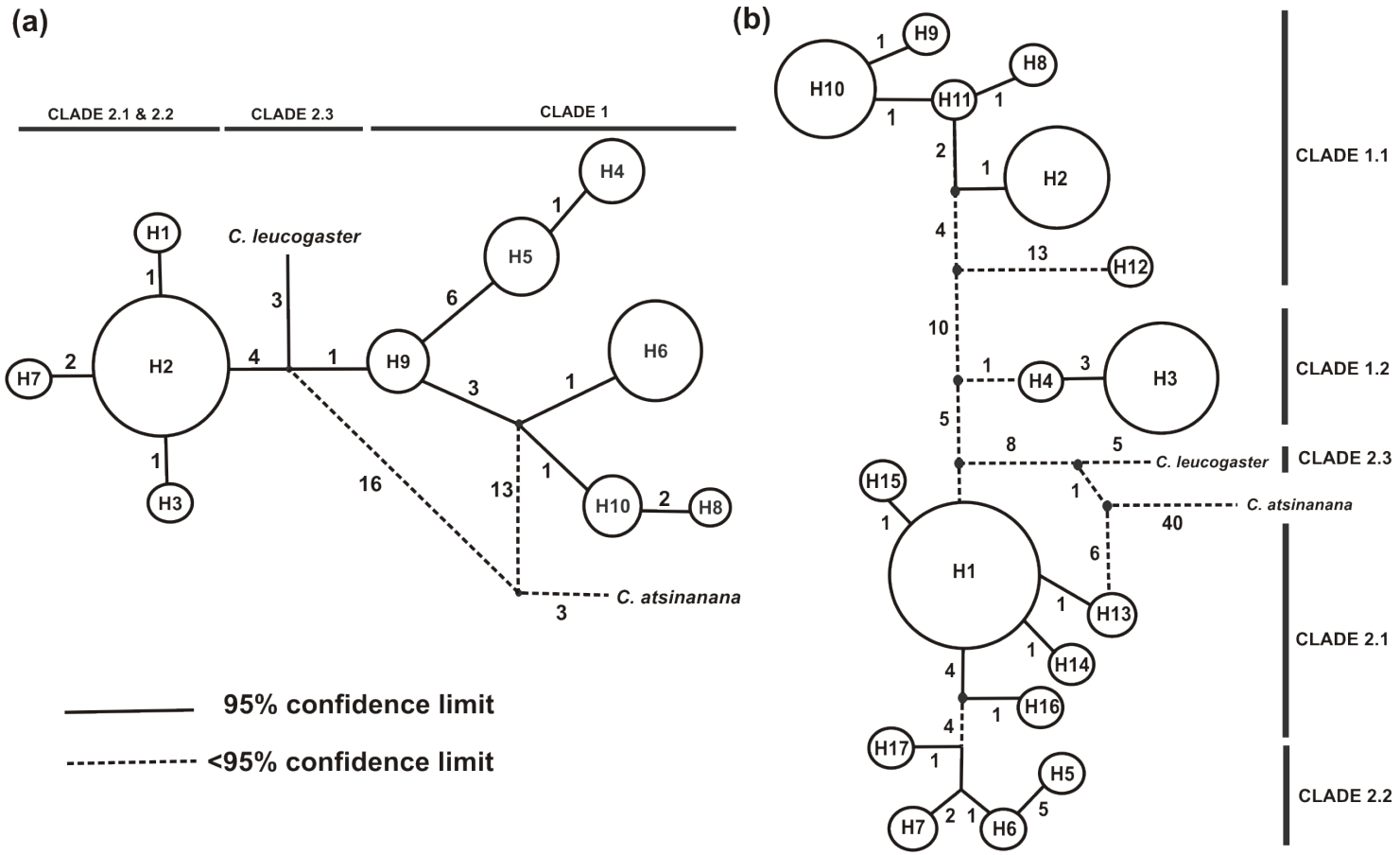

Figure 6. Statistical parsimony networks illustrating mutational relationships among haplotypes of *C. pumilus* s.l., *C. leucogaster* and *C. atsinanana* for (a) 830 bp of the cytochrome *b* region and (b) 306 bp of the control region.

Table 3. Ages of *Chaerephon pumilus* s.l. (south eastern Africa) control region clades (estimated using BEAST; Drummond *et al*. 2005). BP – before present.Tree not shown (refer to Fig. 2).

Node on tree	Age (Thousands of years BP)	95% confidence limits	
		Lower	Upper
Clade 1.1	13.0	0.08	74.0
Clade 1.2	51.2	0.07	61.4
Clade 1.3	32.4	0.06	72.0
Clade 2.1	60.0	0.09	69.3
Clade 2.2	36.5	0.11	73.9

Can echolocation characteristics predict phylogenetic structure?

Preliminary t-tests showed no evidence for sexual dimorphism in males and females hence the data for both the sexes were combined.

We recorded echolocation calls of 36 individuals of *C. pumilus* s.l. from KwaZulu-Natal. A phylogenetic tree for these individuals, based on a concatenated cytochrome *b* and control region dataset (1178 bp), was congruent with cytochrome *b* and control region phylogenies in Figs 1 and 2. There was much overlap in the five echolocation parameters across individuals within the different clades (Table 4). Consequently, the DFA was not able to assign individuals based on echolocation parameters to clades (Wilks' Lambda 0.381, $F_{(12,36)} = 6.24$, $P < 0.005$) (Table 5). Clades 1.1, 1.2, 2.1 and 2.2 showed marked overlap along Factor 1 and Factor 2 (Table 4, Fig. 7). The classification success for these lineages ranged from 52.9% (clade 2.2) to 87.5% (clades 1.2 and 1.1).

There were no significant differences in PF among the four clades (all $P > 0.05$). Conversely, there was a significant difference in Dur and BW (MANOVA, $F_{(3;\ 36)} = 6.24$, $P = 0.002$); $F_{(3;\ 36)} = 4.02$, $P = 0.016$), respectively. According to the post hoc Tukey's test, Dur and BW

of clade 2.2 were significantly shorter than Dur of clade 1.2 and BW of clade 1.1 ($P< 0.01$), respectively. There was no significance between PF, BW and Dur ($P> 0.05$).

There was no significant difference in mass or forearm length among the clades ($P> 0.05$) and considerable overlap in these parameters exists among individuals of clades (Table 4).

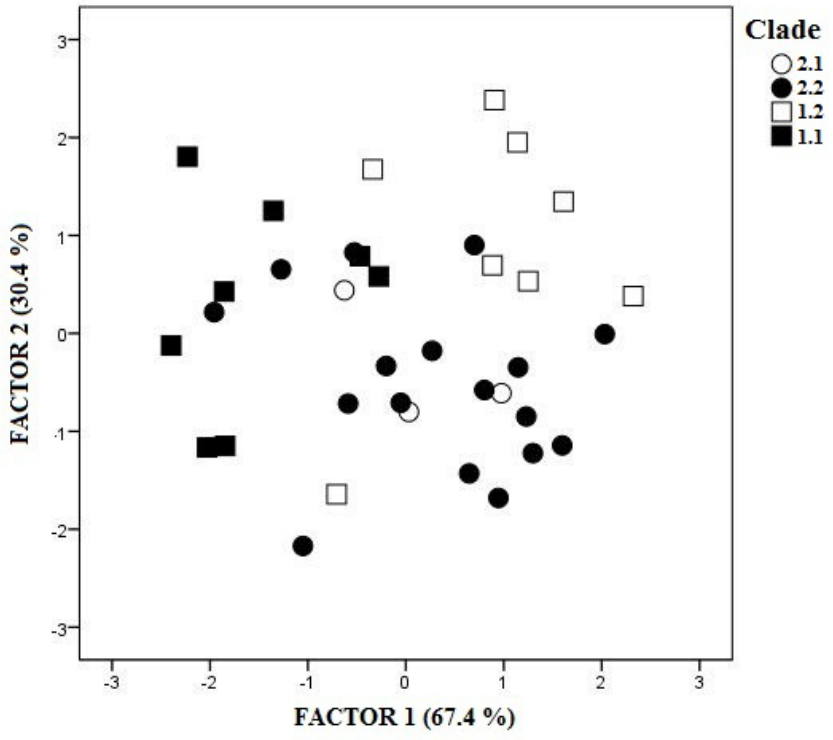

Figure 7. Plot of canonical scores from discriminant function analysis on echolocation parameters of four clades of *Chaerephon pumilus* s.l. from south eastern Africa (clades 1.1, 1.2, 2.1, 2.2 from Fig. 3)

Table 4. Four echolocation parameters (peak frequency, bandwidth, duration, Fmin), mass and forearm length of 36 individuals of *Chaerephon pumilus* s.l. distributed across four genetic clades (1.1 – 2.2). Refer to Appendix (Table 1) for further details of samples. SD – standard deviation.

Sample name	Lineage	Peak frequency (kHz)	Bandwidth (kHz)	Duration (ms)	Fmin (kHz)	Fmax (kHz)	Mass (g)	Forearm length (mm)
CH1	2.1	26.2	5.1	14.0	24.0	29.1	11.0	35.8
QB12	2.1	24.9	2.6	12.8	23.0	25.6	8.5	35.0
QB13	2.1	23.3	3.4	15.1	22.0	25.4	10.5	38.0
Mean ± SD		**24.8 ± 1.5**	**3.7 ± 1.3**	**13.9 ± 1.2**	**23.0 ± 1.0**	**26.7 ± 2.1**	**10.0 ± 1.3**	**36.3 ± 1.6**
PNT1	2.2	24.5	2.6	14.6	23.0	25.6	10.0	38.3
PNT2	2.2	26.9	4.2	11.1	24.0	28.2	13.0	37.6
QB3	2.2	25.4	4.2	12.0	22.0	26.2	9.0	35.4
QB5	2.2	25.1	4.7	14.6	24.0	28.7	8.0	34.5
QB6	2.2	23.5	3.1	12.5	24.0	27.1	8.0	36.7
QB8	2.2	23.3	3.7	13.2	22.0	25.7	10.0	38.4
QB9	2.2	23.5	3.1	15.9	23.0	26.1	12.0	35.7
QB11	2.2	25.8	5.1	14.0	23.0	28.1	10.5	39.2
URPV1CP1	2.2	26.6	3.0	13.2	24.0	27.0	8.0	37.4
URPV1CP3	2.2	26.8	3.6	14.0	23.0	26.6	9.5	36.7
URPV2CP1	2.2	26.2	3.0	13.0	24.4	27.4	10.0	36.5
URPV2CP3	2.2	26.1	2.1	14.2	24.8	26.9	9.0	35.7
UWWWCP1	2.2	24.7	2.6	14.0	24.0	26.6	10.0	36.4
UWWWCP3	2.2	26.0	2.2	13.7	23.3	25.5	10.0	37.2
UWWWCP4	2.2	26.9	2.4	15.0	24.0	26.4	9.5	37.7
UWWWCP5	2.2	24.7	3.0	15.0	24.8	27.8	9.0	37.1
UWWWCP6	2.2	24.9	3.2	14.5	23.3	26.5	11.0	37.8
Mean ± SD		**25.4 ± 1.2**	**3.3 ± 0.9**	**13.8 ± 1.2**	**23.6 ± 0.8**	**26.9 ± 0.9**	**9.8 ± 1.4**	**36.9 ± 1.2**
QB4	1.2	23.9	3.6	13.3	24.0	27.6	10.0	34.7
QB14	1.2	24.1	5.1	14.0	20.0	25.1	11.5	36.6
QB16	1.2	22.4	3.6	16.9	20.0	23.6	11.0	37.0
EH1	1.2	28.7	3.3	13.7	23.0	26.3	9.0	37.4
EH2	1.2	27.3	3.3	14.6	24.0	27.3	9.5	37.9
EH3	1.2	28.9	2.9	14.2	24.0	26.9	8.5	37.9
EH4	1.2	29.8	4.2	14.1	24.0	28.2	10.0	36.5
EH5	1.2	28.7	3.3	13.9	25.0	28.3	10.0	38.7
Mean ± SD		**26.7 ± 2.8**	**3.7 ± 0.7**	**14.3 ± 1.1**	**23.0 ± 1.9**	**26.7 ± 1.6**	**9.9 ± 0.9**	**37.1 ± 1.2**
PH1	1.1	25.2	3.4	11.1	24.0	27.4	10.0	41.2
PH2	1.1	26.4	5.7	11.7	22.0	27.7	11.0	40.3
PH4	1.1	26.2	4.2	11.2	23.0	27.2	13.5	40.1
PH5	1.1	26.9	4.9	12.3	23.0	27.9	12.0	39.5
PH6	1.1	24.5	4.3	11.9	24.0	28.3	12.0	39.6
PH8	1.1	26.2	4.5	13.4	23.0	27.5	12.0	39.6
PH9	1.1	25.8	4.5	13.8	23.0	27.5	12.0	39.5
PH11	1.1	26.2	4.5	11.0	24.0	28.5	11.0	37.8
Mean ± SD		**25.9 ± 0.8**	**4.5 ± 0.7**	**12.1 ± 1.1**	**23.3 ± 0.7**	**27.8 ± 0.5**	**11.7 ±1.0**	**39.7 ± 1.0**

Table 5. Results of Discriminant Function Analysis based on five bioacoustic variables (Dur – duration, PF – peak frequency, BW – bandwidth, Fmin – minimum frequency, Fmax – maximum frequency. 'Sig' – significant value) and membership of genetic lineages.

Function	Dur	PF	BW	Fmin	Fmax	Eigenvalues	Cumulative (%)	Wilks' lambda	d.f.	Sig
1	0.825	0.052	- 0.555	- 0.009	- 0.402	0.849	67.4	0.381	12	0.003
2	0.029	0.593	0.530	- 0.307	0.069	0.383	97.8	0.704	6	0.092

DISCUSSION

Molossid bats from south eastern Africa, referred to as *Chaerephon pumilus* s.l., are taxonomically complicated as they comprise a number of well-supported sympatric genetic lineages, and exhibit paraphyly, with the Malagasy *C. leucogaster* nested among them. Individuals of *C. pumilus* s.l. have been reported to echolocate at peak frequencies between 16.3 kHz and 23.9 kHz (Taylor 1999b; Fenton *et al*. 2004), leading to the hypothesis that it comprises a number of cryptic 'sono' species (Taylor *et al*. 2009) or lineages.

One of the aims of this study was to re-examine the phylogenetic structure of *C. pumilus* s.l. from south eastern Africa by extending the sample set used in the phylogenetic study of Taylor *et al*. (2009) both numerically and geographically. Overall, our phylogenetic analyses are congruent with those previously reported. The cytochrome *b* sequence data revealed two major clades separated by a genetic distance of 0.7% (0.1% - 1.4%), consistent with intraspecific distances for bats (Baker and Bradley, 2006). In addition to the four control region lineages identified by Taylor *et al*. (2009), we identified an additional clade comprising samples from the greater Durban area in KwaZulu-Natal. This well-supported lineage, comprising two haplotypes and 20 samples, was not fully resolved in the cytochrome *b* analyses. We conclude that *C. pumilus* s.l. from south eastern Africa comprises a number of sympatric mitochondrial lineages, representative of intraspecific variation.

However the *C. pumilus* s.l. clade, whilst well-supported, is paraphyletic, consistent with Taylor *et al*. (2009), as it contains a nested *C. leucogaster* lineage separated by a mean genetic distance of 0.95% (0.9% - 1.0%). This is consistent with intraspecific level distances reported by Baker and Bradley (2006), making the classification of *C. leucogaster* and *C. pumilus* s.l. as distinct genetic species somewhat questionable.

We investigated the hypothesis of Taylor *et al*. (2009) that the mitochondrial lineages of *C. pumilus* s.l. are distinguishable by different echolocation calls, and were unable to predict cladal designation based on a DFA of the echolocation calls. A wide range of PFs (23 – 29 kHz) was recorded in individuals of all four clades, and this range was consistent with published frequencies, except the 16 kHz sonotype was not recorded (Taylor 1999b; Fenton *et al*. 2004; Schoeman & Waddington 2011).We also found no significant difference in mean size of bats among lineages, which is consistent with the strong correlation between PF and body size observed in many species of insectivorous bats (Jones 1996). Although we found a significant difference in Dur between clades 1.2 and 1.1, acoustic divergence among cryptic species and populations typically involves PF or BW rather than Dur (Heller & von Helversen 1989; Russo & Jones 2000; Kingston *et al*. 2001; Jacobs *et al*. 2006, 2007; Furman *et al*. 2010; Ramasindrazana *et al*. 2011). However; none of these examples include molossid taxa. The difference of ~2.7 ms in Dur between lineages reduces the minimum detection range of prey by 45.9 cm for clade 1.2 (Schnitzler & Kalko 2001), and, hence, is unlikely to be important from an ecological or sensory perspective, especially for a fast-flying open-air forager. Instead, the Dur differences might be attributed to different recording conditions where bats from clade 1.1 perceived the habitats of release as more cluttered, despite our efforts to control for this by releasing the bats in open habitat.

This, in combination with the low inter-clade genetic distances, leads us to reject the hypothesis of Taylor *et al*. (2009) that these clades represent cryptic sono-species. Thus, based on current data, *C. pumilus* s.l. from south eastern Africa represents a single species with distinct lineages identified at the intraspecific level.

Indices of diversity and neutrality combined with a ragged multimodal mismatch distribution are inconsistent with the notion of demographic expansion of a single overall *C. pumilus* s.l. population in south eastern Africa (Hull & Girman 2005), and are consistent with a number of ancient stable populations at demographic equilibrium (Slatkin & Hudson 1991; Rogers

& Harpending 1992; Schneider & Excoffier 1999). Further, mismatch distributions based on both cytochrome *b* and the control region do not show the characteristic star-shape expected of an expanding population (Excoffier *et al*. 2009). Rather they are consistent with the existence of a number of stable co-existing lineages, as revealed by the phylogenetic analyses. There was some evidence of ancestor/descendant relationships; haplotype 2 (Fig. 6a, cytochrome *b*) appeared to be ancestral to haplotypes 1 and 3; haplotype 1 (Fig. 6b, control region) appeared ancestral to haplotypes 13, 14 and 15, and haplotype 10 to haplotypes 9 and 11. However many of the haplotypes were separated by more than one mutational step, and could not easily be inferred as ancestors or descendants of other haplotypes. A similarly-structured haplotype network has been reported for *C. atsinanana* from eastern Madagascar (Lamb *et al.* 2012), consistent with the existence of ancient stable populations of this *Chaerephon* species. These authors hypothesise that the highly structured populations of *C. atsinanana* are maintained by female philopatry, a possible explanation for the similar structuring in *C. pumilus* s.l. observed here. Our Bayesian skyline analysis indicates that the population size of *C. pumilus* s.l. has been constant for the last ~ 400 000 years of the Quaternary. Although there is an apparent decrease in size from ~40 000 years ago to the present, the 95% credibility range in this part of the plot indicates a high degree of uncertainty and should be viewed with caution. Dating analyses indicate that the independent *C. pumilus* s.l. lineages were established during the late Pleistocene, between 60 000 and 13 000 years ago. Consistent with this, Taylor *et al*. (2009) suggested that two lineages (Clade 1.1 and 2.1 in our analyses) underwent population expansion 12 956 - 3 360 yr BP and 59 434 - 14 726 yr BP, respectively, possibly associated with expansion from Pleistocene glacial refugia, a process shown to be influential in shaping patterns of genetic diversification among different taxa (Matthee & Robinson 1997; Hewitt 2000; Flagstad *et al*. 2001; Muwanika *et al*. 2003; Hewitt 2004; Anthony *et al*. 2007; Brown *et al*. 2007; Voelker *et al*. 2010; Miller *et al*. 2011).

The timing of the origin of the south eastern African *C. pumilus* s.l. lineages corresponds to the existence of scarp forests along the eastern seaboard (10-15 km inland), which may have acted as major refugia for a number of organisms during the last glacial maximum (Lawes *et al.* 2007). These forests could have acted as refugia for *C. pumilus* s.l. directly by providing roosts in narrow cracks of trees, or indirectly by influencing the distribution of their insect prey. Open-air bats including *Chaerephon* will forage on insects above the trees in the forests. Forest habitats probably harbour more insect abundance and diversity than more homogenous grassland habitats.Water bodies and rivers may also have facilitated the maintenance and expansion of lineages as they would have acted as corridors to suitable foraging areas, but our data do not distinguish between these hypotheses.

To conclude, we confirmed that *Chaerephon pumilus* s.l. comprises a number of stable sympatric lineages whose demographic history may have been shaped by behavioural and/or climatic factors. We found that the balance of evidence does not support the existence of cryptic lineages of *C. pumilus* s.l. with distinct echolocation characteristics. We confirmed that *Chaerephon pumilus* s.l. comprises a number of stable sympatric lineages whose demographic history may have been shaped by behavioural and/or micro-climatic factors rather than geographic processes. One potential explanation for these stable sympatric lineages is that they reflect a behavioural system of female philopatry, or roost faithfulness, as revealed by the maternally-inherited mitochondrial markers, consistent with similar findings for the Australian ghost bat, *Macroderma gigas* (Worthington-Wilmer *et al*. 1999). Further, in the Durban area *C. pumilus* s.l. has been observed to emit social calls during emergence from roosts and in flight (M. Corrie Schoeman & Peter J. Taylor, unpubl. data). We hypothesise that the *C. pumilus* s.l. populations are characterised by differing social calling systems, however this will need to be verified by detailed ecological work on the social dynamics of these bats.

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Appendix

Details of captured bats and museum specimens used in this study. Most localities are within the KwaZulu-Natal Province of South Africa, unless otherwise noted (SZ –Swaziland). The code used in the analyses are the codes assigned to each specimen (DM - Durban Natural Science Museum; CROW - Centre for Rehabilitation of Wildlife, Durban, South Africa; FMNH – Field Museum of Natural History). Abbreviations Cyt *b* – Cytochrome *b*;CR – Control region

Field number	Museum number	Locality	Genbank number		Latitude	Longitude	Sex	Haplotype number	
			Cyt *b*	CR				Cyt *b*	CR
C. pumilus s.l.									
UWWW1CP1	-	Umbilo Waste Water	JX976488	JX976432	29.846S	30.890E	F	1	1
UWWW1CP3	-	Umbilo Waste Water	JX976490	JX976431	29.846S	30.890E	F	2	1
UWWW1CP4	-	Umbilo Waste Water	JX976489	JX976430	29.846S	30.890E	F	2	1
UWWW1CP5	-	Umbilo Waste Water	JX976491	JX976433	29.846S	30.890E	F	2	1
UWWW1CP6	-	Umbilo Waste Water	JX976492	JX976434	29.846S	30.890E	F	2	1
URPV1CP1	-	Paradise Valley	JX976493	JX976436	29.831S	30.892E	M	2	1
URPV1CP2	-	Paradise Valley	JX976494	JX976438	29.831S	30.892E	F	2	1
URPV1CP3	-	Paradise Valley	JX976495	JX976437	29.831S	30.892E	F	2	1
URPV1CP4	-	Paradise Valley	JX976496	JX976439	29.831S	30.892E	F	2	1
URPV1CP5	-	Paradise Valley	JX976497	JX976435	29.831S	30.892E	F	2	1
URPV2CP6	-	Paradise Valley	JX976498	JX976442	29.831S	30.892E	F	2	1
URPV2CP7	-	Paradise Valley	JX976499	JX976441	29.831S	30.892E	F	2	1

URPV2CP8	-	Paradise Valley	JX976500	JX976440	29.831S	30.892E	F	2	1
PNT1	-	Pinetown	JX976501	JX976486	29.828S	30.866E	F	2	1
PNT2	-	Pinetown	JX976502	JX976487	29.828S	30.866E	F	2	1
PH1	-	Phinda: Swilles	JX976503	JX976448	27.695S	32.356E	M	4	2
PH2	-	Phinda: Swilles	JX976504	JX976450	27.695S	32.356E	F	4	2
PH3	-	Phinda: Swilles	JX976505	JX976452	27.695S	32.356E	F	4	2
PH4	-	Phinda: Swilles	JX976506	JX976443	27.695S	32.356E	F	4	2
PH5	-	Phinda: Swilles	JX976507	JX976444	27.695S	32.356E	F	4	2
PH6	-	Phinda: Swilles	JX976508	JX976445	27.695S	32.356E	M	4	2
PH7	-	Phinda: Swilles	JX976509	JX976446	27.695S	32.356E	M	5	2
PH8	-	Phinda: Swilles	JX976510	JX976447	27.695S	32.356E	F	5	2
PH9	-	Phinda: Swilles	JX976511	JX976449	27.695S	32.356E	F	4	2
PH11	-	Phinda: Swilles	JX976512	JX976451	27.695S	32.356E	F	5	2
EH1	-	Effingham Heights	JX976513	-	29.769S	31.010E	F	6	-
EH2	-	Effingham Heights	JX976514	JX976463	29.769S	31.010E	F	6	3
EH3	-	Effingham Heights	JX976515	JX976465	29.769S	31.010E	M	6	3
EH4	-	Effingham Heights	JX976516	JX976467	29.769S	31.010E	M	6	3
EH5	-	Effingham Heights	JX976517	JX976453	29.769S	31.010E	F	6	3
EH6	-	Effingham Heights	JX976518	JX976455	29.769S	31.010E	F	6	3
EH7	-	Effingham Heights	JX976519	JX976457	29.769S	31.010E	F	6	3

EH8	-	Effingham Heights	JX976520	JX976459	29.769S	31.010E	F	6	3
EH9	-	Effingham Heights	JX976521	JX976461	29.769S	31.010E	F	6	3
EH10	-	Effingham Heights	JX976522	JX976464	29.769S	31.010E	F	6	3
EH11	-	Effingham Heights	JX976523	JX976466	29.769S	31.010E	F	6	3
EH12	-	Effingham Heights	JX976524	JX976468	29.769S	31.010E	F	6	3
EH13	-	Effingham Heights	JX976525	JX976454	29.769S	31.010E	F	6	3
EH14	-	Effingham Heights	JX976526	JX976456	29.769S	31.010E	F	6	3
EH15	-	Effingham Heights	JX976527	JX976458	29.769S	31.010E	F	6	3
EH16	-	Effingham Heights	JX976528	JX976460	29.769S	31.010E	M	6	3
EH17	-	Effingham Heights	JX976529	JX976462	29.769S	31.010E	F	6	3
QB1	-	Queensburgh	-	JX976469	29.857S	30.899E	F	-	1
QB2	-	Queensburgh	JX976530	-	29.857S	30.899E	M	2	-
QB3	-	Queensburgh	JX976531	JX976472	29.857S	30.899E	M	2	1
QB4	-	Queensburgh	JX976532	JX976476	29.857S	30.899E	F	2	4
QB5	-	Queensburgh	JX976533	JX976482	29.857S	30.899E	M	8	1
QB6	-	Queensburgh	JX976534	JX976483	29.857S	30.899E	F	2	1
QB7	-	Queensburgh	-	JX976475	29.857S	30.899E	F	-	4
QB8	-	Queensburgh	JX976535	JX976485	29.857S	30.899E	M	2	1
QB9	-	Queensburgh	JX976536	JX976470	29.857S	30.899E	M	2	1
QB10	-	Queensburgh	JX976537	JX976471	29.857S	30.899E	F	2	1
QB11	-	Queensburgh	JX976538	JX976473	29.857S	30.899E	F	2	1
QB12	-	Queensburgh	JX976539	JX976480	29.857S	30.899E	F	9	6
QB13	-	Queensburgh	JX976540	JX976478	29.857S	30.899E	M	2	5
QB14	-	Queensburgh	JX976541	JX976474	29.857S	30.899E	F	10	4
QB15	-	Queensburgh	JX976542	JX976484	29.857S	30.899E	M	2	1
QB16	-	Queensburgh	JX976543	JX976477	29.857S	30.899E	F	10	4
CH1	-	Chatsworth	JX976544	JX976479	29.930S	30.925E	M	9	6
D1	DM 7363	Durban Int.	FJ415813	FJ415824	29.967S	30.942E	F	5	8

		Airport							
D2	DM 7367	Hell’s Gate	FJ415814	FJ415826	28.067S	32.421E	F	5	9
D4	DM 7369	Hell’s Gate	-	FJ415837	28.067S	32.421E	F	-	10
D5	DM 7370	Hell’s Gate	-	FJ415838	28.067S	32.421E	F	-	10
D6	DM 7371	Hell’s Gate	-	FJ415839	28.067S	32.421E	F	-	10
D7	DM 7372	Hell’s Gate	-	FJ415827	28.067S	32.421E	M	-	9
D8	DM 7373	uMkhuze	FJ415815	FJ415828	27.583S	32.217E	F	5	2
		Game Reserve							
D9	DM 7374	uMkhuze	FJ415816	FJ415829	27.583S	32.217E	M	4	2
		Game Reserve							
D10	DM 7377	Amanzimtoti	-	FJ415846	30.05S	30.883E	F	-	13
D11	DM 7378	Amanzimtoti	-	FJ415830	30.05S	30.883E	M	-	10
D12	DM 7379	Morningside	FJ415817	FJ415848	29.833S	31.00E	F	2	14
D13	DM 7380	CROW	FJ415818	FJ415849	Unknown		F	2	1
D14	DM 7381	Hell’s Gate	-	FJ415841	28.067S	32.421E	F	-	10
D15	DM 7382	Hell’s Gate	-	FJ415831	28.067S	32.421E	F	-	10
D16	DM 7383	CROW rehab	-	FJ415850	Unknown		M	-	15
D17	DM 7384	Hell’s Gate	-	FJ415832	28.067S	32.421E	M	-	10
D18	DM 7385	Bluff	-	FJ415836	29.933S	31.017E	F	-	10
D19	DM 7386	Ballito	-	FJ415847	29.533S	31.217E	M	-	13
D20	DM 7387	Bluff	-	FJ415840	29.933S	31.017E	M	-	10
D22	DM 7401	Amanzimtoti	-	FJ415843	30.05S	30.883E	-	-	11
D23	DM 7525	Charters Creek	FJ415819	-	28.2S	32.417E	M	5	-
D26	DM 7851	Umbilo	-	FJ415844	29.833S	31.00E	-	-	12
D27	DM 7905	Athlone Park	-	FJ415851	30.016S	30.917E	-	-	1
D28	DM 7907	Carrington Heights	-	FJ415852	29.883S	30.967E	M	-	1
D29	DM 7910	Pinetown	-	FJ415853	29.817S	30.85E	F	-	1
D30	DM 7913	Illovo	-	FJ415833	30.1S	30.833E	F	-	10
D31	DM7922	SZ: Mlawula	FJ415820	-	26.192S	32.005E	-	5	-
D34	DM 8030	Park Rynie	-	FJ415854	30.317S	30.733E	M	-	16
D35	DM 8036	SZ: Mlawula	FJ415821	FJ415834	26.192S	32.005E	M	5	11
D36	DM 8042	SZ: Wylesdale	FJ415822	FJ415856	25.819S	31.292E	F	9	17
D37	DM 8348	Durban City Hall	-	FJ415855	29.858S	31.025E	M	-	1
D38	DM 8437	SZ: Rosecraft	-	-	26.632S	31.293E	-	2	-
D39	-	Durban	-	FJ415842	29.867S	31.00E	-	-	10

D40	-	Yellowwood Park	-	FJ415845	29.917S	30.933E	-	-	12
D43	-	Durban	-	FJ415857	Unknown		-	-	6
PT 2011-2	-	Limpopo	-	JX976481	23.059S	30.067E	M	-	7
C. leucogaster									
-	FMNH 184924	Madagascar	EU716007	-	16.701S	46.072E	M	11	-
-	FMNH 184979	Madagascar	EU716024	-	16.116S	46.757E	F	12	-
-	FMNH 188644	Madagascar	EU716035	-	13.443S	48.348E	F	13	-
-	FMNH 184974	Madagascar	-	EU727524	16.414S	46.765E	F	-	18
-	FMNH 188500	Madagascar	-	EU727463	13.353S	48.192E	F	-	19
-	FMNH 184608	Madagascar	-	EU727517	15.713S	44.530E	M	-	20
C. atsinanana		Madagascar	JN867806	JN867854					
M. leucostigma		Madagascar	EF474049	FJ546305					
M. midas		Madagascar	EF474049	EF474034					

CHAPTER THREE

Discordance between mitochondrial and nuclear genetic structure in the bat, *Chaerephon pumilus* s. l. (Chiroptera: Molossidae) from South Africa

ABSTRACT

The aim of this study was to investigate the evolutionary determinants of genetic structure in the molossid bat, *C. pumilus* s.l. from South Africa based on 306 nucleotides of the mitochondrial control region and 6 microsatellite markers. We recovered strong mitochondrial genetic structure, with 90% of the molecular variance occurring among four phylogenetically-defined groups, and a high significant Fst (0.897). Mismatch distributions and Bayesian skyline analyses of mitochondrial data indicated that the sample comprised subgroups which were at demographic equilibrium over the Late Pleistocene era. Analyses based on microsatellite data contrasted strongly with the mitochondrial data, as three admixed populations were recovered. Only 3% of the nuclear variance occurred among populations, and global (Fst=0.037) and pairwise Fst values among populations were low and not significant. This is indicative of little nuclear genetic structure among the groups of *C. pumilus* s.l., which appear to comprise a single interbreeding population. We recovered significant Fis values for three of the six microsatellite markers, but not globally, indicative of some population inbreeding. Such high levels of mitochondrial genetic structure in the absence of significant nuclear structure are consistent with social isolation mechanisms such as female philopatry.

Keywords: microsatellites, mitochondrial, population structure, genetic variation

Introduction

The use of molecular genetic tools offers a powerful approach for elucidating the historical (i.e. isolation in geographic refugia, bottlenecks, range expansion and colonization events) and contemporary (i.e. gene flow, breeding structure and reproductive success) processes that might influence the genetic variation and population genetic structure of taxa (Neubaum *et al*., 2007). These tools are particularly useful in bats, which are difficult to observe due to their nocturnal nature and high mobility (Bryja *et al*., 2009). Recent work within the Order Chiroptera has revealed patterns of cryptic diversity (e.g. Kingston & Rossiter, 2004; Thabah *et al*., 2006; Racey *et al*., 2007; Ramasindrazana *et al*., 2011) as well the existence of distinct species (Li *et al*., 2006; Larsen *et al*., 2007; Baker *et al*., 2009; Goodman *et al*., 2009; Goodman *et al*., 2010) using mitochondrial DNA markers. Understanding the relative roles of historical and contemporary processes requires comparing phylogenetic patterns across a multitude of genetic characters which evolve at different rates (Karl & Avise, 1992; Palumbi & Baker, 1994; Ballard *et al*., 2002; Wiens & Penkrot, 2002). For example, Turmelle (2002) discovered two divergent mitochondrial lineages of the big brown bat (*Eptesicus fuscus*) occurring in sympatry in North America, but subsequent work using nuclear DNA demonstrated hybridization between these two lineages (Neubaum *et al*., 2007). Several other studies have also found greater population differentiation using mitochondrial markers (Wilmer *et al*. 1999; Piertney *et al*. 2000; Wirth & Bernatchez 2001).

The little free-tailed bat, *Chaerephon pumilus* (Cretzchmar 1830-31) (Chiroptera: Molossidae), has a broad distribution across sub-Saharan Africa, extending to the Arabian Peninsula and islands in the western Indian Ocean (Peterson *et al*., 1995; Bouchard, 1998; Simmons, 2005). Goodman *et al*. (2010) used mitochondrial sequence data to show that *C. p. pumilus* from Massawa formed a separate clade from other forms of this species elsewhere on the African continent, henceforth referred to as *C. pumilus* sensu lato (s.l.). Mitochondrial

DNA analyses have revealed several well supported, morphologically homogeneous, sympatric clades within *C. pumilus* s.l. in eastern South Africa and neighbouring Swaziland, separated by cytochrome *b* genetic distances of ~0.7 % (Naidoo *et al*., 2013). These are likely to reflect intraspecific genetic variability (Baker & Bradley, 2006) even though divergence values between molossid bat species (Lamb *et al*. 2008, Ratrimomanarivo *et al.* 2008, Goodman *et al*. 2010) are often lower than the range of interspecific values (3.3% - 14.7%) reported by Baker & Bradley (2006).

There are a number of potential explanations for the existence of discrete sympatric mitochondrial lineages of *C.pumilus* s.l. in South Africa. Although many bat populations show near panmixia, there is always some degree of population structure due to behavioural barriers such as those created by female philopatry (Worthington-Wilmer *et al.*, 1994; Lowe, Harris & Ashton, 2004). Worthington-Wilmer (1994) reported control region divergences of up to 6% among populations of the Australian Ghost Bat, *Macroderma gigas*, with 8% of the variance occurring among divergent lineages. This extreme structuring is proposed to be the result of long-term isolation accentuated by female philopatry. Lamb *et al.* (2012) reported similar levels of structuring of the control region in the recently described *C. atsinanana* from eastern Madagascar, with female philopatry as a potential explanation.

Alternatively, the observed mitochondrial genetic structuring in *C. pumilus* s.l. may have been the result of expansion from glacial refugia. During the glacial cycles of the late Pleistocene southern Africa experienced cooler, drier conditions that could have lead to refugial use by regional biodiversity. Glaciation events were influential in shaping patterns of genetic diversification among taxa (Matthee & Robinson 1997; Hewitt 2000; Flagstad *et al*. 2001; Muwanika *et al*. 2003; Hewitt 2004; Anthony *et al*. 2007; Brown *et al*. 2007; Voelker *et al*. 2010; Miller *et al*. 2011). Cyclical periods of glaciation would have resulted in animals retreating into refugia, from which recolonisation of partially genetically differentiated

populations may have occurred. Mitochondrial and microsatellite data have provided information on how post-glacial events have influenced current population structure in a range of species (Heckel *et al*. 2005; Howes *et al*. 2006; Jadwiszczak *et al*. 2006; Brito, 2007). Furthermore, refugia may have indirectly influenced the distribution of insect prey. This may have facilitated the maintenance and expansion of *C. pumilus* s.l. lineages in South Africa (Naidoo *et al*., 2013).

Introgression of mitochondrial DNA resulting from past hybridization events may also account for the presence of genetically diverse clades (Bachtrog *et al*., 2006); this has been documented in several bat genera, but not yet within the Molossidae (Hoffman, Owen & Baker, 2003; Berthier, Excoffier & Ruedi, 2006; Neubaum *et al*., 2007; Artyushin *et al*., 2009; Mao *et al*., 2010; Sztencel-Jablonka & Bogdanowicz, 2012).

Hypervariable markers such as nuclear microsatellites, which evolve faster than mitochondrial sequences such as control region sequences are useful in determining differences in allele frequencies within and between populations and inferring the mode and frequency of dispersal and gene flow in highly mobile mammals (Proctor *et al*., 2004). Microsatellites have been used *inter alia* to study the effects of geographic barriers (Castella *et al*., 2000; Bilgin *et al*., 2008), dispersal patterns (Castella, Ruedi & Excoffier, 2001; Kerth *et al*., 2002; Bryja *et al*., 2009) and philopatry (Weyandt *et al*., 2005; Dixon *et al*., 2011; Kerth & Van Schaik, 2012) on the population genetic structure of bats.

Our aim is to investigate population genetic structure in South African *C. pumilus* s.l. based on the maternally-inherited mitochondrial control region and biparentally-inherited microsatellite markers, with a view to identifying the evolutionary determinants of genetic structure. Interpretation of the results is complicated by the different mutation rates of mitochondrial sequences vs microsatellites, a problem in all studies based on more than one marker type. A finding of strong mitochondrial genetic structure and little nuclear structure

would be consistent with female philopatry or introgression. Significant mitochondrial and nuclear structure could be explained by incipient speciation, expansion from Pleistocene-era glacial refugia or ecological factors such as prey selection.

Materials and Methods

Collection of samples

A total of 92 *Chaerephon pumilus* s.l. individuals were genotyped. Our sample of 57 bats was supplemented with tissues from 35 individuals captured by Taylor *et al*. (2009). *C. pumilus* s.l. individuals were captured for this study using mist nets placed at their points of entry to and exit from house roofs and in their flight paths over rivers. Groups of bats sampled from a single roof space were regarded as potential colonies. The South African sampling included various localities in the Durban area (KwaZulu-Natal Province), Mpumalanga Province and neighbouring Swaziland (Appendix 1) (Fig. 1). Tissue samples were obtained using a non-lethal method described by Worthington-Wilmer & Barratt (1996), and preserved in 90% ethanol. All procedures were in accordance with the guidelines of the American Society of Mammalogists (Sikes *et al*., 2011) and approved by the Animal Ethics Committee of the University of Kwa-Zulu Natal.

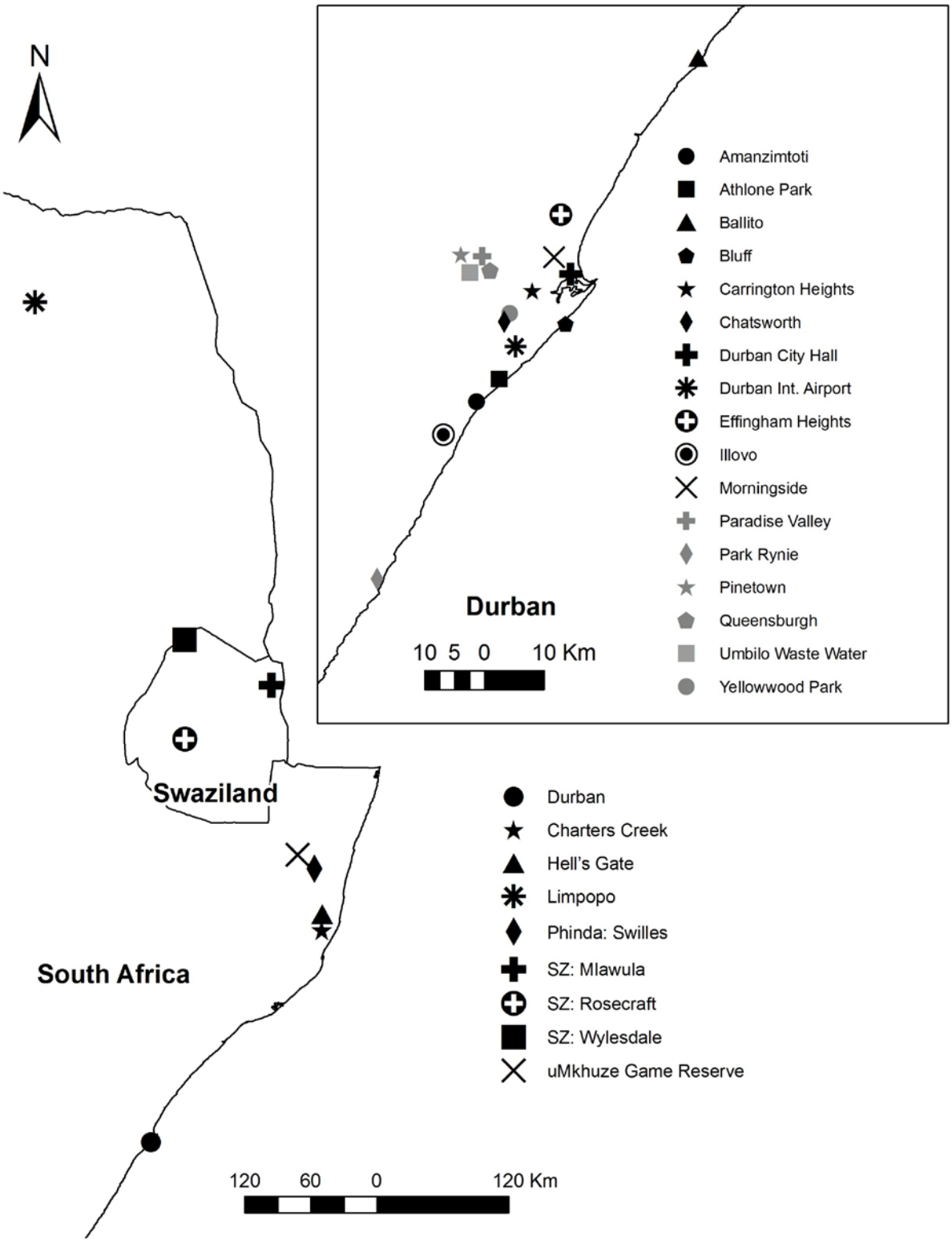

Figure 1: Map of southeastern Africa showing distribution of samples of *Chaerephon pumilus* s.l. used in this study

Isolation of DNA and amplification of the mtDNA control region

The DNeasy® Blood and Tissue Kit (QIAGEN Inc.) was used to isolate genomic DNA. Polymerase Chain Reaction (PCR) amplifications were performed in 25 µl volumes with each reaction containing: 3 ngµl^{-1}DNA, 1.0mM 10 X reaction buffer (Super-Therm), 25 mM $MgCl_2$ (Super-Therm), 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Fermentas), 5 u/µl*Taq* polymerase (Super-Therm) and 6 µM of each primer (forward and reverse) per reaction.

The mitochondrial control region was PCR-amplified as a single fragment using primers P (5'-TCCTACCATCAGCACCCAAAG C –3') and E (5'-CCTGAAGTAGGAACCAGA TG -3') (Wilkinson & Chapman, 1991; Wilkinson *et al*., 1997). The thermal cycling parameters used were: 94°C for 4 min, followed by 94°C for 1 min, 55°C for 1.30 min and 72°C for 2 min (40 cycles) and followed by 72°C for 7 min.

Amplified fragments were purified from excised gel bands using the ZymoClean Gel Extraction Kit (Zymogen Inc.) and sequenced at Inqaba Biotechnical Industries, Muckleneuk, Pretoria, South Africa. Sequences were aligned using the CLUSTAL W option (Thompson,Higgins & Gibson, 1994) of the BioEdit programme (Hall, 1999) and further adjusted by eye. Aligned sequences were cut to a common length of 306 base pairs. All new sequences were deposited in GenBank (Appendix 1).

Network analysis of control region sequences

A statistical parsimony analysis of haplotypes was carried out in TCS version 1.13 (Clement, Posada & Crandall, 2000) to determine the number of networks formed at a 95% parsimony connection. Networks were overlaid with information relating to roost membership. In order to look for patterns consistent with possible female philopatry, separate networks were created for each roost, overlaid with gender composition.

Sequence analysis of the control region

Phylogenetic analyses were carried out on haplotype data generated in DnaSP 5.10 (Librado & Rozas, 2009). The HKY sequence-evolution model (Hasegawa, Kishino& Yano, 1985) with invariant sites (HKY+I) was selected as the most appropriate model of sequence evolution under the AIC criterion in jModelTest (Posada, 2008). PAUP* 4.0 (Swofford, 2002) was used to generate maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) trees. Nodal support was estimated by bootstrap resampling analysis using 1000 pseudoreplicates. The molossid bats *Chaerephon atsinanana* (FMNH 185315) and *Mops leucostigma* (FMNH 185098) were used as outgroups.

Mismatch distributions and Bayesian skyline analysis of the control region

Mismatch distributions and Bayesian Skyline analyses were carried out for each of the mitochondrially-defined sample groups in order to search for evidence of past population expansion, possibly from Pleistocene-era glacial refugia. Mismatch distributions were plotted with DnaSP version 5.1.0 (Librado & Rozas, 2009) and compared with the expectations for populations in growth/decline or stasis. Past population dynamics of *C. pumilus* s. l. were also estimated with a Bayesian skyline plot implemented in BEAST 1.5.4 (Drummond & Rambaut, 2010). The parameter m (the number of grouped intervals) was set to five in order not to over-parameterise the model. The Markov Chain Monte Carlo (MCMC) analysis was run for 10 000 000 generations (sampled every 1000 iterations), of which the first 10% was discarded as burn-in. The substitution model used was HKY. The plot was created using TRACER 1.2.1 (Rambaut & Drummond, 2009).

Microsatellite amplification

Microsatellite analysis was carried out in order to investigate genetic structure in *C. pumilus* s.l. based on biparentally-inherited nuclear data, to compare with patterns generated using maternally-inherited mitochondrial control region data. We used 6 microsatellites originally developed for the American molossid, *Tadarida brasiliensis* (Russell *et al*., 2005) and optimised for use in *C. pumilus* s.l. (Naidoo, MacDonald & Lamb, 2013). All loci were amplified under reaction conditions specified above for the control region. The thermal cycling parameters were: 95˚C for 1 min, followed by 95˚C for 30 sec, annealing temperature for 30 sec, 72˚C for 2 min (for 39 cycles), followed by 72˚C for 10 min (optimal annealing temperature for each primer in Table 1). STRs, labelled with the dyes 5' 6-FAM and 5' HEX, were genotyped on an ABI 3500 Genetic Analyzer (Applied Biosystems) at the South African Sugar Research Institute (SASRI), Mount Edgecombe, South Africa. The presence of simple sequence repeats was confirmed by sequencing of microsatellite bands.

Table 1: Characteristics of six microsatellite loci for *Chaerephon pumilus* s.l.

Locus	Repeat motif	Genbank Accession Number	Number of Repeats	Ta (°C)	Allele size range	Primer sequences (5'-3')
Tabr A10	TAGA	KC896691	8 - 23	60	182 – 254	F:AAGTGGTTGGGCGTTGTC
	TGGA		3 - 8			R:GCGATGCACTGCCTTGAGA
Tabr A30	GA	KC896690	5 - 33	65	250 – 295	F:AGTCGCGGGTTTGATTCCAGTTA
						R:ACCCCTTCCCTTTGTTCCTTCAG
Tabr D10	GATA	KC896693	2 - 14	60	343 – 379	F:CCCCACTCATTTATCCATCCACA
						R:ATCTCGCAGCTATTGAAGTA
Tabr D15	GATA	KC896692	4 - 38	60	156 – 192	F:AGTCCTGGCTCCTATTCTCATTG
						R:CTATCCGTCTACCTGTCCGTCTAT
Tabr E9	GA	KC896694	6 - 24	60	339 – 359	F: GTTTGTCTTCCCCACTGA R: CTTAGGACAGGAGAAGTCA
Tabr H6	TAGA	KC896695	4 - 49	60	143 – 318	F:ATCTCTCCAGTCCTTACCA
						R:TTTACCCTCCACAGTCTCA

Microsatellite Analysis

Microsatellites from 74 individuals were successfully amplified and analysed according to groups defined in the control region sequence analysis. Data were scored manually using STRand v. 2.2.30 (Locke, Baacke & Toone, 2000) and checked for errors in scoring due to stuttering, large allele dropout or null alleles using Micro-checker (van Oosterhout *et al*., 2004). Data were further explored using GenAlex 6 (Peakall & Smouse, 2006). Microsatellite analyser (Dieringer & Schlotterer, 2003) and Genepop (Raymond & Rousset, 1995) were used to calculate genetic diversity, including the number of alleles (N), the mean (Na) and effective (Ne) number of alleles per population, the Shannon-information index (I), the expected (He) and observed (Ho) heterozygosity, departures from Hardy-Weinberg equilibrium (HWE) and pairwise G'st values. Allelic Richness (R), pairwise Fsts and linkage disequilibrium amongst populations were determined in F-Stat version 2.9.3 (Goudet, 1995).

Population structure was inferred using a model-based clustering method implemented in STRUCTURE v2.2.4 (Pritchard, Stephens & Donnelly, 2000). The programme determines the most likely number of genetic clusters (K) in the dataset by using a Markov Chain Monte Carlo procedure to estimate Pr(X/K), the posterior probability that the data fit the hypothesis of K clusters. The fractional membership of each individual in each cluster is calculated. As there are complexities in determining the optimal value of K, we calculated ΔK, a measure of the second order rate of change in the likelihood of K (Evanno, Regnaut & Goudet, 2005). The modal value ofΔK corresponds to the most apparent genetic subdivision present in the dataset. Burnin length was fixed to 100 000 and 1500 000 iterations were computed for each value of K (the number of populations) from1 to 5.

Indirect methods of measuring gene flow are often inappropriate because natural sets of populations may not be at equilibrium (Whitlock & McCauley, 1999). Therefore,

GENECLASS 2.0 (Piry *et al*. 2004) was used to conduct self-assignment and population simulations to test for first generation migrants. The assignment test was performed using the Bayesian method of Rannala and Mountain (1997). The detection of migrants was conducted using the L_home/L_home_max criterion with Monte Carlo resampling (Paetkau *et al*. 2004) and an alpha level of 0.01.

AMOVA

Analysis of molecular variance was carried out in Arlequin 3.11 (Excoffier, Laval & Schneider, 2005) for both mitochondrial and microsatellite datasets in order to determine levels of genetic structuring among groups identified in phylogenetic analysis of the control region dataset. Fixation indices were calculated and significance tested using a 1000 permutation approach described by Excoffier, Smouse & Quattro (1992), consisting of permuting haplotypes and loci among groups and within groups.

Results

Mitochondrial control region

The control region alignment was trimmed to a length of 306 base pairs. Of the 242 variable sites, 46 were parsimony informative and 77 were singletons. Control region sequences of 92 individuals yielded 19 haplotypes – 10 samples that did not sequence correctly were excluded from the analysis. The ingroup dataset comprised 17 haplotypes with a haplotype diversity of 0.837 and nucleotide diversity of 0.036 (Appendix 1).

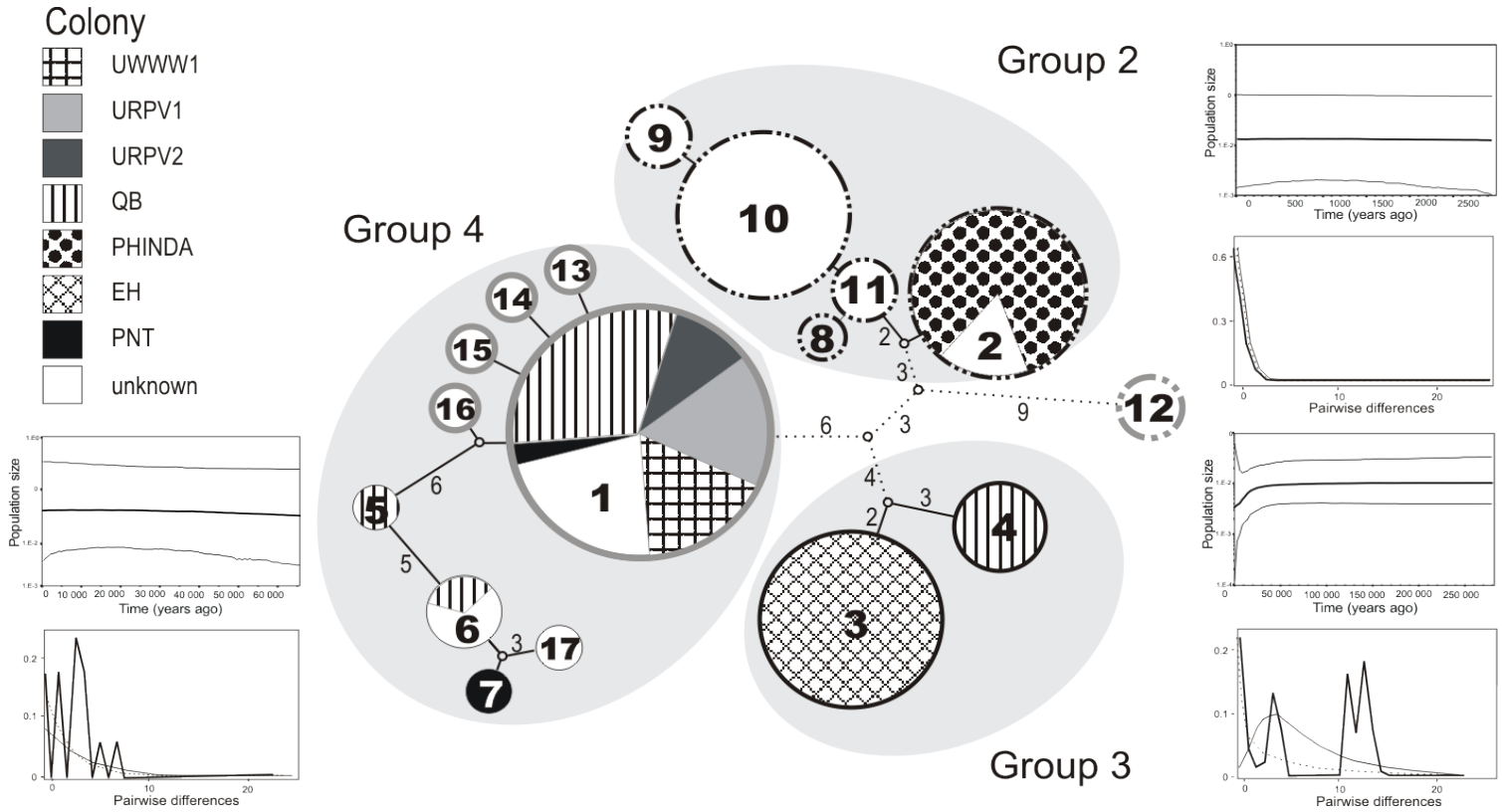

Figure 2. A statistical parsimony haplotype network based on analysis of 306 nucleotides of the mitochondrial control region of *Chaerephon pumilus* s.l. from South Africa. Mismatch distributions and Bayesian skyline plots are shown for groups 2, 3 and 4. For the Bayesian skyline plots, the central line shows the estimate of median past population size, and the outer lines bound the 95% confidence intervals. For the mismatch distributions, the dotted line indicates the observed distribution, the fine solid line indicates the expected distribution under a scenario of population expansion and the thick solid line indicates the expected distribution for a static population.

Statistical parsimony analysis of the haplotype data carried out in TCS (Clement *et al*., 2000) yielded four separate networks at the 95% connection limit (Figs 2 and 4), corresponding to Groups 1 to 4 from the phylogenetic analysis (Fig. 3). The haplotype groups are separated by between 12 and 18 mutational steps, whereas adjacent haplotypes within groups were separated by between 1 and 7 mutations.

Maximum likelihood, maximum parsimony and neighbour joining analyses of the control region dataset yielded a well-supported monophyletic *C. pumilus* s.l. clade distinct from the *Mops* outgroups (Fig. 3). The ingroup samples formed a basal trichotomy, nested within which were a number of well- to strongly-supported clades. To create grouping structures as the basis for comparison in this study, we defined four groups (Groups 1-4, Fig. 2) based on groups identified by the statistical parsimony haplotype analysis which corresponded with the separate control region clades (Fig. 3). The strongly supported clade comprising haplotypes 3 and 4 was defined as Group 3; the well-supported clade comprising haplotypes 2, 8, 9, 10 and 11 formed Group 2; haplotype 12 formed Group 1; and the remaining nine haplotypes (1, 5, 6, 7, 13, 14, 15, 16, 17) formed strongly supported Group 4.

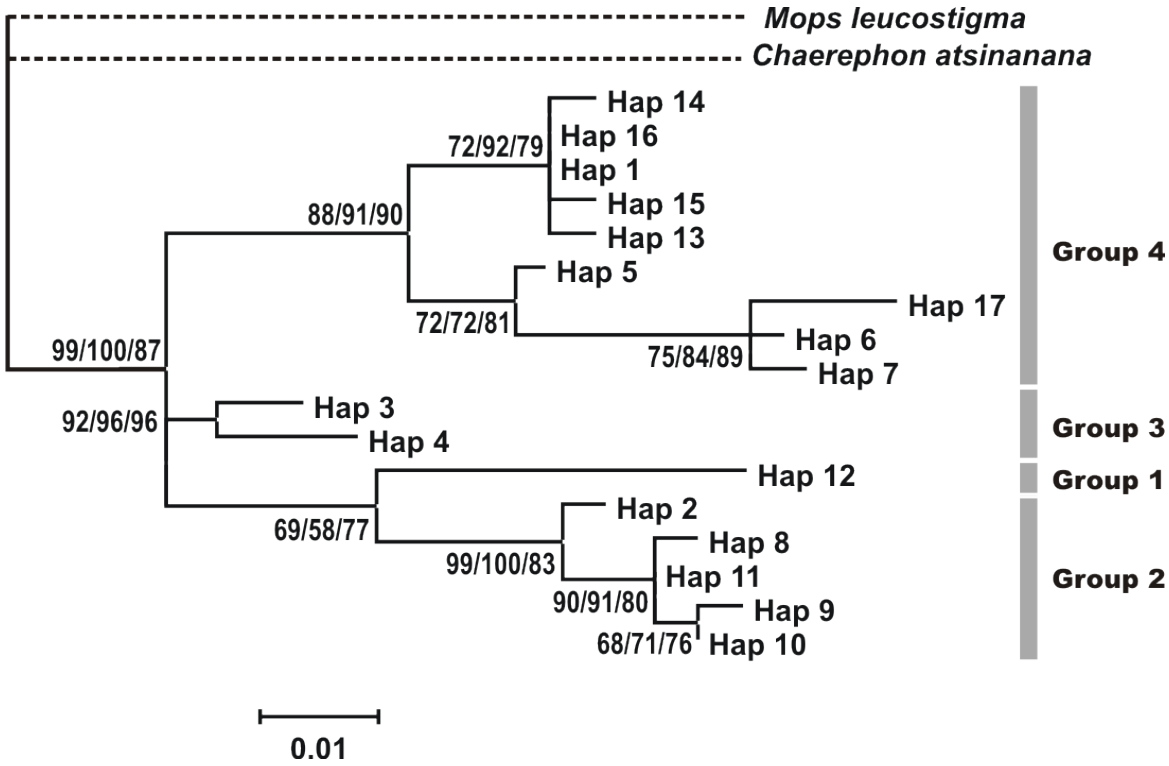

Figure 2. Maximum likelihood tree based on analysis of 306 nucleotides of the mitochondrial control region of 92 individuals of *Chaerephon pumilus* s.l. illustrating relationships between 17 haplotypes. Values at nodes represent bootstrap support for maximum likelihood, maximum parsimony and neighbour-joining respectively. Scale bar units are substitutions per site.

Mean HKY+I control region genetic distances among the four groups ranged from 5.1% (between Groups 1 and 3) to 7.8% (between Groups 2 and 4) (Table 2). The mean genetic distances between Groups 1 and 4 and Groups 2 and 3 were 6.2% and 6.4% respectively.

Table 2. Mean pairwise genetic distances using the HKY+I model among the four phylogenetically defined groups of *Chaerephon* haplotypes based on 306 bp of the control region.

Groups	**1**	**2**	**3**	**4**
1	-			
2	0.055	-		
3	0.051	0.064	-	
4	0.062	0.078	0.056	-

Information on colony membership was available only for the samples trapped specifically for this study (Appendix 1). Individual haplotypes represented between one and 29 samples. The most common haplotype (H1) was present in five colonies, whereas all other haplotypes were restricted to only one of our sampled colonies. Whereas most colonies (UWWW1, URPV1, URPV2, PH, EH) comprised only one haplotype, colonies QB and PNT comprised three and two haplotypes, respectively (Figs. 2 and 4). With the exception of one juvenile, all of the colony members trapped for this study were adults; 75% were females and 25% males. Individuals sampled from colonies UWWW1, URPV2 and PNT were all female, whereas URPV1, PH, EH were predominantly female and QB contained similar proportions of both males and females. A female-bias could be an indication of a system of female philopatry.

Mismatch distributions and Bayesian skylines were plotted for sample groups 2, 3 and 4 (Fig. 3). The mismatch distributions for groups 3 and 4 were multimodal and ragged, but not significantly so (Table 3), and conformed neither to the pattern expected for populations in growth/decline or stasis. Such patterns are indicative of ancient, stable population subdivisions (Rogers & Harpending, 1992). The corresponding Bayesian skyline plots were consistent with an essentially constant past population size for the past 250 000 (Group 3) or 60 000 (Group 4) years, and showed no evidence of past population expansion. The mismatch

distribution for Group 2 showed no evidence of raggedness, but declined steadily before reaching a value of zero, consistent with the pattern predicted for both population growth/decline and stasis. The Bayesian skyline for this group indicated a constant population size over the past 3 000 years.

Table 3. Indices of diversity, neutrality, and historical demography based on an analysis of control region sequences for four *Chaerephon pumilus* s.l. groups and the total population.

Parameter	**Group 2**	**Group 3**	**Group 4**	**Total population**
Nucleotide diversity	0.001	0.028	0.012	0.036
Haplotype diversity	0.275	0.200	0.800	0.837
Raggedness statistic	0.293	0.119	0.253	0.081*
Mismatch distribution	unimodal	multimodal	multimodal	multimodal

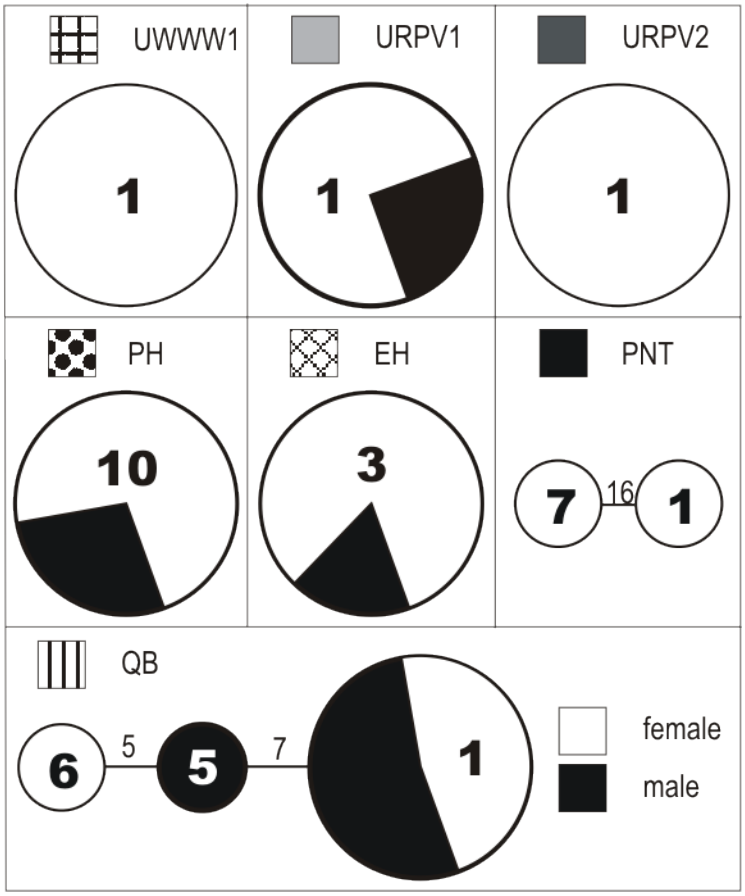

Figure 4. Haplotype networks based on 306 nucleotides of the mitochondrial control region for individual colonies of *Chaerephon pumilus* s.l. collected from different sites (as seen in Figure 1), illustrating the proportion of males and females trapped. Acronyms are defined as follows – UWWW: Umbilo waste water works, URPV: Paradise Valley, QB: Queensburgh, PH: Phinda; EH: Effingham Heights and PNT: Pinetown.

The AMOVA based on the control region dataset revealed significant genetic variation ($p < 0.05$); 90 % of the variance occurred among groups 1-4 ($p<0.05$) and 10% within groups (Table 4). The Fst value (0.897) was also high and significant ($p< 0.05$).

Table 4. Components of variance from AMOVAs based on analysis of six microsatellite loci and mitochondrial control region sequences of *Chaerephon pumilus* from Southeastern Africa. *denotes statistically significant values ($p<0.05$).

	Source of variation	d.f	Sum of squares	Variance components	Percentage variance
nuclear microsatellites	Among populations	4	18.3	0.08	3
	Within populations	143	327.7	2.29	97
	TOTAL	147	345.9	2.38	
	Fst 0.037				
mitochondrial control region	Among populations	4	411.8	6.30*	90*
	Within populations	87	62.5	0.72	10
	TOTAL	91	474.3	7.02	
	Fst 0.897				

Nuclear DNA

Genotyping of 74 individuals at six microsatellite loci identified a total of 70 alleles. No null alleles or large allele dropout were identified. All microsatellite loci were polymorphic, with the number of alleles per locus ranging from 9 to 14. There was no linkage disequilibrium between pairs of microsatellite loci after standard Bonferroni correction (Rice, 1989).

Allelic richness (R) was similar among the four mitochondrially-defined groups (mean 2.7). The Shannon Information Index ranged from 0.92 to 1.77 across the four groups (mean 1.47). The expected heterozygosity (He) ranged from 0.54 to 0.78 (mean 0.58) and the observed heterozygosity (Ho) was 0.53 to 0.66 (mean 0.58) (Table 5). Neither the overall sample nor any of the four groups showed significant deviation of Ho from values expected under HW equilibrium.

Table 5. Indices of genetic diversity based on analysis of six microsatellite loci for 74 *Chaerephon pumilus* individuals divided into four groups. N - number of samples, Na - mean number of alleles per population, Ne - expected number of alleles per population. I - Shannon-information index, He – Expected heterozygosity, Ho - observed heterozygosity and R – Allelic Richness.

GROUP	N	Na	Ne	I	Ho	He	R
1	28.0	8.83	4.51	1.65	0.53	0.73	2.61
2	2.00	2.83	2.72	0.92	0.66	0.54	2.60
3	17.0	6.33	4.12	1.53	0.56	0.73	2.70
4	27.0	8.83	5.10	1.77	0.55	0.78	2.70
Mean	18.5	6.70	4.11	1.47	0.58	0.69	2.65
SE (+/-)	2.17	0.71	0.37	0.10	0.06	0.03	0.02

Significant Fis values indicated departures from HWE at loci 1, 3 and 5 (Table 6), indicating the presence of some inbreeding. Loci 2 and 6 had a negative Fis values, indicating heterozygote excesses, although these were not significant. Values of Fit and Fst were not significant for any locus.

Table 6. Fixation indices based on six microsatellite loci calculated for four *Chaerephon pumilus* s. l. Groups from South Africa. Bold values indicate significant differences from assumptions of HWE.

Locus	Fis	Fit	Fst
1 (Tabr A10)	**0.321**	0.355	0.051
2 (Tabr D10)	**-0.011**	0.048	0.058
3 (Tabr D15)	**0.867**	0.891	0.178
4 (Tabr E9)	0.018	0.044	0.026
5 (Tabr H6)	**0.280**	0.386	0.146
6 (Tabr A30)	**-0.246**	-0.203	0.034
Mean	0.205	0.253	0.082
SE (+/-)	0.157	0.156	0.026

No significant genotypic differentiation was detected among groups; pairwise between-group Fst values ranged from 0.023 to 0.116 (mean 0.06) whereas between-group Gst values ranged from 0.003 to 0.032 (mean 0.003) respectively (Table 7). Consistent with this, AMOVAs carried out on the microsatellite dataset showed no significant genetic structure among the same groups (Fst = 0.037), with only 3% of the variance occurring among the four mitochondrially-defined groups.

Table 7. Pairwise Fst values (below diagonal) and Gst values (above diagonal) calculated for four groups of *Chaerephon pumilus* from South Africa based on six microsatellite loci.

GROUPS	**1**	**2**	**3**	**4**
1	-	0.032	0.016	0.032
2	0.116	-	0.003	0.068
3	0.023	0.094	-	0.029
4	0.029	0.067	0.031	-

Analyses in STRUCTURE (Pritchard *et al*., 2000) indicated the highest likelihood of 3 genetic clusters (Fig. 5). The structure plot indicates considerable admixture, and little unambiguous assignment of individuals to particular populations (Appendix 2). Analysis with GENECLASS (Piry *et al*., 2004) identified eleven individuals (D35, D30, D39, D26, D40, EH1, U3, D27, D10, D43, D36) as potential migrants, or of migrant ancestry ($P<0.01$). Assignment tests revealed that 58% of the individuals were residents of the sampled groups (at K=3).

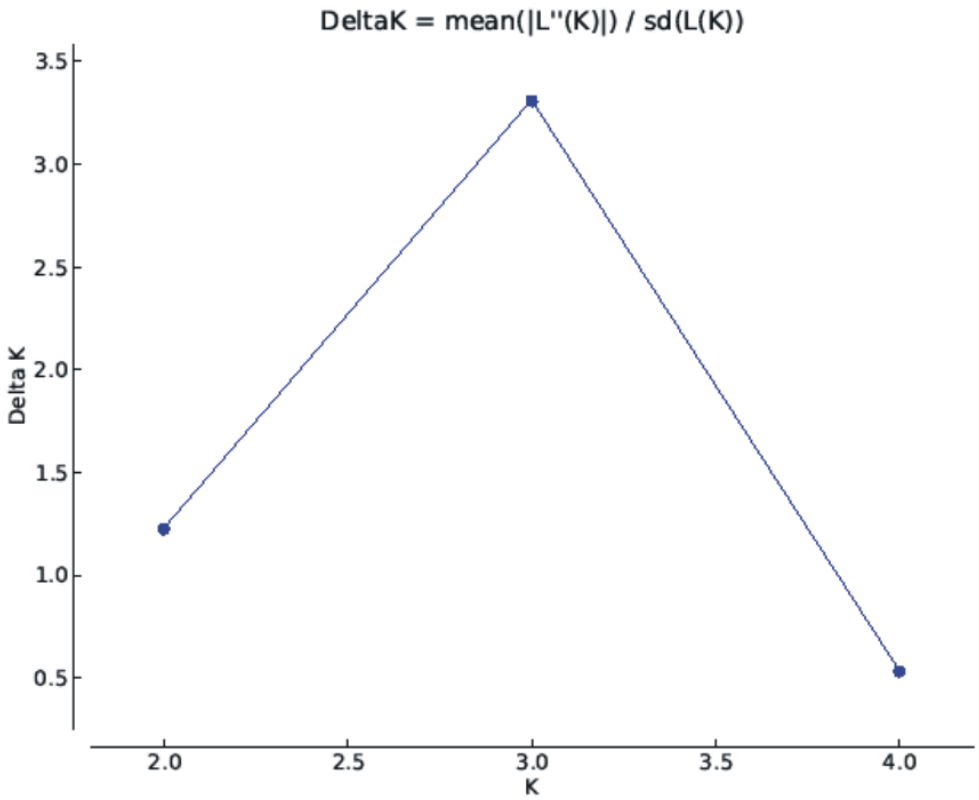

Figure 5. ΔK (a measure of the rate of change in the STRUCTURE likelihood function) values as a function of K, the number of putative populations.

Discussion

Our analyses of the genetic structure of *Chaerephon pumilus* s.l. based on nuclear microsatellite data contrast strongly with those derived from the mitochondrial control region. We found strong mitochondrial genetic structure. Phylogenetic and phenetic analyses produced congruent phylogenies containing well-supported genetically distinct clades (Fig. 3) consistent with statistical parsimony analysis, which yielded four independent haplotype networks at a 95% connection limit. Further, Fst values among groups corresponding to the four networks were significant and high (0.897) and AMOVA indicated that 90% of the variance occurred among the four groups. In contrast, analyses of nuclear microsatellite data

recovered a likely three admixed populations with low and non-significant among-population pairwise Fst values (0.023 – 0.116). Consistently, AMOVA revealed only 3% of the nuclear variance among the four mitochondrially-defined groups. This low level of nuclear genetic structure is consistent with a single interbreeding population of *C. pumilus* s.l. from South Africa and Swaziland. These data are less likely to be explained by incipient speciation, expansion from Pleistocene-era glacial refugia or ecological factors such as prey selection, unless very recent, these are likely to manifest as significant mitochondrial and nuclear genetic structure.

The haplotype network based on the mitochondrial control region formed a pattern in which generally fewer mutational steps separated haplotypes within network groups than between them, characteristic of a subdivided overall population and opposite to the star-shaped structure expected of a single expanding population (Excoffier, Foll & Petit, 2009). Consistent with this, neither the Bayesian skyline analyses nor the mismatch distribution analyses produced clear evidence of past population expansion, either of the overall population or of any of the four mitochondirally-defined subgroups. Both global (Naidoo *et al*. 2013) and subgroup-based Bayesian skyline analyses revealed a constant population size over the Late Pleistocene. Furthermore, the ragged mismatch distributions (overall and for groups 3 and 4) were characteristic of subdivided populations at demographic equilibrium (Slatkin & Hudson, 1991; Rogers & Harpending, 1992; Schneider & Excoffier, 1999). The absence of signatures of past population expansion in *C. pumilus* s.l. from South Africa contrasts with the findings of Taylor *et al*. (2009), who suggested that populations of this bat underwent cycles of contraction into and expansion from refugia, corresponding with glacial and interglacial periods in the Late Pleistocene. This difference may be due to the larger sample sizes used in this study. Our finding based on mitochondrial data of subdivided South African *C. pumilus* s.l. populations at demographic equilibrium contrasts with that of

Stoffberg, Schoeman & Matthee (2012), who found Pleistocene-era population expansions in populations of the southern African bat *Rhinolophus clivosus* s.l. Thus the observed strong genetic structuring in *C. pumilus* s.l., is unlikely to be a result of postglacial expansion, and suggests that different processes are driving the evolution of population structure in these two bat species despite their overlapping ranges.

Analyses of the nuclear dataset using STRUCTURE indicated the presence of three weak genetic clusters of *C. pumilus* s. l., whereas four strongly subdivided populations were revealed by analyses of the maternally-inherited mitochondrial dataset. Because one-half of the nuclear DNA is maternally-inherited, some congruence between mitochondrial and nuclear genetic structure is to be expected (Ramos Pereira *et al*., 2009). Assignment tests in GENECLASS assigned 58% of the individuals to the group of origin, but also identified several individuals with migrant ancestry or as potential migrants, providing direct genetic evidence of individual dispersal events between groups (Paetkau *et al*. 2004; Bergl & Vigilant 2007).

Strong mitochondrial genetic structure combined with weak or absent nuclear structure suggests female philopatry and male-biased dispersal (Greenwood, 1980), and has been found in *C. atsinanana* (Lamb *et al.,* 2012), *Nyctalus noctula* (Petit & Mayer, 1999), *Plecotus auritus* (Burland *et al.,* 2001), *Myotis myotis* (Castella*et al*., 2001), *M. bechsteinii* (Kerth, Mayer & Petit,2002) and *M. septentrionalis* (Arnold *et al*., 2007). Mean control region genetic distances between groups of *C. pumilus* s.l. ranged from 5.1% - 7.8%, similar to values reported for other bats such as Malagasy *Myotis goudoti* (mean 4.2%, maximum 9%; Weyeneth *et al*., 2011), *Macroderma gigas* (maximum 6%; Worthington-Wilmer *et al*. 1994) and *Chaerephon atsinanana* (4.95% – 8.14%; Lamb *et al*. 2012), suggested to show female philopatry. Natal female philopatry, if present in *C. pumilus* s.l., may be explained by advantages associated with social structure, including the use of familiar and successful roost

sites, and long-term associations with colony members, which may or may not be genetically related (Wilkinson, 1985; Boughman & Wilkinson, 1998; Kerth, Wagner & Konig, 2001). Further, male-biased natal dispersal may lower levels of inbreeding or competition for mates via extra-colony copulation and low male reproductive skew (Burland *et al.*, 2001). However, little is known about the roosting ecology of *C. pumilus* s l.

Five of the seven *C. pumilus* s.l. colonies that we sampled contained a single control region haplotype (Fig. 4). Under a system of female philopatry, it may be that the males are sub-adult progeny of the females, sharing their mitochondrial genomes. Bats sampled at site PNT comprised two divergent female haplotypes, which may suggest the presence of two separate colonies within this single roost. Bats sampled at site QB comprised 17 individuals exhibiting three divergent control region haplotypes, one of which was all male, one all female, and one of mixed gender. We propose that this might reflect the presence of more than one colony, and of genetically divergent males within this single roost space. In addition, we recovered significant Fis values for three of the six microsatellite markers, but not globally, which indicates a degree of inbreeding. More detailed analyses on larger complete samples of groups of bats roosting in different colonies, in combination with behavioural, echolocation and radio-tracking experiments are necessary to confirm a social structure based on female philopatry.

Low levels of nuclear genetic structure in *C. pumilus* s.l. are an indication of high levels of gene flow and panmixia over the sampled region, such as may be expected strong-flying bats capable of covering considerable distances. Generally, there is a trend showing less phylogeographic structure in more vagile bat species that fly greater distances, such as members of the Molossidae (McCracken, McCracken & Vawter, 1994; Webb & Tidemann, 1996; Russell *et al.*, 2005). Within the Molossidae, there is evidence of a positive correlation between dispersal ability (quantified as wing loading) and gene flow (Fst-Slatkin, 1987;

Slatkin & Barton, 1989) (Taylor *et al*., 2012). Because its wing loading values fall at the intermediate range, *C. pumilus* s.l. might be expected to show more genetic structure than large molossids such as *Otomops,* but less structure than the similarly sized *C. atsinanana*, whose wing loading values are smaller than those of *C. pumilus* s.l. (Taylor *et al*., 2012).

Incomplete lineage sorting and retention of ancestral polymorphisms or gene flow after secondary contact of diverged lineages are possibly better explanations than 'past hybridisation', which would have needed to occur between distinct species.

To summarize, we found significantly differentiated sympatric mitochondrial lineages, in combination with limited nuclear differentiation in *C. pumilus* s.l. in South Africa. These contrasting genetic patterns may indicate social isolation mechanisms such as female philopatry and male dispersal, or possibly introgression of mitochondrial genes due to past hybridisation events with mitochondrially-distinct taxa from outside the sampled area. Alternatively, *C. pumilus* s.l. lineages may be in the early stages of speciation, as mitochondrial lineages are usually not affected by recombination and achieve reciprocal monophyly in about one quarter the time of the average nuclear gene in diploid sexual organisms (Birky, 2013). Regardless, these results reinforce the notion that assessments of genetic structure based on a single marker should be treated with caution.

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Appendix 1. Details of individuals used in this study for mitochondrial and microsatellite analyses. The code used in the analyses are the codes assigned to each specimen (DM - Durban Natural Science Museum; CROW - Centre for Rehabilitation of Wildlife, Durban, South Africa). Haplotype number and group designations are included.

Field Number	Museum Number	Locality	Genbank Number	Latitude	Longitude	Sex	Haplotype Number	Group
***C.pumilus*s.l.**								
UWWW1CP1	-	Umbilo Waste Water	JX976432	29.846S	30.890E	F	1	4
UWWW1CP3	-	Umbilo Waste Water	JX976431	29.846S	30.890E	F	1	4
UWWW1CP4	-	Umbilo Waste Water	JX976430	29.846S	30.890E	F	1	4
UWWW1CP5	-	Umbilo Waste Water	JX976433	29.846S	30.890E	F	1	4
UWWW1CP6	-	Umbilo Waste Water	JX976434	29.846S	30.890E	F	1	4
URPV1CP1	-	Paradise Valley	JX976436	29.831S	30.892E	M	1	4
URPV1CP2	-	Paradise Valley	JX976438	29.831S	30.892E	F	1	4
URPV1CP3	-	Paradise Valley	JX976437	29.831S	30.892E	F	1	4
URPV1CP4	-	Paradise Valley	JX976439	29.831S	30.892E	F	1	4
URPV1CP5	-	Paradise Valley	JX976435	29.831S	30.892E	F	1	4
URPV2CP6	-	Paradise Valley	JX976442	29.831S	30.892E	F	1	4
URPV2CP7	-	Paradise Valley	JX976441	29.831S	30.892E	F	1	4
URPV2CP8	-	Paradise Valley	JX976440	29.831S	30.892E	F	1	4
PNT1	-	Pinetown	JX976486	29.828S	30.866E	F	1	4
PNT2	-	Pinetown	JX976487	29.828S	30.866E	F	1	4
PH1	-	Phinda: Swilles	JX976448	27.695S	32.356E	M	2	1
PH2	-	Phinda: Swilles	JX976450	27.695S	32.356E	F	2	1
PH3	-	Phinda: Swilles	JX976452	27.695S	32.356E	F	2	1
PH4	-	Phinda: Swilles	JX976443	27.695S	32.356E	F	2	1
PH5	-	Phinda: Swilles	JX976444	27.695S	32.356E	F	2	1
PH6	-	Phinda: Swilles	JX976445	27.695S	32.356E	M	2	1
PH7	-	Phinda: Swilles	JX976446	27.695S	32.356E	M	2	1

PH8	-	Phinda: Swilles	JX976447	27.695S	32.356E	F	2	1
PH9	-	Phinda: Swilles	JX976449	27.695S	32.356E	F	2	1
PH11	-	Phinda: Swilles	JX976451	27.695S	32.356E	F	2	1
EH2	-	Effingham Heights	JX976463	29.769S	31.010E	F	3	3
EH3	-	Effingham Heights	JX976465	29.769S	31.010E	M	3	3
EH4	-	Effingham Heights	JX976467	29.769S	31.010E	M	3	3
EH5	-	Effingham Heights	JX976453	29.769S	31.010E	F	3	3
EH6	-	Effingham Heights	JX976455	29.769S	31.010E	F	3	3
EH7	-	Effingham Heights	JX976457	29.769S	31.010E	F	3	3
EH8	-	Effingham Heights	JX976459	29.769S	31.010E	F	3	3
EH9	-	Effingham Heights	JX976461	29.769S	31.010E	F	3	3
EH10	-	Effingham Heights	JX976464	29.769S	31.010E	F	3	3
EH11	-	Effingham Heights	JX976466	29.769S	31.010E	F	3	3
EH12	-	Effingham Heights	JX976468	29.769S	31.010E	F	3	3
EH13	-	Effingham Heights	JX976454	29.769S	31.010E	F	3	3
EH14	-	Effingham Heights	JX976456	29.769S	31.010E	F	3	3
EH15	-	Effingham Heights	JX976458	29.769S	31.010E	F	3	3
EH16	-	Effingham Heights	JX976460	29.769S	31.010E	M	3	3
EH17	-	Effingham Heights	JX976462	29.769S	31.010E	F	3	3
QB1	-	Queensburgh	JX976469	29.857S	30.899E	F	-	-
QB2	-	Queensburgh	-	29.857S	30.899E	M	-	-
QB3	-	Queensburgh	JX976472	29.857S	30.899E	M	1	-
QB4	-	Queensburgh	JX976476	29.857S	30.899E	F	4	-

QB5	-	Queensburgh	JX976482	29.857S	30.899E	M	-	-
QB6	-	Queensburgh	JX976483	29.857S	30.899E	F	-	-
QB7	-	Queensburgh	JX976475	29.857S	30.899E	F	4	-
QB8	-	Queensburgh	JX976485	29.857S	30.899E	M	-	-
QB9	-	Queensburgh	JX976470	29.857S	30.899E	M	-	-
QB10	-	Queensburgh	JX976471	29.857S	30.899E	F	-	-
QB11	-	Queensburgh	JX976473	29.857S	30.899E	F	-	-
QB12	-	Queensburgh	JX976480	29.857S	30.899E	F	6	-
QB13	-	Queensburgh	JX976478	29.857S	30.899E	M	5	-
QB14	-	Queensburgh	JX976474	29.857S	30.899E	F	4	-
QB15	-	Queensburgh	JX976484	29.857S	30.899E	M	-	-
QB16	-	Queensburgh	JX976477	29.857S	30.899E	F	4	-
CH1	-	Chatsworth	JX976479	29.930S	30.925E	M	6	4
D1	DM 7363	Durban Int. Airport	FJ415824	29.967S	30.942E	F	8	1
D2	DM 7367	Hell’s Gate	FJ415826	28.067S	32.421E	F	9	1
D4	DM 7369	Hell’s Gate	FJ415837	28.067S	32.421E	F	10	1
D5	DM 7370	Hell’s Gate	FJ415838	28.067S	32.421E	F	10	1
D6	DM 7371	Hell’s Gate	FJ415839	28.067S	32.421E	F	10	1
D7	DM 7372	Hell’s Gate	FJ415827	28.067S	32.421E	M	9	1
D8	DM 7373	uMkhuze Game Reserve	FJ415828	27.583S	32.217E	F	2	1
D9	DM 7374	uMkhuze Game Reserve	FJ415829	27.583S	32.217E	M	2	1
D10	DM 7377	Amanzimtoti	FJ415846	30.05S	30.883E	F	13	4
D11	DM 7378	Amanzimtoti	FJ415830	30.05S	30.883E	M	10	1
D12	DM 7379	Morningside	FJ415848	29.833S	31.00E	F	14	4
D13	DM 7380	CROW	FJ415849	Unknown		F	1	4
D14	DM 7381	Hell’s Gate	FJ415841	28.067S	32.421E	F	10	1
D15	DM 7382	Hell’s Gate	FJ415831	28.067S	32.421E	F	10	1
D16	DM 7383	CROW rehab	FJ415850	Unknown		M	15	4
D17	DM 7384	Hell’s Gate	FJ415832	28.067S	32.421E	M	10	1
D18	DM 7385	Bluff	FJ415836	29.933S	31.017E	F	10	1
D19	DM 7386	Ballito	FJ415847	29.533S	31.217E	M	13	4
D20	DM 7387	Bluff	FJ415840	29.933S	31.017E	M	10	1
D22	DM 7401	Amanzimtoti	FJ415843	30.05S	30.883E	-	11	1
D23	DM 7525	Charters Creek	-	28.2S	32.417E	M	-	-
D26	DM 7851	Umbilo	FJ415844	29.833S	31.00E	-	12	2
D27	DM 7905	Athlone Park	FJ415851	30.016S	30.917E	-	1	4

D28	DM 7907	Carrington Heights	FJ415852	29.883S	30.967E	M	1	-
D29	DM 7910	Pinetown	FJ415853	29.817S	30.85E	F	1	4
D30	DM 7913	Illovo	FJ415833	30.1S	30.833E	F	10	1
D31	DM7922	SZ: Mlawula	-	26.192S	32.005E	-	-	-
D34	DM 8030	Park Rynie	FJ415854	30.317S	30.733E	M	16	4
D35	DM 8036	SZ: Mlawula	FJ415834	26.192S	32.005E	M	11	1
D36	DM 8042	SZ: Wylesdale	FJ415856	25.819S	31.292E	F	17	4
D37	DM 8348	Durban City Hall	FJ415855	29.858S	31.025E	M	1	4
D38	DM 8437	SZ: Rosecraft	-	26.632S	31.293E	-	-	-
D39	-	Durban	FJ415842	29.867S	31.00E	-	10	1
D40	-	Yellowwood Park	FJ415845	29.917S	30.933E	-	12	2
D43	-	Durban	FJ415857	Unknown		-	6	4
PT 2011-2	-	Limpopo	JX976481	23.059S	30.067E	F	7	-

Appendix 2.

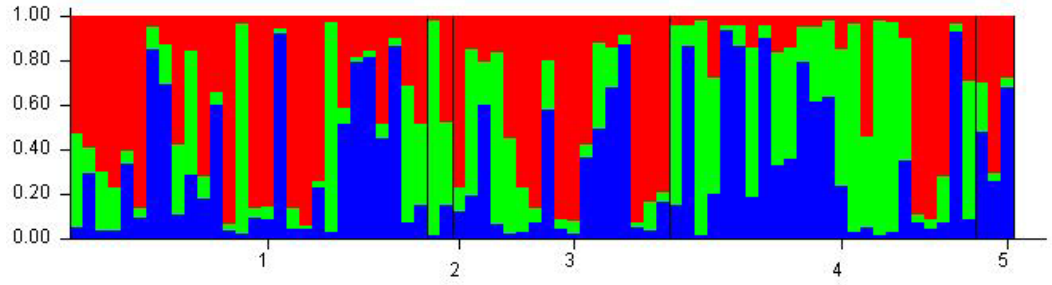

Figure 6. Structure plot v 2.2.4 (Pritchard *et al*. 2004) based on the analysis of six microsatellite loci for *Chaerephon pumilus* s.l. from South Africa which defined three metapopulations (K = 3). The y-axis illustrates the proportion of the genotype of an individual attributed to each genetic cluster.

CHAPTER FOUR

The *Chaerephon pumilus* species complex (Chiroptera: Molossidae) from south eastern Africa and the western Indian Ocean islands is not a classical ring species

ABSTRACT

We examined phylogenetic and phylogeographic relationships (cyt *b*, control region, Rag2) among members of the *Chaerephon pumilus* species complex from eastern Africa and islands in the western Indian Ocean, as well as samples from south eastern Africa and the topotype (*C. naivashae*), holotypes (*C. elphicki* and *C. langi*) and syntypes (*C. limbatus*) of taxa considered junior synonyms of *C. pumilus*. We found that *C. pumilus* from south eastern Africa is specifically distinct from the nominate *C. pumilus* sensu stricto (s.s.) from Massawa, Eritrea, and forms part of the *C. pumilus* species complex defined by Goodman *et al*. (2010). Our molecular evidence does not support that the syntype of *C. limbatus* and the holotypes of *C. elphicki* and *C. langi* and topotype of *C. naivashae* are specifically distinct from *C. pumilus* s.s.

Chaerephon pumilus s.s. (Eritrea and Yemen) diverged from the other members of the *C. pumilus* species complex about 6.24 million years ago (MYA); of these, *C. atsinanana* diverged approximately 5.01 MYA from a well supported but unresolved clade (B1) comprising subclades which appear to have arisen between 1.07 and 2.39 MYA: *C. pusillus* (Comoros and Aldabra; 1 clade); *C. leucogaster* (western Madagascar, Pemba, Zanzibar, Comoros; 2 clades); *C. pumilus* s.l. (south eastern Africa; 3 clades); and *C. pumilus* s.l. (Tanzania; 1 clade). There is evidence of introgression of both *C. pusillus* and *C. pumilus* s.l. (south eastern Africa) mitochondrial haplotypes into *C. leucogaster*. Clade B1 of the *C. pumilus* species complex has several attributes of a ring species, but appears to differ from this model in some important respects. It occurs on the African mainland and western Indian Ocean Islands, including Madagascar, ringing a potential barrier to gene flow, the Mozambique Channel. The taxa within the species complex form a ring in which the differentiated terminal forms, *C. pusillus* and *C. leucogaster*, occur in sympatry on Mayotte (Comoro Islands). Although there is evidence of isolation by distance around the ring, there is also a relatively high degree of genetic structure and limited gene flow. It appears that the

island-based component species may have differentiated in allopatry, with some gene flow by over water dispersal, whereas the African mainland species may have differentiated through isolation by distance.

Key words: *Chaerephon*, taxonomy, mitochondrial, Africa, Madagascar, Comoros

INTRODUCTION

Phylogenetic relationships among species within the Old World genus *Chaerephon* Dobson, 1874 (Family Molossidae, Suborder Vespertilioniformes) remain poorly resolved. *Chaerephon* is distributed across mainland Africa, its offshore and nearshore islands (Zanzibar, Pemba and Mozambique Island), Madagascar and surrounding western Indian Ocean islands (Anjouan, Mayotte, Mohéli, Grande Comore and Aldabra). There are currently 21 recognized species of *Chaerephon* (Simmons, 2005; Goodman and Cardiff, 2004; Goodman *et al*., 2010). Here we examine relationships among members of the *C. pumilus* Cretzschmar 1830-1831 species complex (Goodman *et al*., 2010), and include samples from south eastern Africa, as well as certain holotypes (*C. elphicki* and *C. langi*), topotype (*C. naivashae*) and a syntype (*C. limbatus*) of taxa considered junior synonyms of *C. pumilus*. Analyses are based primarily on mitochondrial markers (cytochrome *b* [*cyt* b] and control region), with some inference from a nuclear marker (Rag2). In the earlier literature this species was referred to *C. pumila*, but the correct species epithet is *pumilus* (Simmons, 2005); hence, inconsistent endings have appeared in the literature.

The systematic arrangement of *Chaerephon* is based on initial studies of cranial and dental morphology (Freeman, 1981; Legendre, 1984) and more recent molecular phylogenetic studies (Ratrimomanarivo *et al*., 2009; Goodman *et al*., 2010; Lamb *et al*., 2011; Ammerman *et al*., 2012). Six of the 21 recognized *Chaerephon* species occur in the southern African subregion - *C. pumilus*; *C. bivittatus* Heuglin 1861; *C. major* Trouessart 1897; *C. ansorgei* Thomas 1913; *C. nigeriae* Thomas 1913 and *C. chapini* Allen 1917-(Monadjem *et al*., 2010) and four in Madagascar and neighbouring western Indian Ocean islands (*C. atsinanana* Goodman *et al.* 2010; *C. leucogaster* Grandidier 1869, *C. pusillus* Miller 1902 and *C. jobimena* Goodman and Cardiff 2004).

The Malagasy form, *C. jobimena*, initially described based on morphological characteristics (Goodman and Cardiff, 2004), is not monophyletic with respect to other *Chaerephon* species based on mitochondrial and nuclear sequence data (Lamb *et al*., 2011). *Chaerephon* populations found in western Madagascar, characterised by pronounced white areas on the wing membrane, are referred to *C. leucogaster* (Ratrimomanarivo *et al*., 2009). Populations on the eastern side of Madagascar, formerly considered *C. pumilus*, were described as a new species, *C. atsinanana* (Goodman *et al*., 2010). The authors of this latter paper also transferred individuals previously considered as *C. pumilus* from the western Seychelles and Comoros Archipelago to *C. pusillus*.

Specimens referred to *C. pumilus* s.l. comprise both lighter- and dark-winged forms (Hayman and Hill, 1971; Meester and Setzer, 1971). The former were originally described as *C. p. limbata* (Koopman, 1965), while *C. p. pumilus* was used for individuals with dark wings. These two forms were considered identical in shape, size and cranial and dental features (Hayman and Hill, 1971). The lighter-winged morph was later classified as *C. limbatus* and considered a distinct species (Rosevear,

1965; Peterson *et al*., 1995), whilst other taxonomists felt that *limbatus* was conspecific with *pumilus* (Meester and Setzer, 1971; Meester *et al.*, 1986; Jacobs *et al*., 2004) and regarded *C. limbatus* as a synonym of *C. pumilus* (Koopman, 1993). Jacobs *et al*. (2004) found less than 0.9% genetic divergence in cyt *b* sequences between light-winged animals from Zambia and Tanzania and dark-winged forms from South Africa, concluding that these two forms are not distinct species. More recent work has helped to elucidate relationships between different members of the *C. pumilus* species complex.

Using mitochondrial data from a specimen of *C. p. pumilus* collected at the type locality (Massawa, Eritrea; USNM 38032), Goodman *et al*. (2010) concluded that this sequence, as well as specimens obtained in the neighbouring Yemen, represented the nominate form, referred to in this report as *C. pumilus* s.s. Naidoo *et al*. (2013) reported five mitochondrial clades *C. pumilus* s.l. from south eastern Africa, separated by a low mean inter-clade genetic distance (0.7%, cyt *b*). Several authors (Taylor, 1999; Aspetsberger *et al*., 2003; Fenton *et al*., 2004; Schoeman and Jacobs, 2008; Schoeman and Waddington, 2011) have reported variation in echolocation calls of *C. pumilus* s.l. from mainland Africa. A subsequent study addressed the question of correlations between genetic lineages and acoustic sonotypes from south eastern Africa and found no support for this hypothesis (Naidoo *et al*., 2013).

Simmons (2005) listed nine synonyms of *C. pumilus* - *dubius* Peters 1852; *limbatus* Peters 1852; *gambianus* de Winton 1901; *C. pusillus*; *hindei* Thomas 1904; *naivashae* Hollister 1916; *elphicki* Roberts 1926; *langi* Roberts 1932; and *faini* Hayman 1951. Peterson *et al*. (1995) had previously proposed based on skull morphology, that three of these (*hindei*, *limbata* and *naivashae*) should be elevated to full species rank.

Phylogenetic inference based on mitochondrial DNA has been commonly used in the taxonomic evaluation of mammal species, including bats (Baker and Bradley, 2006; Goodman *et al*., 2010). Mitochondrial genes are useful in detecting early stages of speciation, because they are usually not affected by recombination and achieve reciprocal monophyly in about one quarter the time of the average nuclear gene in diploid sexual organisms (Birky, 2013). However, as hybridization of bats is likely to result in the introgression of mitochondrial DNA between taxa (Berthier *et al*., 2006, Mayer and von Helversen, 2001; Artyushin *et al*., 2009; Vallo *et al*., 2012), results obtained from mitochondrial data alone should be viewed with caution.

The *C. pumilus* species complex was defined by Goodman *et al*. (2010) to include *C. pumilus* s.s. (Eritrea and Yemen), *C. atsinanana* (eastern Madagascar), *C. pusillus* (Comoros and Aldabra), *C. leucogaster* (western Madagascar, Pemba, Zanzibar, Comoros) and *C. pumilus* s.l. from eastern Africa. These species separated by mean genetic distances of 1.31% - 2.98%, occupy parts of continental Africa and islands separated by a potential barrier to gene flow, the Mozambique Channel. This geographical configuration leads to the hypothesis that they may represent a ring species (Irwin *et al*., 2001).

Ring species comprise two reproductively isolated populations connected by a chain or circle of intergrading populations (Mayr, 1942). These complexes are proposed to have arisen by expansion of a single species in two directions around a geographical barrier, accompanied by gradual divergence into two forms, which, when they eventually meet, behave as separate species (Stegnejer in Jordan (1905) in Irwin *et al*., 2001). Debate over the nature of variation around the ring has led to discussion as to whether the relatively small number (± 23) of proposed cases qualify as ring species (Irwin *et al*., 2001). Various forms of the large tree finch (*Camarhynchus psittacula*), distributed on several Galapagos Islands (a situation similar to the distribution of the *C. pumilus* species complex discussed herein) have been proposed as a ring species (Lack, 1947).

The aim of this study was to further resolve the evolutionary history and taxonomy of the *C. pumilus* species complex by extending the mitochondrial DNA study of Goodman *et al.* (2010), using broader geographical and taxonomic sampling, including new material from previously unrepresented areas, namely Kenya, Botswana, Mozambique, South Africa, Swaziland, Zanzibar and additional samples from the Comoros Archipelago. We include sequences of the syntype of *C. limbatus*, holotypes of *C. elphicki* and *C. langi*, and topotype of *C. naivashae*, all of which are considered junior synonyms of *C. pumilus* (Simmons, 2005). We address the following hypotheses: (1) The junior synonyms of *C. pumilus*, namely *C. limbatus*, *C. naivashae*, *C. elphicki* and *C. langi*, are not genetically distinct at the species level from the nominate form, *C. pumilus* s.s. from Massawa, Eritrea; (2) *C. pumilus* from south eastern Africa is specifically distinct from the nominate form, *C. pumilus* s.s. from Massawa, Eritrea; (3) The *C. pumilus* species complex, which is distributed across a sphere of landmasses surrounding the Mozambique Channel, represents an example of a ring species.

MATERIALS AND METHODS

Specimens Used in this Study

Tissue samples of *Chaerephon* used in this study were obtained from various regions on Madagascar, neighbouring western Indian Ocean islands and the African mainland. These specimens are housed in Field Museum of Natural History (FMNH) – Chicago; Durban Natural Science Museum (DM) – Durban; l'Université d'Antananarivo, Département de Biologie Animale (UADBA) – Antananarivo; The National Museum of Natural History (formerly United States National Museum – USNM) – Washington, D.C; Museum für Naturkunde - Humboldt Universitat zu Berlin (formerly Zoologisches Museum Berlin – ZMB) – Berlin; and The Ditsong National Museum of Natural History (formerly Transvaal Museum – TM) – Pretoria. Genetic analyses included 138 individuals utilized by Goodman *et al*. (2010) and an additional 316 individuals for the present study (Appendix 1).

Tissue samples were obtained from wing punches and liver, heart, kidney or muscle tissue stored in 80% ethanol or lysis buffer. Genetic diversity in *Chaerephon* species was examined using

two mitochondrial DNA regions: cyt *b* (n = 230) and control region (n = 416) (Appendix 1). Outgroup taxa used in the analyses were as follows: *Mops condylurus* (A. Smith, 1833), *M. leucostigma* (G.M. Allen, 1918), *M. midas* (Sundevall, 1843), *Otomops martiensseni* (Matschie, 1897) and *O. madagascariensis* (Dorst, 1953).

DNA Extraction, Amplification and Sequencing

DNA extraction, PCR amplification and sequencing were carried out according to previously-published procedures: mitochondrial cytochrome *b* and control regions after Lamb *et al.* (2012) and nuclear Rag2 region after Lamb *et al*. (2011).

DNA extraction from syntypes, topotype and holotypes

Skull scrapings obtained for each holotype specimen of *C. elphicki* and *C. langi*, topotype of *C. naivashae* and syntype of *C. limbatus* were sent to a Paleo-DNA Laboratory Genetic Testing Service at Lakehead University (Thunder Bay, Ontario, Canada) for cyt *b* sequencing. This was carried out according to the protocol used for the 120-year-old syntype of *C. p. pumilus* from Massawa, Eritrea (Goodman *et al*., 2010).

Sequence Analysis

All sequences were assembled and edited manually and aligned using the Clustal W option (Thompson *et al*., 1997) of the BioEdit program (Hall, 1999). Aligned sequences were trimmed to a common length of 296 nucleotides for the control region. Two cyt *b* alignments were created: the shorter alignment was trimmed to 206 nucleotides in order to include the syntype material of *C. limbatus*, holotype material of *C. elphicki* and *C. langi* and topotype material of *C. naivashae*, and a longer alignment (830 nucleotides) was created to study in more detail the relationships among study samples.

jModeltest (Posada, 2008) was used to select the most appropriate model of nucleotide substitution under the AIC information criterion. The model selected for all datasets was the HKY+I sequence-evolution model (Hasegawa *et al*., 1985). Haplotypes for each alignment were inferred with DnaSP 5.10 (Librado and Rozas, 2009). The phylogenetic trees presented here are based on haplotypes.

Maximum parsimony (MP) and neighbour-joining (NJ) trees were generated using PAUP* 4.0 (Swofford, 2002). Nodal support was estimated using 1000 bootstrap pseudoreplicates. Bayesian analysis was implemented in MRBAYES version 3.1.2 (Ronquist and Huelsenbeck, 2001). Four Markov chains (three hot and one cold) were run for 50 million generations, until the standard deviation of the split frequencies was less than 0.01. The first 5000 000 trees were discarded as

burnin, after an initial run to check that this was sufficient to achieve stationarity. The phylograms were 50% majority-rule consensus trees with nodal support indicated as posterior probabilities.

A statistical parsimony haplotype network was constructed for the control region dataset in TCS (Clement *et al*., 2000). SplitsTree version 4.8 (Huson, 1998; Huson and Bryant, 2006) was used to generate a Neighbour-net network (Bryant and Moulton, 2004) using programme defaults.

Indices of diversity and neutrality (nucleotide diversity, haplotype diversity, mismatch distributions and the raggedness statistic) were estimated in DnaSP 5.10 (Librado and Rozas, 2009) based on the control region. For these analyses, samples were divided into the following groups: *C. atsinanana* -- eastern Madagascar, *C. leucogaster* -- western Madagascar, *C. pumilus* s.l. -- south eastern Africa and Mozambique Island, *C. pusillus* -- Comoros and *C. pumilus* s.s. -- Yemen. There were too few samples from Aldabra, Pemba, Zanzibar and Tanzania to include genetic data from these localities in these analyses.

An analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) implemented in Arlequin v.3.11. (Excoffier *et al.*, 2005) was used to analyse the apportionment of genetic variance within and among populations from the above regions. Pairwise Fst's among populations from these regions were also computed in Arlequin v.3.11 (Excoffier *et al*., 2005). Mantel tests were used to test for a significant correlation between geographic and genetic distances among populations from the sample regions using GenAlex 6 (Peakall and Smouse, 2006).

Molecular Dating Analysis

A time-scale for the divergence of *Chaerephon* species was calculated using a Bayesian inference as implemented in BEAST version 1.5.4 (Drummond and Rambaut, 2007). This was based on the shorter 206 nucleotide cyt *b* alignment, as it included more taxa. The HKY substitution model was used in an analysis that was run for 50 million generations with a 10% burnin. A relaxed molecular clock (uncorrelated lognormal) was employed in conjunction with a Yule species prior. The analysis was calibrated with fossil data for the first known occurrence of *Mops* (11.2-16.4 MYA: Bohme, 2003) – a single calibration point due to unavailability of fossil data. The dates are vague under these circumstances. Time estimates were calculated based on an XML file from BEAST in TreeAnnotator version 1.5.4 (Drummond and Rambaut, 2007). The dated tree viewed and edited in FigTree v.1.3.1.

RESULTS

Phylogenetic Analysis

Analysis based on the 1262 nucleotide Rag2 alignment

An alignment of the Rag2 gene region (1262 nucleotides) yielded 38 variable sites of which 33 were parsimony informative. *Chaerephon* species from southern Africa (*C. pumilus*), Madagascar (*C. leucogaster* and *C. atsinanana*), Pemba, Aldabra and the Comoros (*C. pusillus* and *C. leucogaster*) formed a single haplotype with the exception of *C. jobimena*.

Analysis based on the 206 nucleotide cyt *b* alignment

A 206 nucleotide cyt *b* alignment was created in order to allow comparisons with shorter sequences of *C. pumilus* s.s. from Massawa (Eritrea), the syntype of *C. limbatus*, topotype of *C. naivashae* and holotypes of *C. elphicki* and *C. langi*. Of the 206 nucleotides, 57 sites were variable and 35 were parsimony informative. The 248 samples yielded 22 haplotypes for the ingroup data (Appendix II).

Bayesian inference, maximum parsimony and neighbour joining analyses yielded congruent trees (Fig. 1). Our *Chaerephon* study samples comprised a moderately supported Clade D and within this, the moderately supported Clade D1 composed of *C. pumilus* s.s. from the type locality, Eritrea, and the nearby Yemen, were separated by a genetic distance of 1.4% (Table 1). Sister to D1 was an unsupported clade (D2) comprising haplotypes of *C. atsinanana*, *C. leucogaster*, *C. pusillus*, *C. naivashae* (topotype), *C. langi* (holotype), *C. elphicki* (holotype), *C. limbatus* (syntype) and *C. pumilus* s.l. from Tanzania, Mozambique Island and south eastern Africa. While subclade D3 (*C. atsinanana*) was moderately- to well-supported, there were no other well-supported groupings among haplotypes within D2.

Chaerephon pumilus s.s. was separated from *C. pumilus* s.l. (south eastern Africa) by a mean genetic distance of 3.7% (2.9% - 4.5%) and from *C. pumilus* s.l. (Tanzania) by 2.9% (2.9% - 4.5%). *Chaerephon pumilus* s.l. (south eastern Africa) was separated from the syntype of *C. limbatus* by a mean genetic distance of 2.0% (0.9% -2.9%) (Table 1).

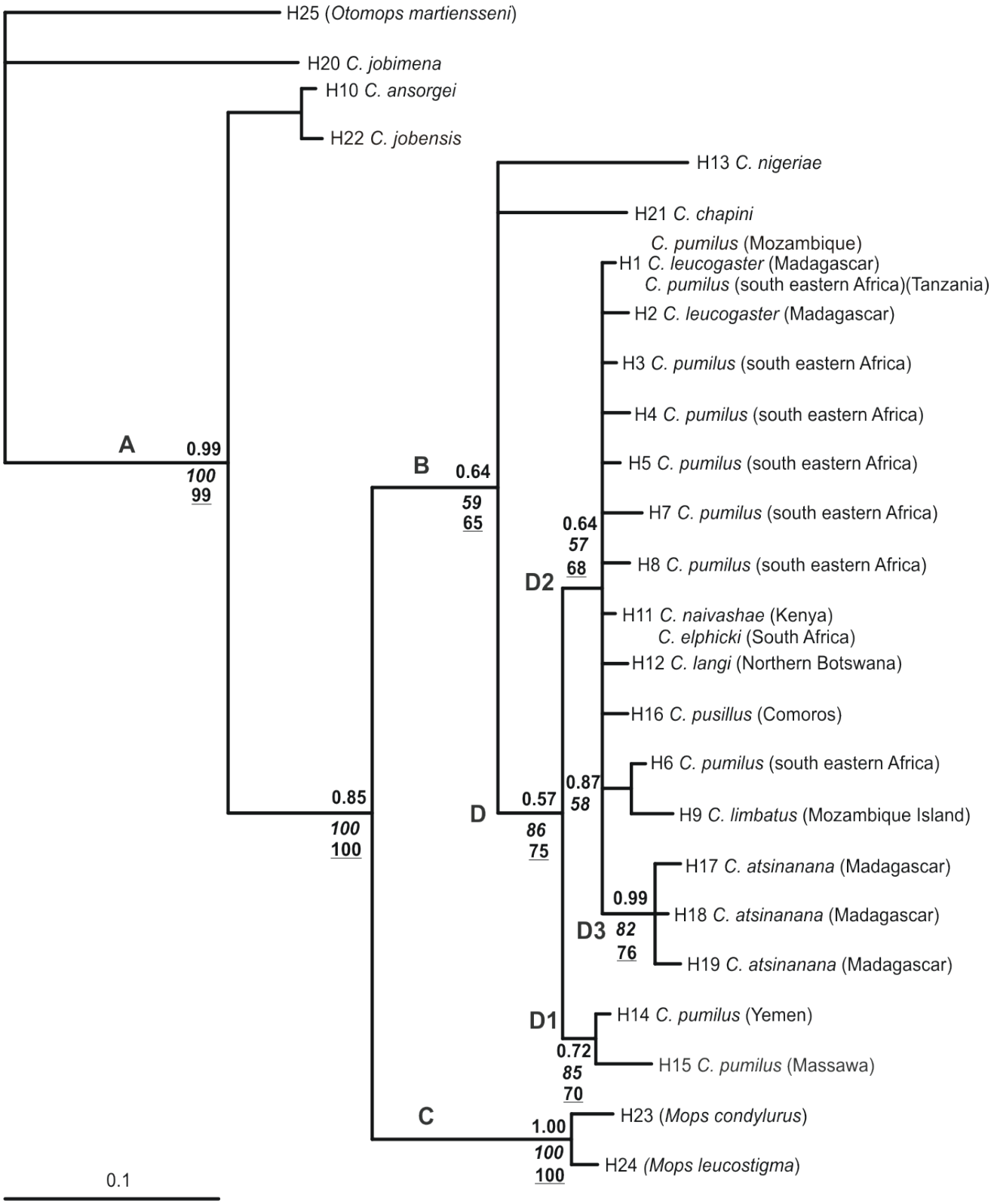

FIG. 1. Phylogenetic tree of haplotypes based on 206 nucleotides of the mitochondrial cyt *b* gene. Nodal support is indicated as follows: Bayesian posterior probabilities (top), bootstrap value for neighbour-joining analysis (middle – italicized) and bootstrap value for maximum parsimony (bottom – underlined).

Table 1: HKY+I genetic distances (%) between haplotypes of the *Chaerephon* ingroup taxa based on 206 nucleotides of the cyt *b* region.

Species	Hap	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
***C. leucogaster/ C. pumilus* s.l. (SEA)**	**1**	-																				
C. leucogaster	**2**	0.4	-																			
***C. pumilus* s.l. (SEA)**	**3**	0.4	0.9	-																		
***C. pumilus* s.l. (SEA)**	**4**	0.9	1.4	0.4	-																	
***C. pumilus* s.l. (SEA)**	**5**	0.4	0.9	0.9	1.4	-																
***C. pumilus* s.l. (SEA)**	**6**	0.9	1.4	1.4	1.9	0.4	-															
***C. pumilus* s.l. (SEA)**	**7**	1.4	1.9	0.9	1.4	0.9	1.4	-														
***C. pumilus* s.l. (SEA)**	**8**	0.9	1.4	0.4	0.9	1.4	0.9	1.4	-													
***C. limbatus* (syntype)**	**9**	1.9	2.4	2.4	2.9	1.4	0.9	2.4	1.9	-												
C. ansorgei	**10**	10.7	11.3	11.3	11.9	11.3	11.2	12.5	11.2	12.4	-											
***C. elphicki/C. naivashae* (holotype/topotype)**	**11**	0.0	0.4	0.4	0.9	0.4	0.9	1.4	0.9	1.9	10.7	-										
***C. langi* (holotype)**	**12**	0.4	0.9	0.9	1.4	0.9	1.4	1.9	1.4	2.4	11.3	0.4	-									
C. nigeriae	**13**	8.4	9.0	7.8	8.4	9.0	9.5	8.9	8.3	10.6	10.0	8.5	9.0	-								
***C. pumilus* (Yemen)**	**14**	1.4	1.9	1.9	2.4	1.9	2.4	2.9	2.4	3.4	10.1	1.4	1.9	9.0	-							
***C. pumilus* (Massawa)**	**15**	2.9	3.4	3.4	4.0	3.4	4.0	4.5	4.0	4.0	10.6	3.0	3.5	10.6	1.4	-						
C. pusillus	**16**	0.4	0.9	0.9	1.4	0.9	1.4	1.9	1.4	2.4	11.3	0.4	0.9	9.0	1.9	3.4	-					
C. atsinanana	**17**	1.9	2.4	2.4	2.9	2.4	2.4	3.4	2.4	3.4	10.6	1.9	2.4	9.5	3.5	5.0	2.4	-				
C. atsinanana	**18**	1.4	1.9	1.9	2.4	1.9	1.9	2.9	1.9	2.9	10.0	1.4	1.9	8.9	2.9	4.5	1.9	0.4	-			
C. atsinanana	**19**	1.9	2.4	2.4	2.9	2.4	2.4	3.4	2.4	3.4	9.5	1.9	2.4	8.3	2.4	4.0	2.4	0.9	0.4	-		
C. jobimena	**20**	16.4	16.4	16.4	17.2	17.2	16.5	17.8	15.8	17.9	12.8	16.5	17.2	15.1	17.2	17.7	16.4	16.3	16.4	17.1	-	
C. chapini	**21**	12.0	12.6	11.4	12.0	11.4	11.3	11.3	11.3	12.5	8.4	11.4	12.0	9.5	11.4	13.1	12.6	11.9	11.3	10.7	16.5	-
C. jobensis	**22**	6.7	7.3	7.3	7.9	6.2	6.7	7.3	7.8	7.2	12.5	6.8	7.3	12.6	6.7	7.8	7.3	9.0	8.4	7.8	20.0	9.5

Analysis based on 830 nucleotide cyt *b* alignment

Analyses based on 830 nucleotides of cyt *b* (Fig. 2) comprised 44 parsimony informative sites and 33 haplotypes (Appendix III). Results were congruent with those based on 206 nucleotides, with better support for some groups. *Chaerephon* is monophyletic and forms a strongly supported clade (A) (1.00 BI, 100% MP, 100% NJ) with respect to the *Mops* outgroup. Within A, strongly supported clade B2 (*C. atsinanana*) is sister to a strongly supported clade (B1) comprising *C. leucogaster*, *C. pusillus*, *C. pumilus* s.l. (south eastern Africa) and *C. pumilus* s.l. (Tanzania).

Clade B1 comprises five subclades (B1a, B1b, B1c, B1d, B1e) separated by mean genetic distances between 0.66% and 2.25% (Table 2). Moderately supported Clade B1a further comprises three subclades, B1a.1 (*C. leucogaster* –Madagascar/Pemba; moderate to weak support), B1a.2 (*C. pumilus* s. l. – south eastern Africa; moderate support) and B1a.3 (*C. leucogaster* – Zanzibar; good support). In addition to Clade B1a.2, *C. pumilus* s.l. from south eastern Africa comprises two further clades, B1b (good support) and B1c (strong support).

Chaerephon pusillus from Aldabra and the Comoros Archipelago comprises one strongly-supported Clade, B1d. The mean genetic distances separating clade B1d from the other clades range from 0.97% (B1a.3) to 2.97% (B2). Clade B1e from Bukoba, Tanzania is well-supported and separated from the other members of the *C. pumilus* species complex by genetic distances of 2.34 – 2.97%.

TABLE 2: Mean HKY+I genetic distances (%) between and within clades based on 830 nucleotides of the cyt *b* gene. Cladal assignments are based on Figure 2: Clade B1a.1 (*C. leucogaster*: western Madagascar/Pemba), Clade B1a.2 (*C. pumilus*: south eastern Africa), Clade B1a.3 (*C. leucogaster*: Zanzibar), Clade B1b (*C. pumilus*: south eastern Africa), Clade B1c (*C. pumilus*: south eastern Africa), Clade B1d (*C. pusillus*: Aldabra/Comoros), Clade B1e (*C. pumilus*: Tanzania) and Clade B2 (*C. atsinanana*: eastern Madagascar).

	Mean genetic distances between clades (%)								**Mean within clade genetic distance (%)**
Clades	**B1a.1**	**B1a.2**	**B1a.3**	**B1b**	**B1c**	**B1d**	**B1e**	**B2**	
B1a.1	-								0.18
B1a.2	0.66	-							0.35
B1a.3	0.46	0.80	-						0.08
B1b	0.77	0.96	0.79	-					0.12
B1c	1.11	1.24	1.13	1.03	-				0.30
B1d	1.30	1.54	0.94	1.13	1.73	-			0.24
B1e	1.81	2.01	1.12	1.65	2.25	2.06	-		0.72
B2	2.34	2.54	2.36	2.43	2.53	2.97	2.55	-	0.55

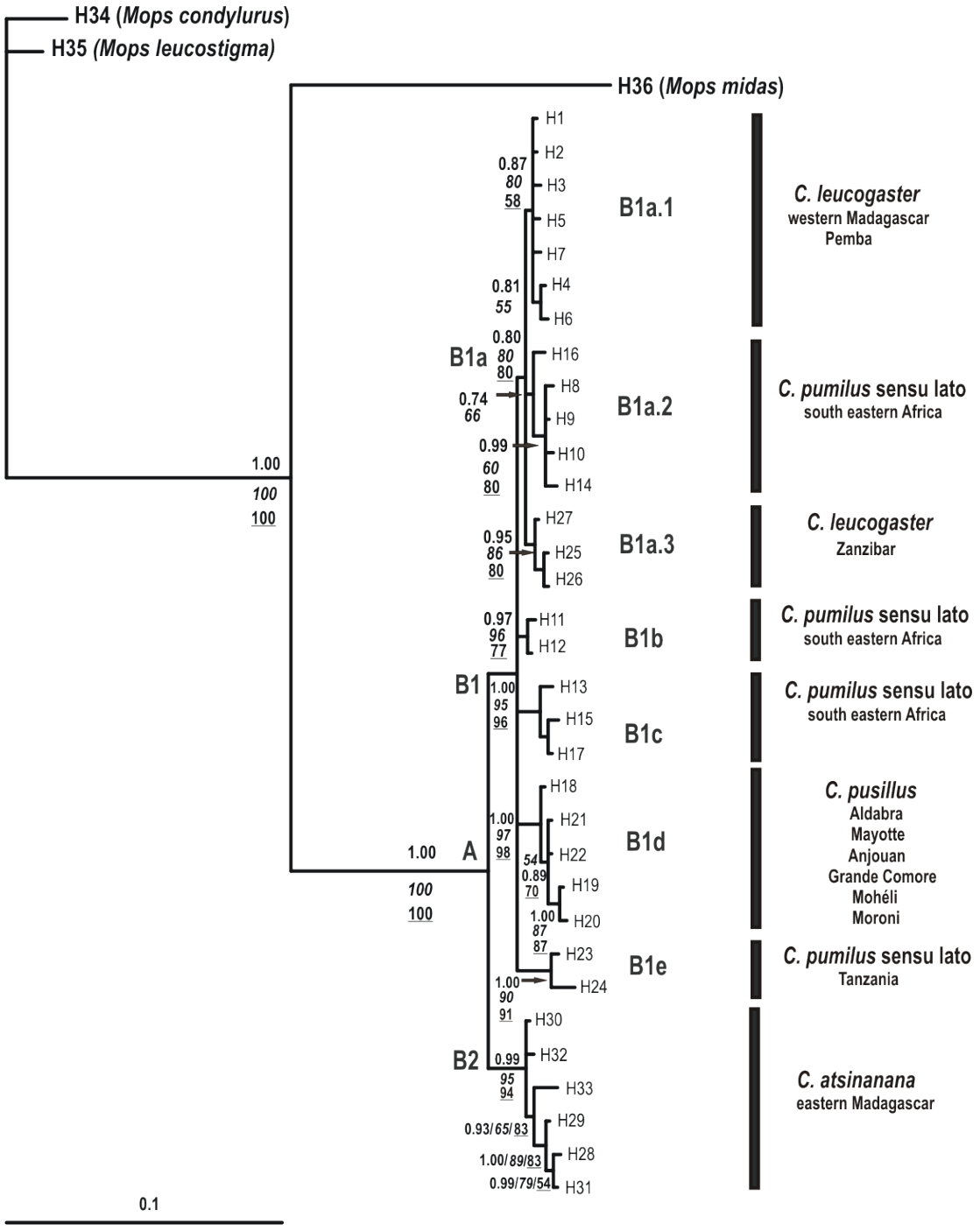

FIG. 2. Phylogenetic tree of haplotypes based on 830 nucleotides of the mitochondrial cyt *b* gene. Nodal support is indicated as follows: Bayesian posterior probabilities (normal font), bootstrap value for neighbour-joining analysis (italicized) and bootstrap value for maximum parsimony (underlined). Posterior probabilities < 0.5 and bootstrap values <50% are not indicated.

Dating Analysis

Chaerephon pumilus s.s. (Massawa and Yemen) appears to have diverged from other forms of *C. pumilus* s.l. 6.24 million years ago (MYA) (node A: 95% HPD: 0.32 – 11.99 MYA) (Fig. 3). The split between individuals of *C. pumilus* from Massawa and Yemen is dated at 2.07 MYA (node B: 95% HPD: 0.26 – 8.26 MYA). *Chaerephon atsinanana* last shared a common ancestor with members of the *C. pumilus* species complex 5.01 MYA (node C: 95% HPD: 0.01 – 5.15 MYA). The split between H9 (syntype of *C. limbatus*) and H6 (*C. pumilus*- south eastern Africa) is at 1.07 MYA (node D: 95% HPD: 0.0 - 3.92 MYA).

Analysis based on 296 nucleotides of the control region

Analysis of 296 nucleotides of the control region dataset yielded 115 variable characters of which 100 were parsimony-informative. The 405 sequences yielded 105 haplotypes with a haplotype diversity of 0.97 (Appendix IV).

Bayesian inference, maximum parsimony and neighbour-joining analyses yielded results which were congruent with each other, and with the results of the cyt *b* analysis. We present these results as a series of statistical parsimony networks (Fig. 4).

Network Analysis

Statistical parsimony analysis carried out at a 95% connection limit produced eight networks, and at a 90% connection limit produced seven networks, as two *C. leucogaster* networks were joined (Fig. 4). *Chaerephon pumilus* s.s. from Yemen formed a distinct network of five haplotypes, of which H55 was separated from H104 (*C. pusillus*, Comoros) by 19 mutations (Network 1, Fig. 4).

Chaerephon atsinanana, endemic to eastern Madagascar, formed three networks comprising 15, seven and one haplotypes respectively (networks 2, 3, 4, Fig. 4). Haplotype 62 (network 1) was separated from H5 (*C. pusillus*, Comoros) by 23 mutations. Networks 2 and 3 were separated by 35 mutational steps (H19 – H54), whereas network 4 (H18) was separated from each of the other two *C. atsinanana* networks by 31 and 6 mutations.

Samples morphologically identified as *C. pusillus* formed networks 5 and 6 comprising four and three haplotypes, respectively, separated by 13 mutational steps (H48 – H104). H104 was also separated from H55 (*C. pumilus* s. s. from Yemen) by 19 mutations. Haplotypes of *C. pusillus* also formed part of a third ring-shaped network (network 7). Nested within the *C. pusillus* section of network 7 are two samples (haplotypes 4 and 5), which were morphologically identified as *C. leucogaster*.

Overall, ring-shaped network 7 contained haplotypes of *C. pumilus* s. l. (south eastern Africa), *C. pumilus* s. l. (Tanzania), *C. pusillus* and *C. leucogaster*. *Chaerephon pumilus* s.l. haplotypes from south eastern Africa are central within this network. On one side they connect

through unsampled haplotypes ancestral to H100 (Comoros) and H2 (Mozambique Island) via H8 (*C. pumilus* s.l., Tanzania) to a terminal cluster of *C. leucogaster* haplotypes. The *C. leucogaster* haplotypes are primarily from western Madagascar, but H6, H28, H38, H90 and H101 are represented on the Comoros, and H46 is present in western Madagascar and on Pemba Island. To the other side, the central *C. pumilus* s.l. haplotypes connect to a terminal group of 18 haplotypes from the Comoros Archipelago. The most common haplotype in this grouping (H84) is represented in samples from Grand Comore, Mohéli, Anjouan or Mayotte, whereas other haplotypes are from fewer islands, or have been found on only one of these four islands. The two terminal ends of network 7 connect to form a circle via H87 and H26, and are separated by 26 mutational steps.

A neighbour-net distance network of the control region dataset (Fig. 5) showed 287 splits and diversification into eight major groups: *C. atsinanana* – eastern Madagascar; *C. pumilus* s.l. – south eastern Africa; *C. pusillus* – Comoros and Aldabra; *C. pumilus* s.l. – Mozambique Island; *C. leucogaster* – western Madagascar; *C. leucogaster* – Mayotte and Pemba; *C. pumilus* s.l. – Tanzania; and *C. pumilus* s.l. - Yemen.

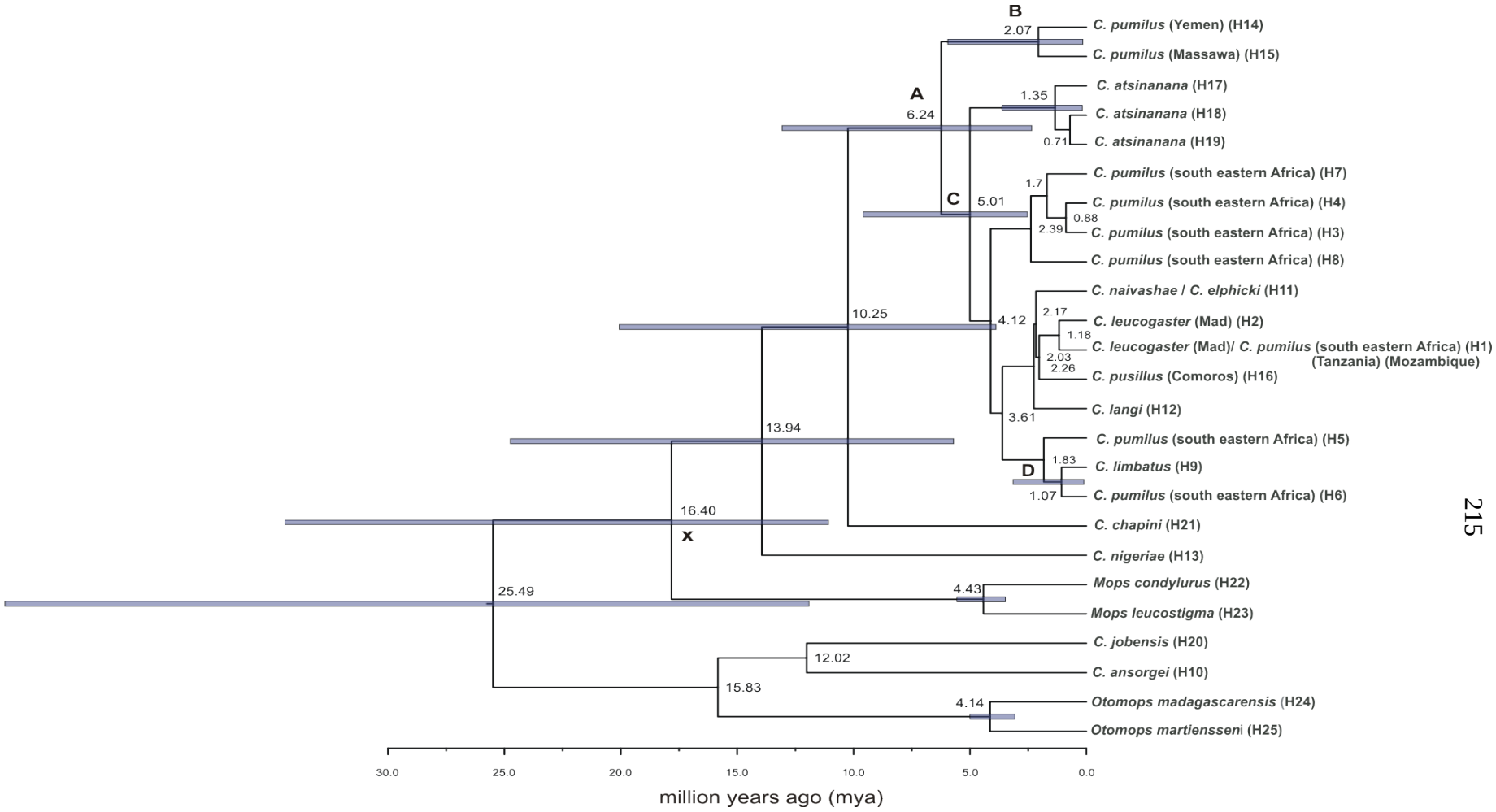

FIG. 3.Chronogram based on 206 nucleotides of the cyt *b* gene showing (**x** indicates the fossil date used to calibrate the tree).

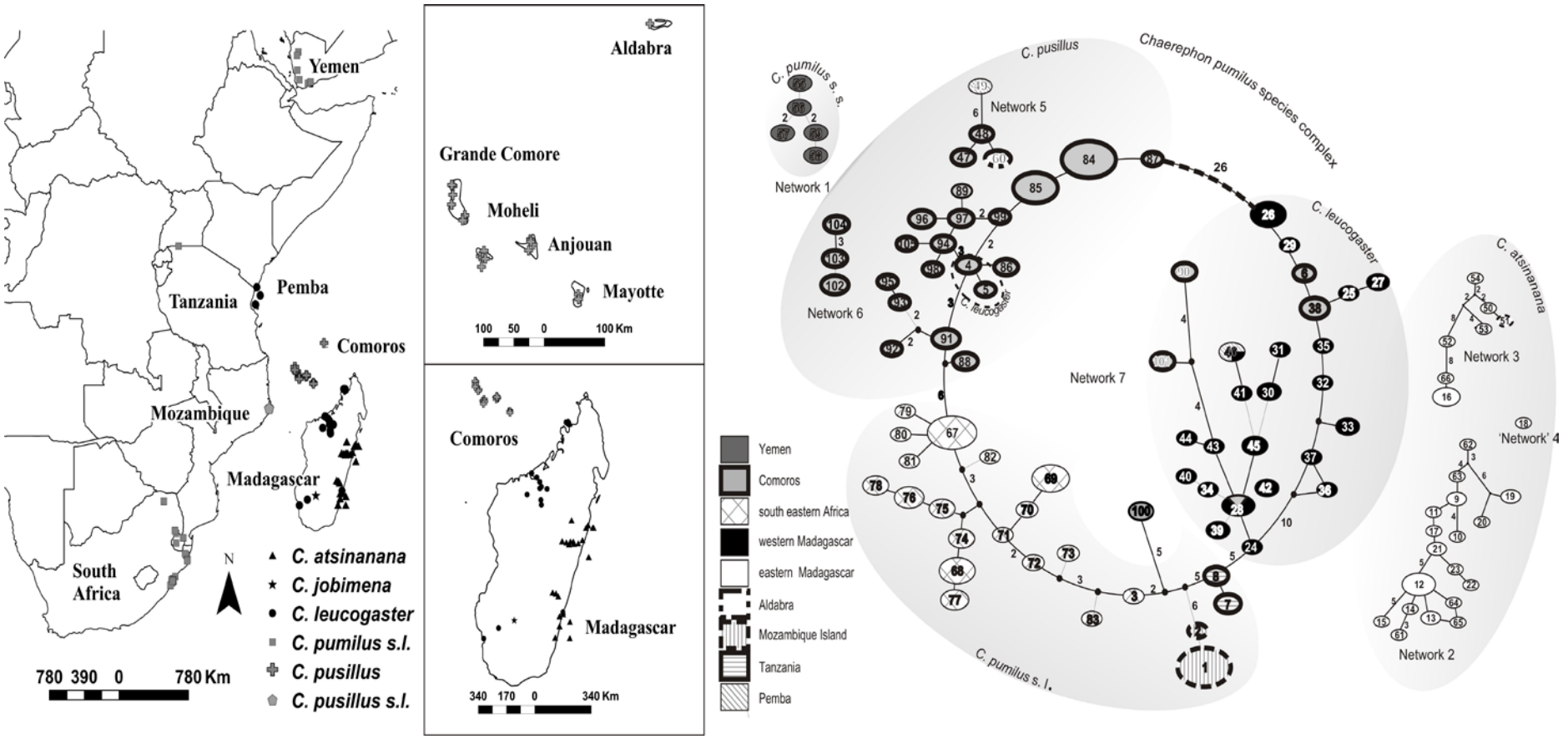

FIG. 4. Map showing distribution of samples of *Chaerephon pumilus* s.l. used in this study. Haplotype network based on 296 nucleotides of the control region illustrating relationships between 105 haplotypes.

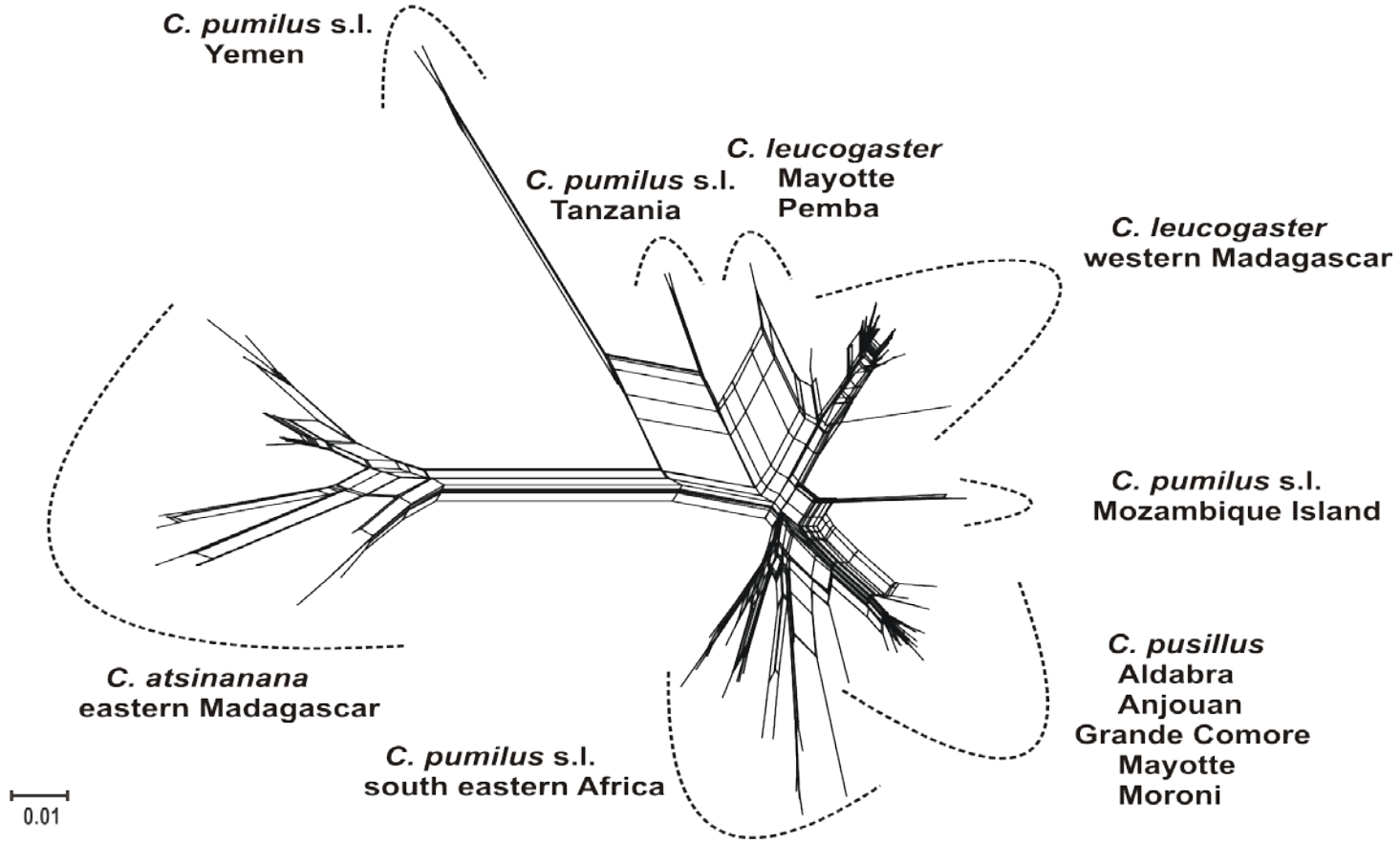

FIG. 5.Neighbour-network based on the HKY+I distance model for the mitochondrial control region dataset. Scale bar units are substitutions per site.

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Demographic Analysis

The nucleotide diversity of populations from eastern Madagascar, western Madagascar, south eastern Africa, Comoros, Yemen and Mozambique Island was low (0.004 – 0.034), whereas the haplotype diversity was high (0.700 – 0.838) with the exception of the population on Mozambique Island (0.011) (Table 3). Fu's Fs was not significant for any population, consistent with a neutral dataset. Mismatch distributions were plotted for each of the six regions (Fig. 6). Individuals from eastern Madagascar, south eastern Africa, the Comoros and Yemen showed a multimodal distribution which was significantly ragged for populations from the first mentioned two regions. Mismatch distributions of populations from western Madagascar and Mozambique Island were unimodal, consistent with population expansion. The low negative value of Fu's Fs for the Mozambique Island population was also indicative of expansion.

TABLE 3: Indices of diversity, neutrality, and historical demography based on an analysis of control region sequences across six regions (*C. atsinanana*: eastern Madagascar, *C. leucogaster*: western Madagascar, *C. pumilus* s.l.: south eastern Africa, *C. pusillus*: Comoros, *C. pumilus* s.l.: Mozambique Island and *C. pumilus* s.l.: Yemen).

Parameter	Eastern Madagascar	Western Madagascar	Mozambique Island	South eastern Africa	Comoros	Yemen
Nucleotide diversity	0.0343	0.0073	0.0040	0.0346	0.0273	0.0064
Haplotype diversity	0.779	0.885	0.111	0.837	0.838	0.700
Raggedness statistic	0.2240*	0.0741	0.6173	0.0724*	0.0274	0.1700*
Fu's (1997) Fs	4.346	2.353	-0.794	3.257	2.250	0.276
Mismatch distribution	multimodal	unimodal	unimodal	multimodal	multimodal	multimodal

*$P < 0.05$

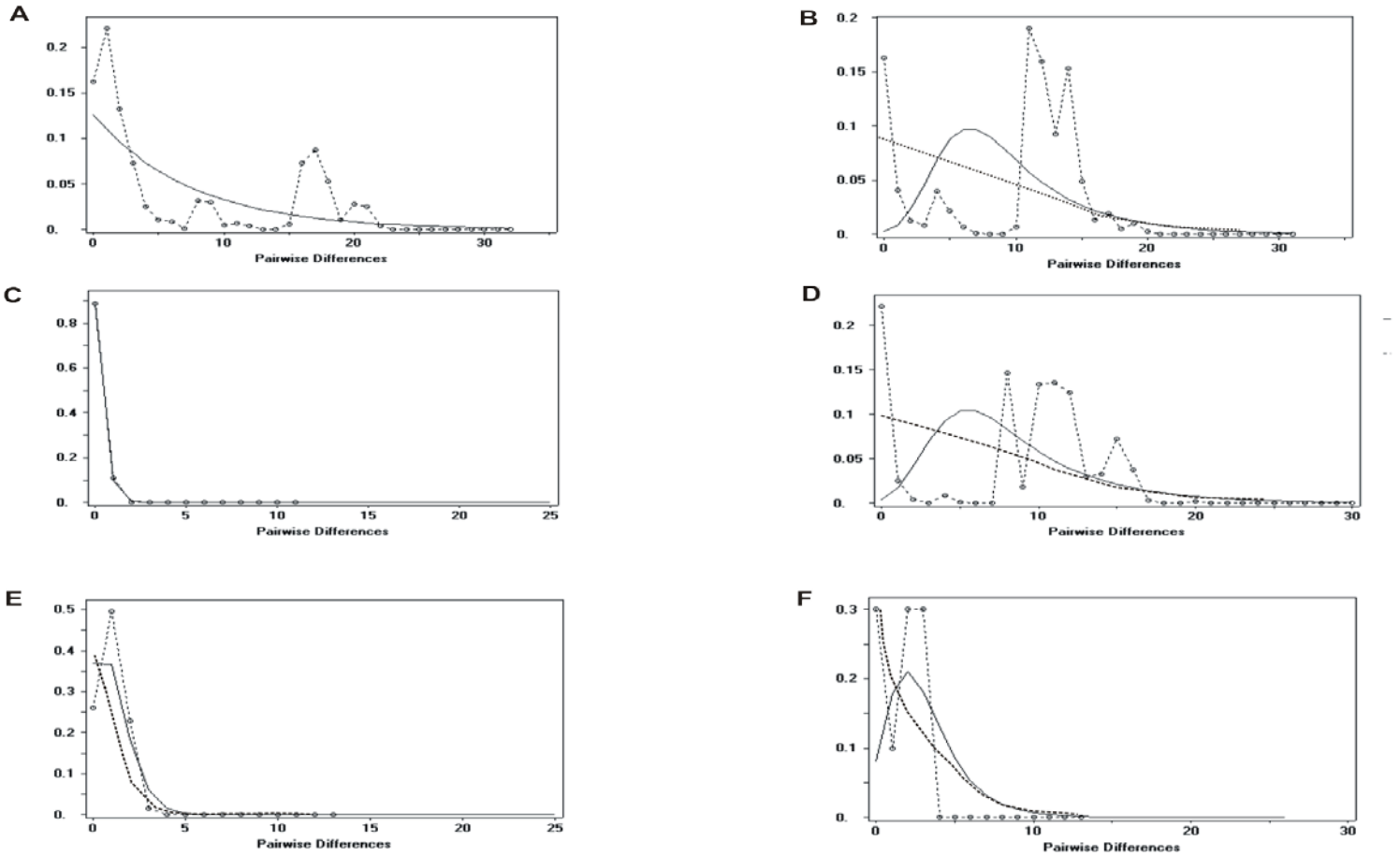

FIG. 6.Mismatch distribution for populations of *Chaerephon* from six regions: (A = *C. pusillus*, Comoros; B = *C. pumilus* s.l., south eastern Africa; C = *C. limbata*, Mozambique island; D = *C. atsinanana*, eastern Madagascar; E = *C. leucogaster*, western Madagascar and F = *C. pumilus* s.l., Yemen). Dashed line = observed (multimodal) distribution; solid line = expected distribution under a model of population growth/decline; dotted line = expected distribution under a model of constant population size.

Analyses of genetic structure

The AMOVA revealed that a significant ($P < 0.01$) 68.2% of the variance occurred among localities (Table 4). Pairwise Fst values were significant ($P < 0.05$) and high (0.296 – 0.959) (Table 5), consistent with significant structuring among localities. *C. pumilus* s.s. from Yemen was most significantly different from species/ populations in all other regions.

TABLE 4: Analysis of molecular variance (AMOVA) of *Chaerephon* individuals among and within groups.

Source of variation	Df	Sum of squares	Percentage variation
Among regions	8	3669.036	68.52*
Within regions	469	2069.391	31.48
Total	477	5738.427	

*$P < 0.05$

Isolation by distance

Mantel tests revealed positive correlations between genetic and geographic distances ($r^2 = 0.3294$, $P < 0.01$), indicative of isolation by distance within the overall sample.

TABLE 5: Pairwise Fsts of *Chaerephon* species across nine regions (eastern Madagascar, western Madagascar, south eastern Africa, Mozambique Island, Mohéli, balance of the Comoros, Tanzania, Pemba, Yemen).

Region	Eastern Madagascar (EM)	Western Madagascar (WM)	South eastern Africa (SEA)	Mozambique Island (MI)	Mohéli (MO)	Balance of the Comoros (CM)	Tanzania (TZ)	Pemba (PB)	Yemen (YM)
EM	-								
WM	0.749	-							
SEA	0.687	0.623	-						
MI	0.702	0.828	0.625	-					
MO	0.607	0.584	0.485	0.593	-				
CM	0.721	0.709	0.546	0.587	0.463	-			
TZ	0.652	0.617	0.423	0.987	0.334	0.642	-		
PB	0.650	0.640	0.456	0.866	0.296	0.648	0.882	-	
YM	0.721	0.906	0.759	0.959	0.548	0.811	0.889	0.780	-

DISCUSSION

This study complements that of Goodman *et al*. (2010) on genetic variation in members of the *Chaerephon pumilus* species complex from the western Indian Ocean region, including eastern Africa (Tanzania and Eritrea) and Yemen. We included all of the samples used by these authors, and additional samples from south eastern Africa and nearshore and offshore islands (South Africa, Swaziland, Mozambique Island, Zanzibar) for broader geographical coverage. Also included in our dataset were the holotypes (*C. elphicki* and *C. langi*), topotype (*C. naivashae*) and syntypes (*C. limbatus*) of taxa considered junior synonyms of *C. pumilus*.

Our findings support the genetic and phylogenetic relationships reported by Goodman *et al*. (2010), specifically the distinctness of: *C. atsinanana* from eastern Madagascar; populations on the Comoros and Aldabra, referred to *C. pusillus*; samples from western Madagascar, Pemba, and the Comoros, referred to *C. leucogaster*; and samples from Tanzania, referred to *C. pumilus* s.l.

We recovered three clades of *C. pumilus* s.l. from south eastern Africa, and find that these also form part of the *C. pumilus* species complex reported by Goodman *et al*. (2010). Consistent with Taylor *et al*. (2009) and Naidoo *et al*. (2013), we find a clade of *C. pumilus* s.l. from south eastern Africa nested within a *C. leucogaster* clade. In phylogenies based on mitochondrial markers, paraphyly among species may be explained by introgression due to past hybridization. This has been reported for several vespertillionid genera including *Myotis* (Berthier *et al*., 2006), *Eptesicus* (Mayer and von Helversen, 2001; Artyushin *et al*., 2009) and *Scotophilus* (Vallo *et al*., 2012), but not yet for the Molossidae. This hypothesis could be further tested by analyses based on nuclear markers such as microsatellites.

Taylor *et al*. (2009) have hypothesised that the distinct cyt *b* clades of *C. pumilus* s.l. from south eastern Africa might represent cryptic species with different echolocation characteristics, although recent research suggests that this is not the case (Naidoo *et al*., 2013). Initial results of an ongoing study examining population genetic relationships among these lineages using microsatellite markers, indicates that the clades of *C. pumilus* s.l. from south eastern Africa form one interbreeding population, and are therefore one species.

Chaerephon leucogaster consists of two cyt *b* clades, one located on Zanzibar and the other on Madagascar and Pemba. In analyses based on the control region, a further clade comprising two *C. leucogaster* haplotypes from Mayotte (Comoros) and Pemba (H4, H5) (Fig. 4) forms a strongly supported group with *C. pusillus* from Aldabra and the Comoros. As *C. leucogaster* is morphologically distinct from *C. pusillus* based on presumed-adaptive craniofacial and dental characters (Goodman *et al*., 2010), this could be explained by introgression of *C. pusillus* genes into *C. leucogaster* due to past hybridization between these taxa. The next step in resolving the taxonomy of *C. leucogaster*, which was described from western Madagascar, would be to determine if the various small morphologically described west African forms are genetically associated with *C.*

leucogaster, and also to carry out phylogenetic investigations based on nuclear markers to determine the likelihood that the paraphyly observed in *C. leucogaster* is due to introgression.

Chaerephon langi, *C. elphicki*, *C. limbatus* and *C. naivashae*

We investigated the taxonomic status of four forms of *C. pumilus* currently considered as junior synonyms (Simmons, 2005), by including sequence data from the holotypes of *C. langi* (TM 6544) and *C. elphicki* (TM 2488) and a topotype *C. naivashae* (FMNH 152967), and the syntypes of *C. limbatus* (ZMB 537, 538) in our analyses. Analyses show that these samples form three haplotypes within an unresolved lineage (D2) (Fig. 1) of the *C. pumilus* species complex. They are separated from *C. pumilus* s.s. by species level genetic distances of 3.0% - 4.0% (Baker and Bradley, 2006), and we therefore regard them as possibly specifically distinct from *C. pumilus.*

Chaerephon naivashae (type locality: Naivasha Station, Kenya) and *C. elphicki* (type locality: Malelane Estate, Barberton District, Mpumalanga Province, South Africa) do not appear to be genetically distinct from each other as they fall within the same haplotype (H11) based on 206 nucleotides of cyt *b*. They are separated from other D2 haplotypes by distances of 0.0% to 1.9% (vs. *C. limbatus*). Although Peterson *et al*. (1995) proposed the elevation of *naivashae* to species rank based on skull morphology, its distinctness is not supported by our genetic data and it would be premature to implement this proposed taxonomic change based on one type of evidence (Zachos *et al.*, 2013). *Chaerephon langi* (type locality: Tsotsoroga Pan, Northern Botswana) is separated from other Clade D2 haplotypes by genetic distances of 0.4% - 2.4%).

The syntype specimen of *C. limbatus* (type locality: Mozambique Island) is separated from *C. leucogaster* and three *C. pumilus* s.l. haplotypes from south eastern Africa by genetic distances of 0.9% - 2.9% and greater than the distance between *C. pusillus* and *C. leucogaster*, possibly consistent with the description of *C. limbatus* as a genetic species. *Chaerephon limbatus* is also morphologically distinct based on skull morphology and light-coloured wings, and therefore was considered a distinct species by Rosevear (1965) and Peterson *et al*. (1995). There is thus morphological and molecular evidence to support the status of *C. limbatus* as a distinct species. However in some analyses (see Fig. 3), *C. limbatus* is nested within *C. pumilus* s.l. lineages from south eastern Africa, from which it is separated by a genetic distance of 0.9%. It would therefore seem unwarranted to regard this syntype as clearly representative of an independent species, and perhaps more appropriate to regard it as a genetic variant of *C. pumilus* s.l. from south eastern Africa.

Is the C. pumilus species complex a ring species?

The distribution of network 7 of the *C. pumilus* species complex is suggestive of a ring species. Haplotype groups are distributed on a series of islands and continental landmasses surrounding the Mozambique Channel, which, at 1600 km long and 400-950 km wide between mainland Africa and Madagascar and the Comoros Archipelago, forms a putative geographic barrier

to dispersal of these bats. The Comoros Archipelago is situated toward the north of the channel, equidistant from northern Mozambique and north western Madagascar, and separated from each of them by ~ 300 km. The Comoro islands have been reported for bats to act as 'stepping stones' across the channel (Weyeneth *et al*., 2011).

It is proposed that ring species arise by expansion of a single species in two directions around a geographical barrier, accompanied by gradual divergence into two forms, which, when they eventually meet, behave as separate species (Stegnejer in Jordan (1905) in Irwin *et al*., 2001). Afro-Malagasy molossids share a wing morphology adapted for fast-flying aerial feeding, and are capable of covering scores of kilometres whilst foraging (Taylor *et al*., 2012). Based on their size, it would appear unlikely that the smaller molossids, *C. pumilus* and *C. leucogaster*, which have relatively weaker flight ability than larger molossids, could naturally traverse the Mozambique Channel on a regular basis, or perhaps the inter-island gaps. Migration of these bats could be associated with seasonal wind patterns of the Inter-Tropical Convergence Zone (ITCZ) (Dijkstra, 2007; Anderson, 2009). Recent evidence suggests that such exchanges do occur in bats (Weyeneth *et al*., 2008; Goodman *et al*., 2009; Weyeneth *et al*. 2011).

Chaerephon atsinanana and the *C. pumilus* species complex diverged from *C. pumilus* s.s. (type locality Eritrea) about 6.24 MYA (Fig. 3). *Chaerephon atsinanana* and its sister group, comprising *C. leucogaster*, *C. pusillus* and *C. pumilus* s.l. from south eastern Africa and Tanzania, last shared a common ancestor 5.01 MYA. As the predominant pattern of dispersal of bats is from the African mainland to Madagascar and not vice versa (Eger and Mitchell, 2003; Ratrimomanarivo *et al*., 2007; Russell *et al*., 2007), it is likely that the lineage which led to *C. atsinanana*, whose current range is eastern Madagascar, colonised this island by over-water dispersal from the mainland.

The *C. pumilus* species complex appears to have diversified primarily during the Pleistocene era of the Quaternary (Fig. 3). Although Pleistocene era glacial cycling may have played a role, we speculate that their evolution has been strongly influenced by distribution on a ring of landmasses bordering the Mozambique Channel, as discussed below.

An 'ideal' ring species would have the following characteristics (Irwin *et al*., 2001): (1) The chain forms a complete ring; (2) The two distinct terminal forms occur in sympatry; (3) Gene flow through a chain of populations has connected them before sympatry; and (4) The terminal differentiates are connected by gradual geographical differentiation. To determine if the *C. pumilus* species complex represents such a ring species, we evaluate it based on these four criteria.

Criterion 1: All members of network 7 of the *C. pumilus* species complex are part of the same 90% parsimony network (Fig. 4). Although there are some missing (unsampled) haplotypes, they form a genetic ring in which the terminal taxa are *C. pusillus* (Comoros and Aldabra) and *C. leucogaster* (western Madagascar), separated by a relatively large gap of 26 nucleotides between H87 and H26 (Fig. 4). In the case of a ring species, an ancestral form should undergo a range expansion from one side of the barrier to the other along two pathways. We postulate that the ancestral taxa were

forms of *C. pumilus* s.l., differentiated along a north/south gradient on the African continent (southern Africa through Mozambique to Tanzania), and that these independently underwent over-water range expansions to the Comoros and Aldabra.

One pathway appears to have been from south east Africa (H38) to the Comoros and Aldabra (H88) (Fig. 4) resulting in colonization of the Comoros Archipelago and subsequently Aldabra and differentiation into the terminal form *C. pusillus*, endemic to these islands. The other pathway appears to have been from south east Africa (H3) via the ancestors of H100 (Mohéli) and H2 (Mozambique Island) through Tanzania (H8) to western Madagascar (H24, *C. leucogaster*) (Fig. 4).

Chaerephon leucogaster appears to have diversified on Madagascar, where it underwent a Pleistocene-era population expansion, as indicated by a unimodal mismatch distribution (Fig. 5, Ratrimomanarivo *et al*., 2009). *Chaerephon leucogaster* haplotypes 4 and 5 are found on the Comoros, sympatric with *C. pusillus*, possibly indicative of further long distance colonization from Madagascar back to the Comoros, completing the geographic ring (Fig. 4). *Chaerephon leucogaster*, although currently partially sympatric with *C. pusillus* on Mayotte, is likely to have diverged in allopatry on Madagascar, an isolated landmass, rather than by circular overlap of populations without vicariant barriers to prevent interbreeding, as in a ring species (Irwin *et al*., 2001).

Criterion 2. The terminal forms of the *C. pumilus* species complex ring, *C. pusillus* and *C. leucogaster*, are morphologically and genetically distinct (Goodman *et al*., 2010), and are sympatric on Mayotte (Fig. 4), consistent with expectations for a ring species.

Criterion 3. Significant pairwise Fst values among localities for the *C. pumilus* species complex (0.296 – 0.882) are indicative of high levels of genetic structure and relatively low levels of gene flow. Consistent with this, AMOVA reveals a significant 68.52% of the variance among localities. Further, analyses of gene flow using Migrate indicate essentially no migration between localities. However, there are two instances of haplotype sharing between localities (H46 - Pemba Island and Madagascar; H28 - Madagascar and Mayotte), consistent with some contemporary gene flow or incomplete lineage sorting. However, the overall prediction for a ring species, namely high levels of gene flow though circular overlaps, is not supported.

Criterion 4. Contrary to the high levels of differentiation between localities, discussed above, a significant Mantel test statistic is indicative of isolation by distance; consistent with a level of gradual genetic differentiation between the terminal differentiates.

SUMMARY AND CONCLUSIONS

Our first hypothesis is supported, as we find that the junior synonyms of *C. pumilus*, represented by the syntype of *C. limbatus*, topotype of *C. naivashae*, and holotypes of *C. elphicki* and *C. langi*, are genetically distinct at species level from the nominate form, *C. pumilus* s.s. from Massawa, Eritrea.

Our second hypothesis is also supported, as analyses based on the mitochondrial cyt *b* and control regions indicate that forms of *C. pumilus* s.l. from south eastern Africa are possibly distinct at the species level from *C. pumilus* s.s. We further find that they form part of the *C. pumilus* species complex, also comprising *C. pumilus* s.s., *C. atsinanana*, *C. leucogaster*, *C. pusillus* and *C. pumilus* s.l. (Tanzania) (Goodman *et al*., 2010). Pending confirmation from other forms of data, we suggest that *C. pumilus* s.l. from south eastern African be renamed.

With respect to our third hypothesis, we find that network 7 within the *C. pumilus* species complex has several of the attributes of a ring species, although it appears to differ from this model in some important respects. *Chaerephon leucogaster*, *C. pumilus* s.l. (south eastern Africa) and *C. pusillus* form a ring in which haplotypes of the last two species represent terminally differentiated forms occurring in sympatry on Mayotte. Although there is evidence of isolation by distance, there was also a relatively high degree of structuring and limited gene flow around the ring; it appears that the island based species may have differentiated in allopatry, with some gene flow associated with over water dispersal. In contrast, differentiation of the species located along the south east coast of Africa, from South Africa northwards to Tanzania, may have been according to the isolation by distance model.

We conclude there are perhaps three species (see Fig. 4): 1. The 'real' *C. pumilus* (Network 1); 2. *C. atsinanana* (Networks 2,3,4) and 3. the *C. pumilus* species complex (Networks 5.6.7). We agree that Clade C represents at least 2 species, one of which is *C. atsinanana*. Reference to Figure 2 indicates that there is significant support not only for Clade C (Fig. 4.), but also for most subclades within Clade C. Thus, based on the Phylogenetic Species Concept, there are likely to be a number of distinct species within Clade C (*C. atsinanana*, *C. pumilus* (south eastern Africa), *C. leucogaster* and *C. pusillus*). The genetic distance between *C. pumilus* (south eastern Africa), *C. leucogaster* and *C. pusillus* are low (<1.7%), which might indicate that they are really subspecies or incipient species. However, there are morphological differences which are consistent with their being distinct species (Goodman *et al*. 2010). Without additional evidence from nuclear markers, it is not possible to resolve the issue of the number of species present in this complex.

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APPENDIX I. Details of samples used in the data analysis. Museum acronyms are defined in the Methods and Material section. Uncatalogued samples are denoted with the collector name which include PB = Petr Benda, PT = Peter Taylor and JB = Julio Balona.

Taxon	**Geographic co-ordinates**		**Museum/ Collector Number**	**Country/Island**	**Genbank accession number (control region)**	**Genbank accession number (cyt *b*)**
	Latitude	**Longitude**				
C. pumilus s.s.	-	-	USNM 38032	Eritrea	-	GQ 867179
C. pumilus s.l.	13°20'N	43°43'E	NMP PB 3667	Yemen	-	GQ 489112
	15°44'N	43°37'E	NMP PB 3752	Yemen	-	GQ489111
	13°02'N	44°34'E	NMP PB 3626	Yemen	GQ 489128	-
	14°09'N	43°31'E	NMP PB 3685	Yemen	GQ 489129	-
	15°26'N	43°29'E	NMP PB 3154	Yemen	GQ 489130	-
	13°08'N	44°51'E	NMP PB 3606	Yemen	GQ 489131	-
	13°08'N	44°51'E	NMP PB 3619	Yemen	GQ 489132	-
C. pumilus s.l.	1.01751°S	31.54976°E	FMNH 193055	Tanzania	-	GQ 489156
	1.01751°S	31.54976°E	FMNH 192938	Tanzania	-	GQ 489157
C. limbatus	-	-	ZMB 537	Mozambique	-	KF193828
	-	-	ZMB 538	Mozambique	-	KF193829
C. pumilus s.l.	15.03595°S	40.73447°E	FMNH 213633	Mozambique	KF193650	KF193833
	15.03595°S	40.73447°E	FMNH 213634	Mozambique	KF193651	KF193834
	15.03595°S	40.73447°E	FMNH 213635	Mozambique	KF193652	KF193835
	15.03595°S	40.73447°E	FMNH 213636	Mozambique	KF193653	KF193836
	15.00868°S	40.66465°E	FMNH 213638	Mozambique	KF193654	KF193837
	15.00868°S	40.66465°E	FMNH 213639	Mozambique	KF193655	KF193838
	15.00868°S	40.66465°E	FMNH 213640	Mozambique	KF193656	KF193839
	15.00868°S	40.66465°E	FMNH 213641	Mozambique	KF193657	KF193840
	15.00868°S	40.66465°E	FMNH 213642	Mozambique	KF193658	KF193841
	15.00868°S	40.66465°E	FMNH 213643	Mozambique	KF193659	KF193842
	15.00868°S	40.66465°E	FMNH 213644	Mozambique	KF193660	KF193843
	15.00868°S	40.66465°E	FMNH 213645	Mozambique	KF193661	KF193844
	15.00868°S	40.66465°E	FMNH 213646	Mozambique	KF193662	KF193845
	15.00868°S	40.66465°E	FMNH 213647	Mozambique	KF193663	KF193846

C. pusillus s.l.	15.00868°S	40.66465°E	FMNH 213648	Mozambique	KF193664	KF193847
	15.00868°S	40.66465°E	FMNH 213649	Mozambique	KF193665	KF193848
C. langi	-	-	TM 6544	Botswana (South Africa)	-	KF193831
C. elphicki	-	-	TM 2488	Transvaal (South Africa)	-	KF193832
C. naivashae	-	-	FMNH 152967	Kenya	-	KF193830
C. leucogaster	05°35.400'S	39°13.800'E	FMNH 198093	Zanzibar	-	GQ 489160
	05°35.400'S	39°13.800'E	FMNH 198149	Zanzibar	-	GQ 489158
	05°35.400'S	39°13.800'E	FMNH 198151	Zanzibar	-	GQ 489159
	05°35.400'S	39°13.800'E	FMNH 198152	Zanzibar	-	GQ 489161
	05°35.400'S	39°13.800'E	FMNH 198091	Zanzibar	-	GQ 489162
	05°35.400'S	39°13.800'E	FMNH 198092	Zanzibar	-	GQ 489163
	05°35.400'S	39°13.800'E	FMNH 198150	Zanzibar	-	GQ 489165
	05°35.400’S	39°13.800’E	FMNH 198153	Zanzibar	-	GQ 489164
	12°49.923'S	45°08.215'E	FMNH 194028	Mayotte	KF193706	EU716040
	12°49.923'S	45°08.215'E	FMNH 194023	Mayotte	KF193708	GQ489166
	12°49.923'S	45°08.215'E	FMNH 194019	Mayotte	KF193710	EU716041
	12°49.923'S	45°08.215'E	FMNH 194018	Mayotte	KF193711	-
	12°49.923'S	45°08.215'E	FMNH 194020	Mayotte	KF193704	GQ489153
	12°49.923'S	45°08.215'E	FMNH 194021	Mayotte	KF193705	-
	12°49.923'S	45°08.215'E	FMNH 194022	Mayotte	KF193709	-
	12°49.923'S	45°08.215'E	FMNH 194026	Mayotte	KF193707	-
	04°34.200'S	39°25.200'E	FMNH 192886	Pemba	EU727534	EU716003
	04°34.200'S	39°25.200'E	FMNH 192889	Pemba	-	EU716004
	04°96.487'S	39°71.456'E	FMNH 192891	Pemba	GQ489117	KF193638
	04°96.487'S	39°71.456'E	FMNH 192819	Pemba	GQ489118	KF193639
	15°42.778'S	46°18.752'E	FMNH 184604	western Madagascar	EU727485	-
	15°42.778'S	46°18.752'E	FMNH 184605	western Madagascar	EU727486	-

C. leucogaster	15°42.778'S	46°18.752'E	FMNH 184606	western Madagascar	EU727487	-
	15°42.778'S	46°18.752'E	FMNH 184607	western Madagascar	EU727488	-
	15°42.778'S	46°18.752'E	FMNH 184608	western Madagascar	EU727517	-
	16°42.062'S	46°04.304'E	FMNH 184922	western Madagascar	EU727502	EU716039
	16°42.062'S	46°04.304'E	FMNH 184923	western Madagascar	EU727503	EU716006
	13.4067°S	48.2917°E	FMNH 188575	western Madagascar	KF193670	-
	13.4067°S	48.2917°E	FMNH 188576	western Madagascar	KF193671	-
	13.4067°S	48.2917°E	FMNH 188577	western Madagascar	KF193672	-
	22°54.546'S	44°31.574'E	FMNH 184259	western Madagascar	KF193673	EU716005
	22°54.546'S	44°31.574'E	FMNH 184263	western Madagascar	EU727461	-
	22°54.546'S	44°31.574'E	FMNH 184264	western Madagascar	EU727470	-
	16°42.062'S	46°04.304'E	FMNH 184924	western Madagascar	EU727504	EU716007
	16º26.173'S	47º09.329'E	FMNH 184954	western Madagascar	EU727511	EU716014
	16°20.229'S	46°50.794'E	FMNH 184955	western Madagascar	EU727512	EU716015
	16°20.229'S	46°50.794'E	FMNH 184956	western Madagascar	EU727513	EU716016
	16°20.229'S	46°50.794'E	FMNH 184957	western Madagascar	EU727514	EU716017
	16°20.229'S	46°50.794'E	FMNH 184958	western Madagascar	EU727515	EU716018
	16°20.229'S	46°50.794'E	FMNH 184959	western Madagascar	EU727516	EU716019
	15°54.245'S	46°35.873'E	FMNH 185020	western Madagascar	EU727525	GQ489167
	15°54.245'S	46°35.873'E	FMNH 185021	western Madagascar	EU727526	-
	15°54.245'S	46°35.873'E	FMNH 185022	western Madagascar	EU727527	-
	15°54.245'S	46°35.873'E	FMNH 185027	western Madagascar	EU727528	-
	15°54.245'S	46°35.873'E	FMNH 185028	western Madagascar	EU727529	-
	15°54.245'S	46°35.873'E	FMNH 185029	western Madagascar	EU727530	-
	15°54.245'S	46°35.873'E	FMNH 185030	western Madagascar	EU727531	-
	23°23.704'S	43°43.219'E	FMNH 184239	western Madagascar	EU727483	EU716036
	23°23.704'S	43°43.219'E	FMNH 184240	western Madagascar	EU727474	EU716037
	23°23.704'S	43°43.219'E	FMNH 184238	western Madagascar	EU727471	EU716038
	23°23.704'S	43°43.219'E	FMNH 184237	western Madagascar	EU727462	-
	13°21.095'S	48°11.307'E	FMNH 188496	western Madagascar	GQ489137	GQ489178
	16°42.062'S	46°04.304'E	FMNH 184925	western Madagascar	EU727505	EU716008

C. leucogaster	16°42.062'S	46°04.304'E	FMNH 184926	western Madagascar	EU727506	EU716009
	16º57.452’S	46º49.433'E	FMNH 184915	western Madagascar	EU727496	-
	16º57.452’S	46º49.433'E	FMNH 184916	western Madagascar	EU727497	-
	16º57.452’S	46º49.433'E	FMNH 184917	western Madagascar	EU727498	-
	16º57.452’S	46º49.433'E	FMNH 184919	western Madagascar	EU727500	-
	16º57.452’S	46º49.433'E	FMNH 184920	western Madagascar	EU727519	-
	16º24.807'S	46º45.876'E	FMNH 184973	western Madagascar	EU727518	-
	16º24.807'S	46º45.876'E	FMNH 184974	western Madagascar	EU727524	-
	16º26.173'S	47º09.329'E	FMNH 184950	western Madagascar	EU727507	KF193640
	16º26.173'S	47º09.329'E	FMNH 184951	western Madagascar	EU727508	EU716012
	16º26.173'S	47º09.329'E	FMNH 184952	western Madagascar	EU727509	KF193641
	16º26.173'S	47º09.329'E	FMNH 184953	western Madagascar	EU727510	EU716013
	16º06.961'S	46º45.400'E	FMNH 184975	western Madagascar	EU727521	EU716020
	16º06.961'S	46º45.400'E	FMNH 184976	western Madagascar	KF193674	EU716021
	16º06.961'S	46º45.400'E	FMNH 184977	western Madagascar	EU727522	EU716022
	16º06.961'S	46º45.400'E	FMNH 184978	western Madagascar	KF193675	EU716023
	16º06.961'S	46º45.400'E	FMNH 184979	western Madagascar	EU727484	EU716024
	17º11.000'S	46º50.947'E	FMNH 184896	western Madagascar	EU727489	-
	17º11.000'S	46º50.947'E	FMNH 184897	western Madagascar	EU727490	-
	17º11.036'S	46º51.000'E	FMNH 184898	western Madagascar	EU727491	-
	17º11.036'S	46º51.000'E	FMNH 184899	western Madagascar	EU727492	-
	17º11.036'S	46º51.000'E	FMNH 184900	western Madagascar	EU727493	-
	17º11.036'S	46º51.000'E	FMNH 184901	western Madagascar	EU727494	-
	17º11.036'S	46º51.000'E	FMNH 184902	western Madagascar	EU727495	-
	13°24.308'S	48°18.201’E	FMNH 187750	western Madagascar	EU727464	EU716030
	13°22.012'S	48°18.927’E	FMNH 187751	western Madagascar	EU727478	-
	13°22.012'S	48°18.927’E	FMNH 187753	western Madagascar	EU727467	-

C. leucogaster	13°22.012'S	48°18.927’E	FMNH 187756	western Madagascar	EU727501	-
	13°22.012'S	48°18.927’E	FMNH 187754	western Madagascar	EU727468	EU716026
	13°22.012'S	48°18.927’E	FMNH 187755	western Madagascar	EU727469	EU716027
	13°24.254’S	48°16.425'E	FMNH 188495	western Madagascar	GQ489136	-
	13°21.099’S	48°11.307'E	FMNH 188497	western Madagascar	EU727475	-
	13°21.099’S	48°11.307'E	FMNH 188498	western Madagascar	EU727476	EU716028
	13°21.099’S	48°11.307'E	FMNH 188499	western Madagascar	EU727477	-
	13°21.099’S	48°11.307'E	FMNH 188500	western Madagascar	EU727463	-
	13°24.308’E	48°18.201’S	FMNH 187752	western Madagascar	EU727479	EU716030
	13°26.562'S	48°20.874'E	FMNH 188640	western Madagascar	EU727479	EU716032
	13°26.562'S	48°20.874'E	FMNH 188641	western Madagascar	EU727480	-
	13°26.562'S	48°20.874'E	FMNH 188642	western Madagascar	EU727481	EU716033
	13°26.562'S	48°20.874'E	FMNH 188643	western Madagascar	EU727482	EU716034
	13°26.562'S	48°20.874'E	FMNH 188644	western Madagascar	EU727472	EU716035
	22°09.418'S	48°01.009’E	FMNH 185228	eastern Madagascar	-	EU716031
C. atsinanana	23°21.300'S	47°35.763'E	FMNH 185230	eastern Madagascar	KF193677	GQ489168
	23°21.300'S	47°35.763'E	FMNH 185232	eastern Madagascar	KF193676	GQ489168
	22°82.130'S	47°83.100'E	FMNH 185260	eastern Madagascar	GQ489119	-
	22°36.660'S	47°83.677'E	FMNH 185286	eastern Madagascar	GQ489120	GQ489170
	22°15.700'S	48°01.682'E	FMNH 185315	eastern Madagascar	GQ489121	-
	18°14.070'S	49°37.783'E	FMNH 187797	eastern Madagascar	GQ489122	-
	18°15.020'S	49°41.105'E	FMNH 187799	eastern Madagascar	GQ489123	-
	18°96.060'S	48°84.742'E	FMNH 187834	eastern Madagascar	GQ489124	-
	18°96.060'S	48°84.742'E	FMNH 187836	eastern Madagascar	GQ489125	-
	21°25.760'S	47°45.592'E	FMNH 188088	eastern Madagascar	GQ489126	-
	21°25.760'S	47°45.592'E	FMNH 188089	eastern Madagascar	GQ489127	GQ489176
	22°49.275'S	47°49.860'E	FMNH 185259	eastern Madagascar	KF193678	GQ489169

C. atsinanana	22°09.418'S	48°01.009'E	FMNH 185314	eastern Madagascar	-	GQ489171
	18°49.317'S	49°04.343'E	FMNH 187823	eastern Madagascar	-	GQ489172
	18°57.636'S	48°50.845'E	FMNH 187834	eastern Madagascar	-	GQ489173
	21°18.394'S	47°38.144'E	FMNH 185322	eastern Madagascar	KF193679	GQ489174
	21°18.394'S	47°38.144'E	FMNH 185323	eastern Madagascar	KF193680	-
	18°52.945'S	47°58.245'E	FMNH 184677	eastern Madagascar	KF193681	-
	18°52.945'S	47°58.245'E	FMNH 184678	eastern Madagascar	KF193682	GQ489177
	18°52.945'S	47°58.245'E	FMNH 184680	eastern Madagascar	KF193683	-
	18°52.945'S	47°58.245'E	FMNH 184681	eastern Madagascar	KF193684	-
	19.01915°S	48.34871°E	UADBA 43912	eastern Madagascar	JN867871	JN867794
	19.01915°S	48.34871°E	UADBA 16902	eastern Madagascar	JN867873	JN867796
	19.01915°S	48.34871°E	UADBA 16903	eastern Madagascar	JN867874	JN867797
	19.01915°S	48.34871°E	UADBA 43913	eastern Madagascar	JN867875	JN867798
	19.01915°S	48.34871°E	UADBA 43914	eastern Madagascar	JN867876	KF193642
	19.01915°S	48.34871°E	UADBA 43915	eastern Madagascar	JN867877	JN867799
	19.01915°S	48.34871°E	UADBA 43916	eastern Madagascar	JN867878	JN867800
	19.01915°S	48.34871°E	UADBA 43917	eastern Madagascar	JN867879	JN867801
	19.01915°S	48.34871°E	UADBA 43918	eastern Madagascar	JN867880	JN867802
	17.90000°S	48.48300°E	FMNH 184651	eastern Madagascar	JN867938	JN867841
	17.90000°S	48.48300°E	FMNH 184652	eastern Madagascar	JN867939	JN867842
	17.90000°S	48.48300°E	FMNH 184653	eastern Madagascar	JN867940	JN867843
	17.90000°S	48.48300°E	FMNH 184654	eastern Madagascar	JN867941	JN867844
	17.90000°S	48.48300°E	FMNH 184655	eastern Madagascar	JN867942	JN867845
	23.35500°S	47.59605°E	FMNH 185233	eastern Madagascar	JN867850	JN867804
	23.35500°S	47.59605°E	FMNH 185235	eastern Madagascar	KF193685	JN867805
	23.35500°S	47.59605°E	FMNH 185236	eastern Madagascar	KF193686	-
	22.82125°S	47.83100°E	FMNH 185263	eastern Madagascar	JN867854	JN867806

C. atsinanana	22.82125°S	47.83100°E	FMNH 185265	eastern Madagascar	JN867855	JN867807
	22.36661°S	47.83676°E	FMNH 185287	eastern Madagascar	JN867859	JN867808
	22.36661°S	47.83676°E	FMNH 185288	eastern Madagascar	JN867860	JN867809
	22.15696°S	48.01681°E	FMNH 185317	eastern Madagascar	JN867869	JN867810
	22.15696°S	48.01681°E	FMNH 185318	eastern Madagascar	JN867870	JN867811
	18.88915°S	48.57755°E	FMNH 184509	eastern Madagascar	JN867924	JN867823
	18.88915°S	48.57755°E	FMNH 184510	eastern Madagascar	JN867895	JN867833
	18.88915°S	48.57755°E	FMNH 184512	eastern Madagascar	JN867926	JN867824
	18.88915°S	48.57755°E	FMNH 184513	eastern Madagascar	JN867927	JN867825
	18.25203°S	49.26778°E	FMNH 187822	eastern Madagascar	KF193687	KF193643
	18.25203°S	49.26778°E	FMNH 187931	eastern Madagascar	KF193688	KF193644
	18.25203°S	49.26778°E	FMNH 187816	eastern Madagascar	KF193689	KF193645
	22.15696°S	48.01681°E	FMNH 185318	eastern Madagascar	JN867870	JN867811
	21.30656°S	47.63573°E	FMNH 185319	eastern Madagascar	JN867907	JN867812
	18.88240°S	47.97075°E	FMNH 184681	eastern Madagascar	-	JN867826
	18.88240°S	47.97075°E	FMNH 185682	eastern Madagascar	-	JN867827
	18.93330°S	48.20000°E	FMNH 188113	eastern Madagascar	JN867899	JN867837
	18.93330°S	48.20000°E	FMNH 188115	eastern Madagascar	-	JN867840
	18.93330°S	48.20000°E	FMNH 188116	eastern Madagascar	JN867901	JN867838
	18.93330°S	48.20000°E	FMNH 188117	eastern Madagascar	JN867902	JN867839
	18.98300°S	48.61700°E	FMNH 188142	eastern Madagascar	JN867896	JN867834
	18.98300°S	48.61700°E	FMNH 188143	eastern Madagascar	JN867897	JN867835
	18.98300°S	48.61700°E	FMNH 188144	eastern Madagascar	JN867898	JN867836
	18.89500°S	48.41511°E	FMNH 184491	eastern Madagascar	JN867887	JN867828
	18.89500°S	48.41511°E	FMNH 184492	eastern Madagascar	JN867888	JN867829
	18.89500°S	48.41511°E	FMNH 184493	eastern Madagascar	JN867889	JN867830
	18.89500°S	48.41511°E	FMNH 184494	eastern Madagascar	JN867890	JN867831

C. atsinanana	18.89500°S	48.41511°E	FMNH 184495	eastern Madagascar	JN867891	JN867832
	18.89500°S	48.41511°E	FMNH 184496	eastern Madagascar	JN867892	-
	18.14068°S	49.37783°E	FMNH 187800	eastern Madagascar	KF193691	-
	18.14068°S	49.37783°E	FMNH 187801	eastern Madagascar	JN867903	JN867814
	18.14068°S	49.37783°E	FMNH 187798	eastern Madagascar	KF193690	GQ489172
	18.14068°S	49.37783°E	FMNH 187803	eastern Madagascar	JN867904	JN867815
	18.14068°S	49.37783°E	FMNH 187804	eastern Madagascar	JN867905	JN867816
	18.14068°S	49.37783°E	FMNH 187805	eastern Madagascar	JN867906	JN867817
	18.14068°S	49.37783°E	FMNH 187806	eastern Madagascar	KF193692	JN867818
	18.14068°S	49.37783°E	FMNH 187807	eastern Madagascar	-	JN867819
	18.25203°S	49.26778°E	FMNH 187836	eastern Madagascar	KF193693	-
	18.25203°S	49.26778°E	FMNH 187837	eastern Madagascar	KF193694	-
	18.25203°S	49.26778°E	FMNH 187820	eastern Madagascar	KF193695	-
	18.25203°S	49.26778°E	FMNH 187835	eastern Madagascar	KF193696	-
	18.25203°S	49.26778°E	FMNH 187838	eastern Madagascar	KF193697	-
	23.35500°S	47.59605°E	FMNH 185231	eastern Madagascar	JN867848	-
	22.15696°S	48.01681°E	FMNH 185316	eastern Madagascar	JN867868	-
	22.82125°S	47.83100°E	FMNH 185262	eastern Madagascar	JN867853	-
	22.82125°S	47.83100°E	FMNH 185260	eastern Madagascar	JN867854	JN867806
	23.35500°S	47.59605°E	FMNH 185229	eastern Madagascar	JN867846	-
	22.36661°S	47.83676°E	FMNH 185282	eastern Madagascar	KF193701	-
	22.36661°S	47.83676°E	FMNH 185283	eastern Madagascar	JN867856	-
	22.36661 °S	47.83676°E	FMNH 185284	eastern Madagascar	JN867857	-
	22.36661 °S	47.83676°E	FMNH 185285	eastern Madagascar	JN867858	-
	22.36661 °S	47.83676°E	FMNH 185286	eastern Madagascar	KF193702	-
	23.35500°S	47.59605°E	FMNH 185232	eastern Madagascar	JN867849	-
	23.35500°S	47.59605°E	FMNH 185234	eastern Madagascar	KF193698	-

C. atsinanana	23.35500°S	47.59605°E	FMNH 185231	eastern Madagascar	KF193703	-
	21.25760°S	47.45591°E	FMNH 188090	eastern Madagascar	JN867921	-
	21.30656°S	47.63573°E	FMNH 185320	eastern Madagascar	JN867908	-
	21.30656°S	47.63573°E	FMNH 185321	eastern Madagascar	JN867909	-
	21.25760°S	47.45591°E	FMNH 188091	eastern Madagascar	JN867922	-
	22.15696°S	48.01681°E	FMNH 185315	eastern Madagascar	GQ489121	-
	21.25760°S	47.45591°E	FMNH 188087	eastern Madagascar	KF193699	-
	21.25760°S	47.45591°E	FMNH 188092	eastern Madagascar	KF193700	-
C. pumilus s.l.	29.846°S	30.890°E	UWWW1CP1	south eastern Africa	JX976432	JX976488
	29.846°S	30.890°E	UWWW1CP3	south eastern Africa	JX976431	JX976490
	29.846°S	30.890°E	UWWW1CP4	south eastern Africa	JX976430	JX976489
	29.846°S	30.890°E	UWWW1CP5	south eastern Africa	JX976433	JX976491
	29.846°S	30.890°E	UWWW1CP6	south eastern Africa	JX976434	JX976492
	29.831°S	30.892°E	URPV1CP1	south eastern Africa	JX976436	JX976493
	29.831°S	30.892°E	URPV1CP2	south eastern Africa	JX976438	JX976494
	29.831°S	30.892°E	URPV1CP3	south eastern Africa	JX976437	JX976495
	29.831°S	30.892°E	URPV1CP4	south eastern Africa	JX976439	JX976496
	29.831°S	30.892°E	URPV1CP5	south eastern Africa	JX976435	JX976497
	29.831°S	30.892°E	URPV2CP6	south eastern Africa	JX976442	JX976498
	29.831°S	30.892°E	URPV2CP7	south eastern Africa	JX976441	JX976499
	29.831°S	30.892°E	URPV2CP8	south eastern Africa	JX976440	JX976500
	29.828°S	30.866°E	PNT1	south eastern Africa	JX976486	JX976501
	29.828°S	30.866°E	PNT2	south eastern Africa	JX976487	JX976502
	27.695°S	32.356°E	PH1	south eastern Africa	JX976448	JX976503
	27.695°S	32.356°E	PH2	south eastern Africa	JX976450	JX976504
	27.695°S	32.356°E	PH3	south eastern Africa	JX976452	JX976505

C. pumilus s.l.	27.695°S	32.356°E	PH4	south eastern Africa	JX976443	JX976506
	27.695°S	32.356°E	PH5	south eastern Africa	JX976444	JX976507
	27.695°S	32.356°E	PH6	south eastern Africa	JX976445	JX976508
	27.695°S	32.356°E	PH7	south eastern Africa	JX976446	JX976509
	27.695°S	32.356°E	PH8	south eastern Africa	JX976447	JX976510
	27.695°S	32.356°E	PH9	south eastern Africa	JX976449	JX976511
	27.695°S	32.356°E	PH11	south eastern Africa	JX976451	JX976512
	29.769°S	31.010°E	EH1	south eastern Africa	-	JX976513
	29.769°S	31.010°E	EH2	south eastern Africa	JX976463	JX976514
	29.769°S	31.010°E	EH3	south eastern Africa	JX976465	JX976515
	29.769°S	31.010°E	EH4	south eastern Africa	JX976467	JX976516
	29.769°S	31.010°E	EH5	south eastern Africa	JX976453	JX976517
	29.769°S	31.010°E	EH6	south eastern Africa	JX976455	JX976518
	29.769°S	31.010°E	EH7	south eastern Africa	JX976457	JX976519
	29.769°S	31.010°E	EH8	south eastern Africa	JX976459	JX976520
	29.769°S	31.010°E	EH9	south eastern Africa	JX976461	JX976521
	29.769°S	31.010°E	EH10	south eastern Africa	JX976464	JX976522
	29.769°S	31.010°E	EH11	south eastern Africa	JX976466	JX976523
	29.769°S	31.010°E	EH12	south eastern Africa	JX976468	JX976524
	29.769°S	31.010°E	EH13	south eastern Africa	JX976454	JX976525
	29.769°S	31.010°E	EH14	south eastern Africa	JX976456	JX976526
	29.769°S	31.010°E	EH15	south eastern Africa	JX976458	JX976527
	29.769°S	31.010°E	EH16	south eastern Africa	JX976460	JX976528
	29.769°S	31.010°E	EH17	south eastern Africa	JX976462	JX976529
	29.857°S	30.899°E	QB1	south eastern Africa	JX976469	-
	29.857°S	30.899°E	QB2	south eastern Africa	-	JX976530
	29.857°S	30.899°E	QB3	south eastern Africa	JX976472	JX976531

C. pumilus s.l.	29.857°S	30.899°E	QB4	south eastern Africa	JX976476	JX976532
	29.857°S	30.899°E	QB5	south eastern Africa	JX976482	JX976533
	29.857°S	30.899°E	QB6	south eastern Africa	JX976483	JX976534
	29.857°S	30.899°E	QB7	south eastern Africa	JX976475	-
	29.857°S	30.899°E	QB8	south eastern Africa	JX976485	JX976535
	29.857°S	30.899°E	QB9	south eastern Africa	JX976470	JX976536
	29.857°S	30.899°E	QB10	south eastern Africa	JX976471	JX976537
	29.857°S	30.899°E	QB11	south eastern Africa	JX976473	JX976538
	29.857°S	30.899°E	QB12	south eastern Africa	JX976480	JX976539
	29.857°S	30.899°E	QB13	south eastern Africa	JX976478	JX976540
	29.857°S	30.899°E	QB14	south eastern Africa	JX976474	JX976541
	29.857°S	30.899°E	QB15	south eastern Africa	JX976484	JX976542
	29.857°S	30.899°E	QB16	south eastern Africa	JX976477	JX976543
	29.930°S	30.925°E	CH1	south eastern Africa	JX976479	JX976544
	29.967°S	30.942°E	DM7363	south eastern Africa	FJ415824	FJ415813
	28.067°S	32.421°E	DM7367	south eastern Africa	FJ415826	FJ415814
	28.067°S	32.421°E	DM7369	south eastern Africa	FJ415837	-
	28.067°S	32.421°E	DM7370	south eastern Africa	FJ415838	-
	28.067°S	32.421°E	DM7371	south eastern Africa	FJ415839	-
	28.067°S	32.421°E	DM7372	south eastern Africa	FJ415827	-
	27.583°S	32.217°E	DM7373	south eastern Africa	FJ415828	FJ415815
	27.583°S	32.217°E	DM7374	south eastern Africa	FJ415829	FJ415816
	30.05°S	30.883°E	DM7377	south eastern Africa	FJ415846	-
	30.05°S	30.883°E	DM7378	south eastern Africa	FJ415830	-
	29.833°S	31.00°E	DM7379	south eastern Africa	FJ415848	FJ415817
	unknown		DM7380	south eastern Africa	FJ415849	FJ415818
	28.067°S	32.421°E	DM7381	south eastern Africa	FJ415841	-

C. pumilus s.l.	28.067°S	32.421°E	DM7382	south eastern Africa	FJ415831	-
	unknown		DM7383	south eastern Africa	FJ415850	-
	28.067°S	32.421°E	DM7384	south eastern Africa	FJ415832	-
	29.933°S	31.017°E	DM7385	south eastern Africa	FJ415836	-
	29.533°S	31.217°E	DM7386	south eastern Africa	FJ415847	-
	29.933°S	31.017°E	DM7387	south eastern Africa	FJ415840	-
	30.05°S	30.883°E	DM7401	south eastern Africa	FJ415843	-
	28.2°S	32.417°E	DM7525	south eastern Africa	-	FJ415819
	29.833°S	31.00°E	DM7851	south eastern Africa	FJ415844	-
	30.016°S	30.917°E	DM7905	south eastern Africa	FJ415851	-
	29.883°S	30.967°E	DM7907	south eastern Africa	FJ415852	-
	29.817°S	30.85°E	DM7910	south eastern Africa	FJ415853	-
	30.1°S	30.833°E	DM7913	south eastern Africa	FJ415833	-
	26.192°S	32.005°E	DM7922	south eastern Africa	-	FJ415820
	30.317°S	30.733°E	DM8030	south eastern Africa	FJ415854	-
	26.192°S	32.005°E	DM8036	south eastern Africa	FJ415834	FJ415821
	25.819°S	31.292°E	DM8042	south eastern Africa	FJ415856	FJ415822
	29.858°S	31.025°E	DM8348	south eastern Africa	FJ415855	-
	26.632°S	31.293°E	DM8437	south eastern Africa	-	KF193646
	29.867°S	31.00°E	D39	south eastern Africa	FJ415842	-
	29.917°S	30.933°E	D40	south eastern Africa	FJ415845	-
	unknown		D43	south eastern Africa	FJ415857	-
	23.059°S	30.067°E	PT 2011-2	south eastern Africa	JX976481	-
	25°34.355’S	31°10.866’E	JB 0417-4	south eastern Africa	KF193666	KF193849
	25°34.355’S	31°10.866’E	JB 0417-5	south eastern Africa	KF193667	KF193850
	25°34.355’S	31°10.866’E	JB 0417-7	south eastern Africa	KF193668	-
	25°34.355’S	31°10.866’E	JB 0417-8	south eastern Africa	KF193669	KF193851

C. pusillus	11°41'26.3S	11°41'26.3E	FMNH 194186	Grande Comore	-	GQ489141
	11°41'26.3S	11°41'26.3E	FMNH 194189	Grande Comore	-	GQ489142
	11°82.750’S	43°45.444’E	FMNH 194214	Grande Comore	GQ489113	-
	11°82.750’S	43°45.444’E	FMNH 194215	Grande Comore	KF193712	-
	11°82.750’S	43°45.444’E	FMNH 194217	Grande Comore	GQ489114	-
	11°88.970’S	43°42.000’E	FMNH 194220	Grande Comore	GQ489115	-
	11°88.970’S	43°42.000’E	FMNH 194226	Grande Comore	GQ489116	-
	11°88.970’S	43°42.000’E	FMNH 194227	Grande Comore	KF193713	-
	11°41.263’S	43°15.250’E	FMNH 194250	Grande Comore	-	GQ489138
	11°41.263’S	43°15.250’E	FMNH 194251	Grande Comore	-	GQ489139
	11°41.263’S	43°15.250’E	FMNH 194256	Grande Comore	KF193714	GQ489144
	11°41.263’S	43°15.250’E	FMNH 194258	Grande Comore	KF193715	GQ489145
	11.8275°S	43.45444°E	FMNH 194216	Grande Comore	KF193716	-
	11.8897°S	43.42°E	FMNH 194221	Grande Comore	KF193717	-
	11.8897°S	43.42°E	FMNH 194225	Grande Comore	KF193718	-
	11.8275°S	43.4544°E	FMNH 194210	Grande Comore	KF193719	-
	11.8275°S	43.4544°E	FMNH 194218	Grande Comore	KF193720	-
	11.88°S	43.42°E	FMNH 194226	Grande Comore	KF193721	-
	11.8275°S	43.4544°E	FMNH 194211	Grande Comore	KF193722	-
	11.8275°S	43.4544°E	FMNH 194213	Grande Comore	KF193723	-
	11.8275°S	43.4544°E	FMNH 194212	Grande Comore	KF193724	-
	11.8275°S	43.4544°E	FMNH 194217	Grande Comore	KF193725	-
	11.8897°S	43.4200°E	FMNH 194219	Grande Comore	KF193726	-
	11.8897°S	43.4200°E	FMNH 194220	Grande Comore	KF193727	-
	11.8897°S	43.4200°E	FMNH 194222	Grande Comore	KF193728	-
	11.8897°S	43.4200°E	FMNH 194224	Grande Comore	KF193729	-
	11.8897°S	43.4200°E	FMNH 194223	Grande Comore	KF193730	-

	11.6905°S	43.2569°E	FMNH 194185	Grande Comore	KF193731	-
	11.6905°S	43.2569°E	FMNH 194191	Grande Comore	KF193732	-
	11.4497°S	43.2775°E	FMNH 194209	Grande Comore	KF193733	-
	11.4497°S	43.2775°E	FMNH 194202	Grande Comore	KF193734	-
	11.4497°S	43.2775°E	FMNH 194201	Grande Comore	KF193735	-
	11.4497°S	43.2775°E	FMNH 194208	Grande Comore	KF193736	-
	11.4497°S	43.2775°E	FMNH 194200	Grande Comore	KF193737	-
	11.4497°S	43.2775°E	FMNH 194207	Grande Comore	KF193738	-
	11.4497°S	43.2775°E	FMNH 194206	Grande Comore	KF193739	-
	11.5658°S	43.2752°E	FMNH 194194	Grande Comore	KF193740	-
	11.6905°S	43.2569°E	FMNH 194188	Grande Comore	KF193741	-
	11.5658°S	43.2752°E	FMNH 194195	Grande Comore	KF193742	-
	11.6905°S	43.2569°E	FMNH 194184	Grande Comore	KF193743	-
	11.5658°S	43.2752°E	FMNH 194193	Grande Comore	KF193744	-
	11.6905°S	43.2569°E	FMNH 194192	Grande Comore	KF193745	KF193647
	11.6905°S	43.2569°E	FMNH 194189	Grande Comore	KF193746	-
	11.6905°S	43.2569°E	FMNH 194187	Grande Comore	KF193747	-
	11.6905°S	43.2569°E	FMNH 194183	Grande Comore	KF193748	KF193648
	11.6905°S	43.2569°E	FMNH 194190	Grande Comore	KF193749	-
	11.5658°S	43.2752°E	FMNH 194199	Grande Comore	KF193750	-
	11.5658°S	43.2752°E	FMNH 194197	Grande Comore	KF193751	-
	11.5658°S	43.2752°E	FMNH 194196	Grande Comore	KF193752	-
	12.2002°S	44.4669°E	FMNH 194340	Anjouan	KF193753	-
	12.1591°S	44.4325°E	FMNH 194327	Anjouan	KF193754	KF193649
	12.1591°S	44.4325°E	FMNH 194324	Anjouan	KF193755	-
	12.1352°S	44.4291°E	FMNH 194450	Anjouan	KF193756	-

C. pusillus	12.2002°S	44.4669°E	FMNH 194449	Anjouan	KF193757	-
	12.1352°S	44.4291°E	FMNH 194358	Anjouan	KF193758	-
	12.1352°S	44.4291°E	FMNH 194357	Anjouan	KF193759	-
	12.1352°S	44.4291°E	FMNH 194361	Anjouan	KF193760	-
	12.1352°S	44.4291°E	FMNH 194362	Anjouan	KF193761	-
	12.1352°S	44.4291°E	FMNH 194360	Anjouan	KF193762	-
	12.1352°S	44.4291°E	FMNH 194451	Anjouan	KF193763	-
	12.1352°S	44.4291°E	FMNH 194363	Anjouan	KF193764	-
	12.2002°S	44.4669°E	FMNH 194333	Anjouan	KF193765	-
	12.2002°S	44.4669°E	FMNH 194331	Anjouan	KF193766	-
	12.2002°S	44.4669°E	FMNH 194330	Anjouan	KF193767	-
	12.2002°S	44.4669°E	FMNH 194342	Anjouan	KF193768	-
	12.2002°S	44.4669°E	FMNH 194339	Anjouan	KF193769	-
	12.2002°S	44.4669°E	FMNH 194335	Anjouan	KF193770	-
	12.2002°S	44.4669°E	FMNH 194336	Anjouan	KF193771	-
	12.2002°S	44.4669°E	FMNH 194337	Anjouan	KF193776	-
	12.2002°S	44.4669°E	FMNH 194329	Anjouan	KF193772	-
	12.2002°S	44.4669°E	FMNH 194446	Anjouan	KF193773	-
	12.2002°S	44.4669°E	FMNH 194445	Anjouan	KF193774	-
	12.1591°S	44.4325°E	FMNH 194326	Anjouan	KF193775	-
	12.2002°S	44.4669°E	FMNH 194341	Anjouan	KF193777	-
	12.2002°S	44.4669°E	FMNH 194448	Anjouan	KF193778	-
	12.2002°S	44.4669°E	FMNH 194447	Anjouan	KF193779	-
	12.2002°S	44.4669°E	FMNH 194332	Anjouan	KF193780	-
	12.1591°S	44.4325°E	FMNH 194328	Anjouan	KF193781	-
	12.1591°S	44.4325°E	FMNH 194325	Anjouan	KF193782	-
	12.1355°S	44.4269°E	FMNH 194344	Anjouan	KF193783	-

C. pusillus	12.1355°S	44.4269°E	FMNH 194351	Anjouan	KF193784	-
	12.1355°S	44.4269°E	FMNH 194346	Anjouan	KF193785	-
	12.1352°S	44.4291°E	FMNH 194365	Anjouan	KF193786	-
	12.1355°S	44.4269°E	FMNH 194352	Anjouan	KF193787	-
	12.1352°S	44.4291°E	FMNH 194356	Anjouan	KF193788	-
	12.1352°S	44.4291°E	FMNH 194364	Anjouan	KF193789	-
	12.2708°S	44.3977°E	FMNH 194375	Anjouan	KF193790	-
	12.2708°S	44.3977°E	FMNH 194366	Anjouan	KF193791	-
	12°09.552’S	44°25.952’E	FMNH 194323	Anjouan	-	GQ489146
	12°12.010’S	44°28.014’E	FMNH 194329	Anjouan	-	GQ489147
	12°12.010’S	44°28.014’E	FMNH 194334	Anjouan	KF193792	GQ489148
	12°09.552’S	44°25.952’E	FMNH 194323	Anjouan	KF193793	GQ489146
	12.3475°S	43.6802°E	FMNH 194478	Mohéli	KF193794	-
	12.2802°S	43.7377°E	FMNH 194486	Mohéli	KF193795	-
	12.2802°S	43.7377°E	FMNH 194470	Mohéli	KF193796	-
	12.2802°S	43.7377°E	FMNH 194476	Mohéli	KF193797	-
	12.2802°S	43.7377°E	FMNH 194472	Mohéli	KF193798	-
	12.2802°S	43.7377°E	FMNH 194537	Mohéli	KF193799	-
	12.2802°S	43.7377°E	FMNH 194469	Mohéli	KF193800	-
	12.2802°S	43.7377°E	FMNH 194477	Mohéli	KF193801	-
	12.2802°S	43.7377°E	FMNH 194475	Mohéli	KF193802	-
	12.2802°S	43.7377°E	FMNH 194468	Mohéli	KF193803	-
	12.2802°S	43.7377°E	FMNH 194473	Mohéli	KF193805	-
	12.3647°S	43.7177°E	FMNH 194481	Mohéli	KF193806	-
	12.3647°S	43.7177°E	FMNH 194482	Mohéli	KF193807	-
	12.3647°S	43.7177°E	FMNH 194483	Mohéli	KF193804	-
	12.3647°S	43.7177°E	FMNH 194487	Mohéli	KF193808	-

C. pusillus	12.3647°S	43.7177°E	FMNH 194532	Mohéli	KF193809	-
	12.3647°S	43.7177°E	FMNH 194533	Mohéli	KF193810	-
	12.3647°S	43.7177°E	FMNH 194496	Mohéli	KF193811	-
	12.3647°S	43.7177°E	FMNH 194490	Mohéli	KF193812	-
	12.3647°S	43.7177°E	FMNH 194491	Mohéli	KF193813	-
	12.3647°S	43.7177°E	FMNH 194492	Mohéli	KF193814	-
	12.3647°S	43.7177°E	FMNH 194495	Mohéli	KF193815	-
	12.3647°S	43.7177°E	FMNH 194493	Mohéli	KF193816	-
	12.3647°S	43.7177°E	FMNH 194485	Mohéli	KF193817	-
	11.4405°S	43.2569°E	FMNH 194254	Grande Comore: Moroni	KF193818	-
	11.4405°S	43.2569°E	FMNH 194249	Grande Comore: Moroni	KF193819	-
	11.4405°S	43.2569°E	FMNH 194248	Grande Comore: Moroni	KF193820	-
	11.4405°S	43.2569°E	FMNH 194255	Grande Comore: Moroni	KF193821	-
	11.4405°S	43.2569°E	FMNH 194252	Grande Comore: Moroni	KF193822	-
	11.4405°S	43.2569°E	FMNH 194250	Grande Comore: Moroni	KF193823	-
	11.4405°S	43.2569°E	FMNH 194251	Grande Comore: Moroni	KF193824	-
	12°49.923’S	45°08.215’E	FMNH 194024	Mayotte	KF193827	GQ489149
	12°53.609’S	45°08.550’E	FMNH 194035	Mayotte	KF193825	GQ489150
	12°53.609’S	45°08.550’E	FMNH 194036	Mayotte	KF193826	GQ489151
	09°23.339’S	46°12.142’E	FMNH 205319	Aldabra	GQ489134	GQ489155
	09°23.339’S	46°12.142’E	FMNH 205318	Aldabra	GQ489133	GQ489154
C. jobimena	45.38°S	22.54°E	FMNH 175992	Madagascar	-	HM802932
C. ansorgei	-	-	-	-	-	AY377967
C. nigeriae	-	-	-	-	-	AY591329

C. chapini	-	-	-	-	-	AY591329
C. jobensis	-	-	-	-	-	AY591331
OUTGROUPS						
Mops midas			FMNH 184306			
Mops leucostigma			FMNH 185098			
Mops condylurus			DM 6291			
Otomops martiensseni			DM 8032			
Otomops madagascarensis			FMNH 166073			

APPENDIX II. Haplotypes defined by analysis of 206 nucleotides of the cyt *b* gene of *Chaerephon* species. Museum acronyms are defined in the Methods and Material section.

Haplotype	No of Samples	Sample Codes
1	63	FMNH192886, 192889, 184259, 184924,184925, 184926, 184950, 184952, 184951,184953, 184954,184955, 184956,184957,184958, 184959, 184975, 184976, 184977, 184978, 184979, 185030,187754, 187755,188498,188496, 187750, 185228, 188640, 188642, 188643,188644, 184239, 184240,184238, 193055,192938, 192891, 194028,194019,194023,194251,194250,213633, 213635, 213636, 213638, 213639, 213640, 213641, 213642, 213643, 213644, 213645, 213646, 213647, 213648,DM 8042,QB12, CH1, 0417-4, 0417-5, 0417-8
2	2	FMNH184923, 184922
3	26	UWWW1 - CP1, CP4, CP3, CP5, CP6, URPV1 – CP1, CP2, CP3, CP4, CP5, URPV2- CP7, CP8, PNT1, PNT2, QB3, QB4, QB6, QB8, QB9, QB10, QB11, QB13, QB15, DM 7379, 7380, 8437
4	1	URPV2 – CP6
5	17	PH 1, PH 2, PH3, PH 4, PH 5, PH 6, PH 7, PH 8, PH 9, PH 11, DM 7363, 7367, 7373, 7374, 7525, 7922, 8036
6	20	EH1, EH2, EH3, EH4, EH5, EH6, EH7, EH8, EH9, EH10, EH11, EH12, EH13, EH14, EH15, EH16, EH17, QB14, QB16, 16847
7	1	QB 2
8	1	QB 5
9	2	ZMB 537, 538
10	1	AY 377967
11	2	FMNH 152967, TM 2488
12	1	TM 6544
13	1	AY 591330
14	2	NMP PB 3752, 3667
15	1	USNM 38032
16	21	FMNH194256, 194258, 194323, 194329, 194372,194334,194020, 194024,194035, 194036,194186, 194189, 198149, 198151, 198093, 198152, 198091, 198092, 198153, 198150
17	26	FMNH184651,184652, 184653, 184654, 184655, 185233, 185235, 185263, 185265, 185287, 185288, 185317, 185318, 185230, 185259, 185286, 187834, 185314, 184509, 184512, 184513, 187822, 187931, 187816, 187823, 187834
18	31	FMNH 184510, 185318, 185319, 184681, 184682, 184678, 185322, 188089, 188113, 188115, 188116, 188117, 188142, 188143, 188144, 184491, 184492, 184493, 184494, 184495, UADBA 43912, 16902, 16903, 43913, 43914, 43915, 43916, 43917, 43918, 205318, 205319,
19	7	FMNH187801, 187798, 187803, 187804, 187805, 187806, 187807
20	1	FMNH 1759992
21	1	AY 591329
22	1	AY 591331

APPENDIX III. Haplotypes defined by analysis of 830 nucleotides of the cyt *b* gene of *Chaerephon* species. Museum acronyms are defined in the Methods and Material section.

Haplotype	No of Samples	Sample Codes
1	19	FMNH 192886, 184259, 184924, 184954, 184955, 184956, 184957, 184958, 184959, 185030, 188496, 184239, 184240, 184238, 194028, 194019, 194023, 192891, 188496
2	2	FMNH 192889, 192819
3	2	FMNH 184923, 184922
4	6	FMNH 184925, 184926, 184950, 184952, 184951, 184953
5	4	FMNH 184975, 184976, 184977, 184978
6	1	FMNH 184979
7	9	FMNH 187754, 187755, 188498, 187750, 185228, 188640, 188642, 188643, 188644
8	1	UWWW1- CP1
9	24	UWWW1 - CP4, CP3, CP5, CP6,URPV1 - CP1, CP2, CP3, CP4, CP5,URPV2 - CP7, CP8,PNT1,PNT2,QB3,QB4, QB6,QB8,QB9,QB10,QB11,QB13,QB15, DM 7380, 8437
10	1	URPV2 - CP6
11	8	PH1, PH2, PH3, PH4, PH5, PH6,PH9, DM 7374
12	9	PH7,PH8, PH11, DM 7363, 7367, 7373, 7525, 7922, 8036
13	17	EH1, EH2,EH3,EH4,EH5,EH6, EH7,EH8,EH9,EH10,EH11, EH12, EH13, EH14, EH15, EH16, EH17
14	1	QB2
15	1	QB5
16	3	QB12, CH1, DM 8042
17	2	QB14,QB16
18	2	FMNH 194251, 194250
19	8	FMNH 194256, 194258,194020, 194024, 194035, 194036, 205318, 205319
20	4	FMNH 194323, 194329,194327,194334
21	1	FMNH 194183
22	3	FMNH 194186, 194189, 194192
23	1	FMNH 193055
24	1	FMNH 192938
25	2	FMNH 198149, 198152
26	2	FMNH 198151, 198093
27	4	FMNH 198091, 198092, 198153, 198150
28	13	FMNH 185233, 185235, 185263, 185265, 185287, 185288, 185317, 185318, 185230, 185259,185286,185314, 185260
29	8	FMNH 184509, 184512, 184513,187822,187931, 187816,187823,187834
30	20	FMNH 184510,185318, 185319, 184681, 184682, 185322, 188089,188113,188115, 188116,188117, 188142,188143, 188144, 184491, 184492, 184493, 184494, 184495
31	5	FMNH 184651, 184652, 184653, 184654, 184655
32	10	FMNH 184678, 43912, 16902, 16903, 43913, 43914, 43915, 43916, 43917, 43918
33	7	FMNH 187801, 187798, 187803,187804, 187805, 187806, 187807

APPENDIX IV. Haplotypes defined by analysis of 296 nucleotides of the control region of *Chaerephon* species. Museum acronyms are defined in the Methods and Material section.

Haplotype	No of Samples	Sample Codes
1	14	FMNH 213648, 213643, 213641, 213644, 213633, 213635, 213640, 213636, 213638, 213639, 213642, 213646, 213647, 213649
2	1	FMNH 213645
3	3	JB 0417-8, 0417-4, 0417-7
4	3	FMNH 194217, 194220, 194226
5	1	FMNH 194214
6	3	FMNH 188575, 188576, 188577
7	1	FMNH 194023
8	1	FMNH 192819
9	8	FMNH 187834, 187835, 187931, 184681, 187822, 187816, 187836, 187837
10	1	FMNH 187820
11	6	FMNH 187838, 184651, 184652,184653, 184654, 184655
12	19	FMNH 185260, 185285, 185233, 185235, 185236, 185265, 185287, 185288, 185318, 185314, 185231, 185316, 185262, 185229, 185230, 185283, 185284, 185286, 185315
13	1	FMNH,185317
14	1	FMNH 185232
15	1	FMNH 185234
16	6	FMNH 187797, 187799, 184801, 184803, 184804. 184805
17	1	FMNH 188088
18	10	FMNH 184678, 16902, 16903 UADBA 43912, 43913, 43914, 43915, 43916, 43917, 43918
19	5	FMNH 184677, 185319, 185263, 184681, 184682
20	1	FMNH 184680
21	5	FMNH 188089, 188090, 185320, 185321, 185322
22	1	FMNH 188091
23	4	FMNH 184264, 184263,184259, 184231
24	2	FMNH 184238, 184237
25	23	FMNH 187750, 187751, 187752, 187753, 187754, 187755, 187756, 188495, 188497, 188498, 188499, 188500, 188640, 188643, 188644, 184510, 188113, 188115, 188116, 188117, 188142, 188143, 188144
26	1	FMNH 184239
27	1	FMNH 184240
28	15	FMNH 188496, 185228, 184922, 184923, 184955, 185028, 184509, 184512, 184513, 184491, 184492, 184493, 184494, 184495, 184496
29	2	FMNH 188641, 188642
30	4	FMNH 184604, 184606, 184607, 184608
31	1	FMNH184605
32	3	FMNH 184896, 184897, 184898
33	4	FMNH 184899, 184900, 184901, 184915
34	1	FMNH 184902
35	1	FMNH 184916
36	1	FMNH 184917
37	2	FMNH184919, 184920
38	9	FMNH 184924, 184954, 194021, 194019, 194026, 194020, 194018, 194028, 194022
39	5	FMNH 184925, 184926, 184950, 184951, 184953

40	1	FMNH 184952
41	1	FMNH 184956
42	3	FMNH 184957, 184958, 184959
43	1	FMNH 184973
44	1	FMNH 184974
45	11	FMNH 184975, 184977, 184976, 184978, 184979, 185020, 185021, 185022, 185027, 185029, 185030
46	2	FMNH 192886, 192889
47	1	FMNH 194215
48	3	FMNH194218, 194221, 194227
49	1	FMNH 192891
50	2	FMNH 185260, 185286
51	1	FMNH 185315
52	1	FMNH 187798
53	1	FMNH 187836
54	1	FMNH 188092
55	1	NMP PB -3626
56	1	NMP PB -3685
57	1	NMP PB -3154
58	1	NMP PB -3606
59	1	NMP PB -3619
60	2	FMNH 205318, 205319
61	1	FMNH 188087
62	1	FMNH 188093
63	1	FMNH 187807
64	2	FMNH 185259, 185282
65	1	FMNH 185314
66	1	FMNH 187806
67	29	UWWW1 - CP4,CP3,CP1, CP5,CP6,URPV1 - CP5, CP1, CP3, CP2, CP4, URPV2 - CP8, CP7,CP6 , QB1, QB9, QB10, QB3, QB11, QB5 QB6, QB15, QB8, PNT1, PNT2,DM 7380, 7905, 7907,7910,8348
68	12	PH4, PH5, PH6,PH7,PH8, PH1, PH9, PH2, PH11, PH3,DM 7373, 7374
69	16	EH5, EH13, EH6, EH14, EH7, EH15, EH8, EH16, EH9, EH17, EH2, EH10, EH3, EH11, EH4, EH12
70	4	QB14, QB4, QB7, QB16
71	1	QB13
72	3	CH1, QB12, D43
73	1	PT 2011-2
74	1	DM 7363
75	2	DM 7367, 7372
76	11	DM 7368, 7378, 7382, 7384, 7369, 7370, 7371, 7387, 7381, 7385, D39
77	1	DM 8036
78	2	DM 7851, D40
79	2	DM 7377, 7386
80	1	DM 7379
81	1	DM 7383
82	1	DM 8030
83	1	DM 8042
84	34	FMNH 194216, 194221, 194225, 194226, 194211, 194213, 194212, 194217, 194340, 194219,194220, 194222, 194224, 194223, 194215, 194327, 194324, 194191, 194256, 194258, 194257, 194486, 194450,

		194185, 194449, 194358, 194360, 194483, 194357, 194361, 194362, 194478, 194451, 194363
85	16	FMNH 194333, 194331, 194330, 194334, 194342, 194339, 194335, 194337, 194329, 194446, 194445, 194326, 194323, 194341, 194448, 194447
86	3	FMNH 194332, 194328, 194325
87	1	FMNH 194209
88	6	FMNH 194202, 194201, 194208, 194200, 194207, 194206
89	2	FMNH 194344, 194351
90	6	FMNH 194194, 194254, 194249, 194188, 194195, 194184
91	11	FMNH 194193, 194192, 194248, 194255, 194189, 194252, 194250, 194251, 194187, 194186, 194183
92	1	FMNH 194346
93	2	FMNH 194190, 194199
94	14	FMNH 194197, 194468, 194470, 194476, 194472, 194537, 194469, 194477, 194475, 194472, 194537, 194469, 194477, 194476
95	1	FMNH 194196
96	2	FMNH 194035, 194036
97	5	FMNH 194024, 194475. 194029, 194365, 194352
98	2	FMNH 194356, 194364
99	2	FMNH 194375, 194366
100	1	FMNH 194473
101	6	FMNH 194481, 194482, 194487, 194473, 194532, 194533
102	4	FMNH 194496, 194490, 194491, 194492
103	1	FMNH 194495
104	1	FMNH 194493
105	1	FMNH 194485

SUMMARY AND SYNTHESIS

The focus of this thesis was to study the systematics and evolution of the Afro-Malagasy genus *Chaerephon* (family: Molossidae) using a multifaceted approach that encompassed both molecular and ecological perspectives. The molecular analyses, based on DNA sequence and microsatellite data, included the use of both mitochondrial and nuclear markers, thus encompassing different modes of inheritance and rates of evolution to provide insight into the systematics and population structure of *Chaerephon* taxa (Avise 2004; Flanders *et al*. 2009). This was a significant step in resolving the systematics within the *C. pumilus* complex distributed across Africa, the Arabian Peninsula and the western Indian Ocean islands.

The primary aims of this study were to examine:

(1) The phylogenetics and phylogeography of a *C. leucogaster*, a previously unstudied member of the *C. pumilus* species complex from Madagascar and the western Indian Ocean islands.

(2) Phylogenetics, population genetics and aspects of the ecology of *C. pumilus* sensu lato (s.l.) from south eastern Africa.

(3) The evolutionary history and taxonomy of the *Chaerephon pumilus* species complex distributed across Africa, the Arabian Peninsula and the western Indian Ocean islands.

Working at the subspecific level, Chapter 1 was a pilot study undertaken that examined the phylogenetic and phylogeographic position of *C. leucogaster* from Madagascar based on the mitochondrial cytochrome *b* and control regions. Cytochrome *b* genetic distances among individuals tended to be low (max 0.35%). In contrast, those between *C. leucogaster* and another Malagasy *Chaerephon* species, *C. atsinanana* (formerly referred to as *C. pumilus*), ranged from 1.77% to 2.62%. Mitochondrial D-loop data for the Malagasy samples of *C. leucogaster* revealed significant but shallow phylogeographic structuring into three latitudinal groups (15-17˚S, 17-22˚S, 22-23˚S) showing exclusive haplotypes which correlated with regions of suitable habitat defined by Ecological Niche modelling. This was supported in a combined morphological and genetic study by Ratrimomanarivo *et al*. (2009a). This led to further determine the position of *C. leucogaster* relative to *C. pumilus* from south eastern Africa.

A study done in a similar context was carried out by Taylor *et al*. (2009), focusing on *C. pumilus* sensu lato (s.l.) from south eastern Africa and Swaziland and *C. leucogaster* from Madagascar. Analyses of the mitochondrial cytochrome *b* and control regions revealed genetically distinct lineages (at least four clades from the greater Durban area (South Africa)) separated by inter-clade cytochrome *b* genetic distances of 0.6 - 0.9%. Although *C. leucogaster* formed a paraphyletic clade, it was defined as a separate species based on morphological and genetic data. These authors further hypothesised that the southern African *C. pumilus* clades may represent cryptic species with distinct echolocation characteristics.

Therefore, Chapter 2 was aimed at determining if echolocation was a driving force in the existence of these genetically distinct lineages. In re-examining the phylogeny of Taylor *et al*. (2009) based on a larger sample set with a wider geographic range,we tested the hypothesis that the sympatric lineages found in south eastern Africa represented cryptic 'sono-species'. The previously reported phylogenetic structure by Taylor *et al*. (2009) was confirmed and an additional strongly supported control region clade was identified. However, Discriminant Function Analysis (DFA) based on four echolocation parameters (namely: peak frequency, bandwidth, duration and Fmin) could not discriminate between the clades. The hypothesised existence of cryptic species with distinct echolocation characteristics was not supported. Indices of diversity and neutrality combined with a ragged multimodal distribution were inconsistent with demographic expansion of a single *C. pumilus* s.l. south eastern African population.

This led to the comparative analyses of population genetic structure of *C. pumilus* s.l. from South Africa using microsatellite data (Chapter 3). Approaches that employ the use of multiple loci in the nuclear genome (such as microsatellites) provide a comprehensive description into the levels of gene flow thus highlighting the subdivision in genetic variation within a population as in the case of *C. pumilus* s.l. (Bergl and Vigilant 2007). We found there was strong mitochondrial genetic structure with 90% of the molecular variance occurring among the phylogenetically-defined groups with significant Fst values in *C. pumilus* s.l. However, nuclear variance accounted for 3% among populations. Pairwise Fst values among populations were low and not significant.

A study by Lamb *et al*. (2012) examined the different levels of population genetic and phylogeographic structuring between *C. atsinanana* and *C. leucogaster*. The Fst (0.994) of *C. atsinanana* showed this population to be highly structured whereas *C. leucogaster* showed lower levels of structure with an Fst of 0.792. The level of structuring in *C. atsinanana* was hypothesised to be due to female philopatry. The Fst (0.897) of *C. pumilus* sensu lato populations falls between those of *C. atsinanana* and *C. leucogaster*. The *C. pumilus* s.l. groups appear to comprise a single interbreeding population. The high levels of mitochondrial genetic structure in absence of significant nuclear structure are consistent with social isolating mechanisms such as female philopatry and may also reflect introgression of mitochondrial genes due to past hybridisation events.

However, the species delimitations of members of the *Chaerephon pumilus* species complex still remained largely unresolved. Analyses of the cytochrome *b* gene was the first step that led to the designation of a clade comprising a museum specimen from Massawa, Eritrea, as well as specimens from neighbouring Yemen, as the nominate *C. pumilus* (Goodman *et al*. 2010) henceforth referred to as *C. pumilus* sensu stricto (s.s.). This enabled other Afro-Malagasy species to be defined relative to *C. pumilus* s.s. *C. leucogaster* occurs on western Madagascar, Pemba, Zanzibar and Comoros. *C. pusillus*was elevated to full species rank and applied initially to animals from Aldabra (Goodman and Ratrimomanarivo 2007), and Goodman *et al*. (2010) assigned animals occurring on the Comoros Archipelago, which were formerly considered *C. pumilus*, to *C. pusillus*. This study also led to the naming of a new species, *C. atsinanana*, from eastern Madagascar. Animals from Tanzania, designated *C. pumilus* s.l., were also identified as members of the *C. pumilus* species complex.

In order to further resolve the evolutionary history and taxonomy of the *C. pumilus* species complex I used a broader geographical and taxonomic sample than was used in Goodman *et al*. (2010) that included the syntypes of *C. limbatus*, holotypes of *C. elphicki* and *langi* and the topotype of *C. naivashae*, all considered junior synonyms of *C. pumilus* (Chapter 4). Analyses based on cytochrome *b* sequence data indicate that these are not distinct from *C. pumilus* s.l. Forms of *C. pumilus* s.l. from south eastern Africa are distinct from the nominate form (*C. pumilus* s.s. from Massawa, Eritrea) and form part of *C. pumilus* species complex comprising *C. atsinanana*, *C. leucogaster*, *C. pusillus* and *C. pumilus* s.l. (Tanzania). *C. pumilus* s.s. (Eritrea and Yemen) diverged from other members of the *C. pumilus* complex approximately 6.24 million years ago (MYA). *C. atsinanana* diverged approximately 5.01 MYA from a well supported but unresolved clade comprising subclades which appear to have diverged between 1.07 and 2.39 MYA: *C. pusillus*, *C. leucogaster*, *C. pumilus* s.l. (south eastern Africa) and *C. pumilus* s.l. (Tanzania). There was evidence of introgression of *C. pusillus* haplotypes into *C. leucogaster* and of *C. leucogaster* haplotypes into *C. pumilus* from south eastern Africa, postulated to be the result of past hybridization within clades forming part of this species complex. The *C. pumilus* species complex was found to have several of the characteristics of a ring species: It occurs on the African mainland and western Indian Ocean islands including Madagascar, ringing a potential barrier to gene flow, the Mozambique channel, and the terminal forms, *C. leucogaster* and *C. pusillus*, occur in sympatry on the Comoros. However, although there is evidence of isolation by distance around the ring, there is also high level of structuring and limited gene flow, which are inconsistent with a ring species.

FUTURE RESEARCH

Our results show strong differentiation of mtDNA in populations of *C. pumilus* from south eastern Africa, consistent with behavioural explanations such as female philopatry. A further hypothesis, yet to be tested, is that the genetic distinctiveness of these mitochondrial clades of *C.*

pumilus s.l. is maintained by their use of different social calling systems. Further studies on social systems could assist in better understanding the dynamics between the mitochondrially-defined populations of *C. pumilus* s.l. from south eastern Africa.

We showed even stronger mitochondrially based population subdivision in *C. atsinanana* (Lamb *et al*. 2012), whereas *C. leucogaster* shows lower, but still significant levels of population structuring (Ratrimomanarivo *et al*. 2009a). In contrast, other molossid species in Africa and the western Indian Ocean islands show lower levels of structure (eg. *Mops leucostigma*, Ratrimomanarivo *et al*. 2008) or panmixia (eg. *Mormopterus jugularis*, Ratrimomanarivo *et al*. 2009b). While there is some indication that levels of structure are inversely related to the size and therefore flight ability of these bats (Taylor *et al*. 2012), it would be interesting to determine what other factors might be driving population structuring. There is very limited information on the behaviour of molossid bats of the Afro-Malagasy region. Studies on mate choice may be informative in this regard.

Our current knowledge of the phylogenetics of members of the *C. pumilus* species complex is based primarily on the maternally-inherited mitochondrial cytochrome *b* and control region markers. It is vital that this information is complemented by studies based on nuclear DNA, which is biparentally-inhertied and therefore also reflects the male contribution to gene flow. Information from both mitochondrial and nuclear marker systems allows cases of introgresssion to be identified, and, in combination with behavioural information, allows us to distinguish between behavioural causes of genetic structuring, such as female vs male philopatry. While our mitochondrial studies indicate that *C. pumilus* s.l. from south eastern Africa and Tanzania, both members of the *C. pumilus* species complex, might warrant description as new species of *Chaerephon*, such a description would need to be based on both mitochondrial and nuclear markers. A search for suitably variable nuclear markers may be a difficult one, as our preliminary studies indicate that nuclear intron markers tend to be less informative than cytochrome *b*. Further, longer length sequence data needs to be obtained from type material or topotypic material to further resolve the taxonomy of these clades. Studies based on nuclear microsatellites would be useful to analyse the fine-scale distribution of genetic variation, and gene flow amongst individuals within the *C. pumilus* species complex located across mainland Africa. Ecological studies (i.e. echolocation and social calls, roosting behaviour, mate choice) will further contribute to the understanding of the dynamics within the system. This in turn will lead to a better understanding of the diversity of *Chaerephon* complex across Africa and the western Indian Ocean islands.

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APPENDICES

Appendix1

RATRIMOMANARIVO, F.H., S.M. GOODMAN, W.T. STANLEY, T. NAIDOO, P.J. TAYLOR, and J. LAMB. 2009. Geographic and phylogeographic variation in *Chaerephon leucogaster* (Chiroptera: Molossidae) of Madagascar and the western Indian Ocean islands of Mayotte and Pemba. Acta Chiropterologica, 11: 25-52.

Appendix 2

TAYLOR, P.J., J. LAMB, D. REDDY, T. NAIDOO, F. RATRIMOMANARIVO, E. RICHARDSON, and S.M. GOODMAN. 2009. Cryptic lineages of little free-tailed bats, *Chaerephon pumilus* (Chiroptera: Molossidae) from southern Africa and the western Indian Ocean islands. African Zoology, 44: 55-70.

Appendix 3

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Appendix 4

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Appendix 5

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Appendix 6

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Geographic and Phylogeographic Variation in *Chaerephon leucogaster* (Chiroptera: Molossidae) of Madagascar and the Western Indian Ocean Islands of Mayotte and Pemba

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Geographic and phylogeographic variation in *Chaerephon leucogaster* (Chiroptera: Molossidae) of Madagascar and the western Indian Ocean islands of Mayotte and Pemba

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We examine patterns of morphological and genetic variation in *Chaerephon leucogaster* (family Molossidae) on Madagascar, Mayotte in the Comoros Archipelago, and the offshore Tanzanian island of Pemba. Five external, 10 cranial, and eight dental measurements of animals from different Malagasy populations (grouped according to bioclimatic regions) show differences in the degree of sexual dimorphism and size variation. Further, the population on Mayotte is largely identical in size to those from western Madagascar, and animals from Pemba are notably larger than those from Madagascar and Mayotte. Cytochrome *b* genetic distances across samples from these islands were low (maximum 0.0035) and animals from Pemba and Mayotte shared cytochrome *b* haplotypes with Malagasy bats. D-loop data showed some concordance between haplotype distribution, geographical position (latitude and island), and the bioclimatic zones. Animals from Pemba and Mayotte formed a unique D-loop haplotype, which was a minimum of six mutational steps different from Malagasy haplotypes. Within Madagascar, certain haplotypes were exclusive to the north (13ºS latitude band) and arid southwest (22º and 23ºS latitudes) regions. In general, there was no clear concordance between variation in haplotype distribution, latitude, altitude or gender. Where concordance occurred, the genetic distances involved were not sufficiently high to warrant the definition of new taxonomic units. Hence, based on current genetic information, patterns of morphological variation of the Madagascar populations and differences between Pemba and Mayotte/Madagascar are best explained as inter-population variation and may be adaptive, associated with different climatic regimes and associated ecological variables.

Key words: *Chaerephon leucogaster*, geographic variation, genetic variation, Madagascar, Mayotte, Pemba

INTRODUCTION

The islands of the western Indian Ocean are excellent natural laboratories to examine the history of over-water dispersal and the colonization of bats, because of their varying size and position with respect to continental Africa and Asia, and very different geological ages. Given that Madagascar has been separated from the African mainland for 170–155 million years (de Wit, 2003), which predates the origin of Chiroptera estimated to be 71–58 Mya (Teeling *et al*., 2005), there is little doubt that the modern bat fauna colonized this island over water. In contrast, the land masses making up the Comoro Archipelago are in situ volcanic islands that range in age from 0.5 to 10–15 million years (Nougier *et al*., 1986); given the lack of land connections since their formation, over water colonization is the only tangible explanation for the presence of bats on these islands. There is some debate as to the history of isolation of Pemba from mainland Africa, and estimates range from as early as the Miocene (roughly 10 million years BP) to as recently as the Pleistocene (1 million years BP). Currently, a deep-water channel of up to 800 m in depth separates the island from the mainland (Pakenham, 1979), a separation thought to be tectonic in origin (Stockley, 1942). Most species of Molossidae bats occurring

on Madagascar and other islands in the western Indian Ocean are presumed to have African origins and to have dispersed to Madagascar across the Mozambique Channel (Ratrimomanarivo *et al*., 2007, 2008; Lamb *et al*., 2008).

Amongst African molossids, the taxon with one of the broadest distributions is *Chaerephon leucogaster* A. Grandidier, 1869. The taxonomic history of this species is rather complex and a number of authors have placed it as a geographical form or synonym of *C. pumilus* (Cretzschmar, 1830) (e.g., Hayman and Hill, 1971; Koopman, 1994). Simmons (2005) considers *C. leucogaster* to be a separate species from *C. pumilus*. Recent phylogenetic work indicates that *C. leucogaster* is nested within a paraphyletic species complex of animals currently assigned to *C. pumilus* (Taylor *et al*., 2009). Numerous geographical forms of *C. leucogaster* have been named from sub-Saharan Africa and this species is reported along a broad band from the northwest (Mali), south to the west (Ghana), through portions of the Congo Basin (Democratic Republic of Congo) to the central east (Ethiopia) (Dollman, 1908; Allen, 1917; Hatt, 1928; Simmons, 2005). The type specimen of the nominate form was collected on the western coast of Madagascar (Grandidier, 1869). The assertion of Hutson *et al*. (2001) that this species is endemic to Madagascar is incorrect given the various geographical forms recognized from the African continent.

Recent morphological and phylogeographical analyses of other molossid bat species or sister taxa with distributions shared between Madagascar and Africa have revealed two distinct patterns. In the case of *Mops midas* (Sundevall, 1843), for example, the African and Malagasy populations show no notable morphological or genetic differences, and this is best explained by a recent colonization of Madagascar or regular exchanges between these disjunct areas (Ratrimomanarivo *et al*., 2007). The second pattern, as found in the species group composed of *Mops condylurus* (A. Smith, 1833) and *Mops leucostigma* G. M. Allen, 1918, is one where the Malagasy population is derived from African stock and has been sufficiently isolated for speciation to have taken place (Ratrimomanarivo *et al*., 2008). Further, distinct morphological differences in eastern and western populations of *M. leucostigma* on Madagascar are not reflected in associated genetic analyses of the same specimen material; these animals show remarkably little haplotypic and genetic diversity, while displaying considerable phenotypic plasticity. In light of these previous regional studies on molossids and the fact that considerable new material of *C. leucogaster* from Madagascar and other islands in the western Indian Ocean has recently been collected, we investigate levels of morphological and genetic divergence between populations of this species across the region to examine their evolutionary history with regards to colonization and dispersal. Insufficient material is currently available to expand this study to *C. leucogaster* from the African mainland.

Materials and Methods

Specimens Used in the Current Study and Geographic Classification

During the course of the past decade, numerous sites on Madagascar and other islands in the western Indian Ocean have been inventoried for bats, and new specimens of *C. leucogaster* have been obtained (Fig. 1, Appendix I). This new material, generally with associated tissue samples, provides the means to study patterns of geographic variation in this species on western Indian Ocean islands. The principal project on Madagascar resulting in new material has been a study by FHR of synanthropic bat species along transects across the island, including two surveys in a largely east-west direction across a notable elevational and longitudinal gradient: (i) from Irondro (21°24'S, 47°59'E) to Toliara (23°23'S, 43°43'E) along Route Nationals (RN) 25 and 7, (ii) from Brickaville (18°49'S, 49°04'E) to Mahajanga (15°43'S, 46°19'E) along RN 2 and RN 4. Two transects were conducted in a north-south direction across a negligible altitudinal gradient and notable latitudinal gradient: (i) from Fénérive Est (17°23'S, 49°25'E) to Brickaville along RN 5, and (ii) from Irondro (21°24'S, 47°59'E) to Vangaindrano (23°21'S, 47°36'E) along RN 12. With the exception of one record in the east, all of the individuals of *C. leucogaster* found during these transects were limited to western lowland localities (sea-level to 230 m). Further, specimens were obtained by SMG in the region between Kirindy Mite (20°53'2''S, 44°04'8''E) and Toliara, across the elevational range of 10 to 90 m above sea level. Specimens have also been examined from other localities on the island (Fig. 1, Appendix I). Finally, during surveys of Mayotte, part of the Comoros Archipelago (by SMG), and Pemba, an offshore Tanzanian island (by WTS), other specimens referable to *C. leucogaster* with associated tissues were collected.

These new specimens are deposited in the Field Museum of Natural History (FMNH), Chicago and a portion of the Madagascar material in the Département de Biologie Animale, Université d'Antananarivo, Antananarivo. Further material of *C. leucogaster* collected on Madagascar has been examined in the Museum of Comparative Zoology (MCZ), Harvard University; The Natural History Museum, London [formerly The British Museum of Natural History (BMNH)]; and the Muséum National d'Histoire Naturelle (MNHN), Paris.

In the context of the current study, specimens were grouped into seven different Operational Taxonomic Units (OTUs). The basis of the OTU classification for Madagascar conforms to Cornet's (1974) bioclimatic classification of the island and in each of these zones the natural forest cover is different

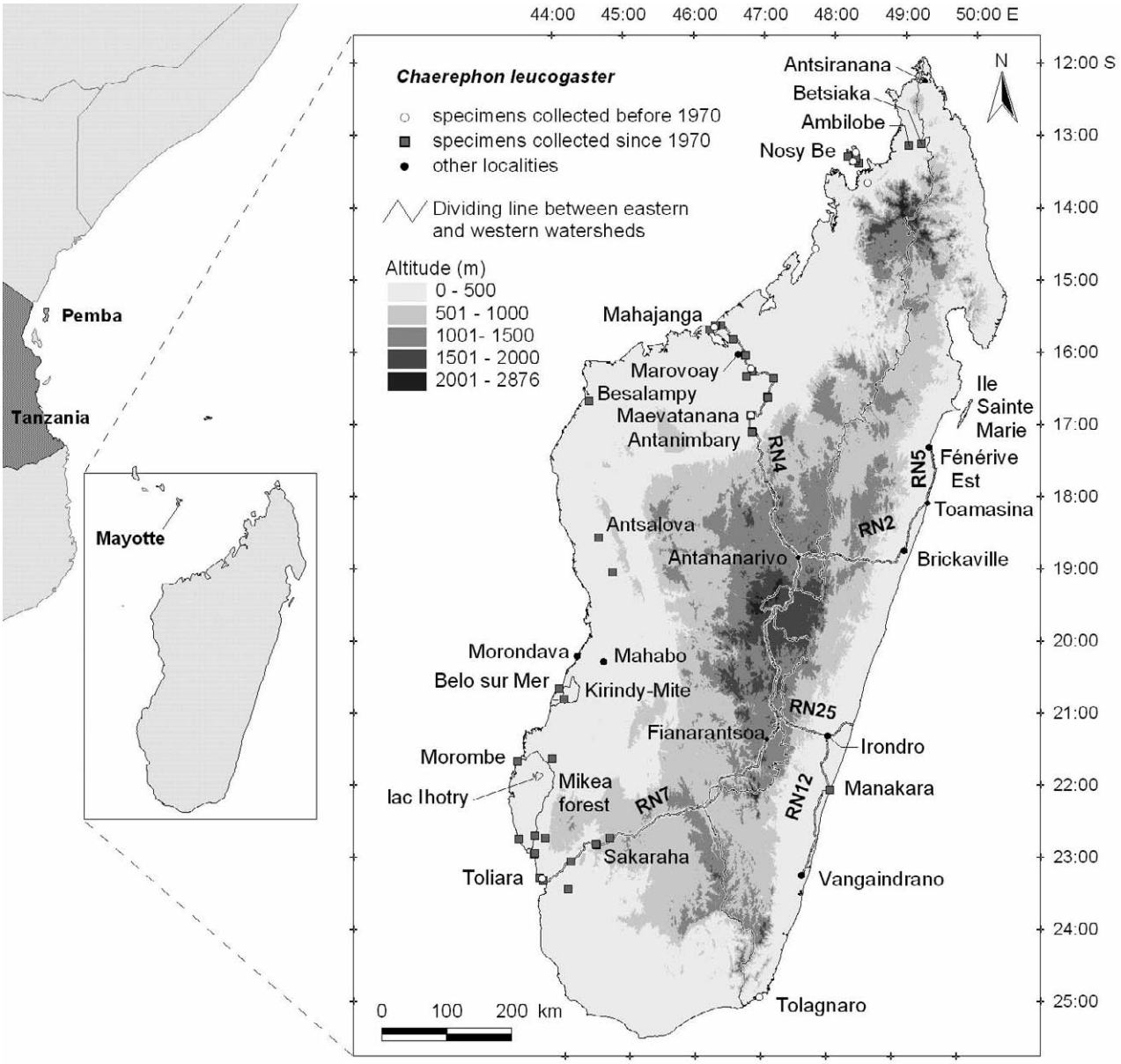

FIG. 1. Map to the left of eastern Africa including Pemba Island of Tanzania, Mayotte Island in the Comoros Archipelago, and Madagascar. The larger map to the right of Madagascar illustrates known collection localities of specimens of *C. leucogaster* obtained before 1970, including those mentioned in Peterson *et al*. (1995), and those after 1970. The different east-west and north-south transects are indicated along the different Routes Nationales (RN)

(see Gautier and Goodman, 2008) [text in bold are the descriptive terms used for each OTU hereafter]: OTU 1 (*n* = 28, 12♂♂, 16♀♀) — sub-arid region of the **extreme south** (south of the Mikea Forest to Toliara and inland to Sakaraha), characterized by a xerophytic vegetation; OTU 2 (*n* = 13, 1♂, 12♀♀) — sub-arid zone of the **southwest** (Kirindy-Mite north to Belo sur Mer), with a zone of transition between xerophytic and dry deciduous forest; OTU 3 (*n* = 129, 50♂♂, 79♀♀) — coastal and slightly inland **central west**ern zone (from Mahajanga to Ambilobe and Betsiaka) with dry deciduous forests; OTU 4 (*n* = 17, 9♂♂, 8♀♀) — from the **northwest** area (including the near shore islands of Nosy Be and the neighboring island of Nosy Komba), composed of subhumid forests; and OTU 5 (*n* = 1♀) — **eastern** Madagascar (Manakara), with lowland humid forest. Extralimital specimens were placed into two separate classes: OTU 6 (*n* = 12, 10♂♂ 2♀♀) — **Pemba;** and OTU 7 (*n* = 2, 1♂, 1♀) — **Mayotte**.

Climate

There is considerable climatic variation among the regions from which specimens of *C. leucogaster* included in this study originated. Most of the western side of Madagascar is in a rain shadow of the eastern north-south aligned mountains. Portions of the east receive between 2 m and 6 m of rainfall per year, and in notable contrast, the extreme southwest rarely receives more than 500 mm. Along the western lowland portion of the

island there is increased annual rainfall following a cline from the extreme southwest to the northwest, with 554 mm at Belo sur Mer, 780 mm at Morondava, 1,503 mm at Mahajanga, 2,193 mm at Nosy Be, and 1,892 mm at Ambilobe (Donque, 1975; Chaperon *et al*., 1993). On Mayotte, in the zone the specimens were collected, annual rainfall approaches 2,000 mm (Louette, 1999), and on Pemba, meteorological records indicate approximately 1,850 mm precipitation per year (Pakenham, 1979).

Morphological Study

From these specimens, five external measurements were taken by FHR, SMG, and WTS using a millimeter ruler, with a precision to 0.5 mm, and include: total length, tail length, hind foot length (excluding claws, with the exception of specimens from Pemba), ear length, and forearm length. Body mass in grams was also recorded with a spring balance at an accuracy of 0.5 g. Herein we have only presented external measurements taken by these three collectors. The mass measurements of females in an advanced state of pregnancy were not used in the descriptive statistics or various analyses. Only adults have been used in the morphological analyses, defined as individuals for whom the third molar has completely erupted and the basi-occipital-basisphenoid suture is ossified.

A series of measurements, derived largely from Freeman (1981), were made by FHR using digital calipers, accurate to the nearest 0.1 mm and include Cranial and Mandibular Measurements — GSL: greatest skull length, from posterior-most part of occipital condyle to anterior-most point of premaxillary bone; CON INCI: condyloincisive length, from occipital condyle to anterior-most point of incisors; ZYGO BR: greatest zygomatic breadth, width taken across zygomatic arches at the widest point; POST ORB: postorbital width, dorsal width at most constricted part of skull; MASTOID: breadth at mastoids, greatest breadth across skull at mastoid processes; PALATE: palatal length, from posterior border of hard palate (not including post-palatal projection) to anterior edge of premaxillary bone; LACR WID: lacrimal width, width across rostrum dorsally at lacrimal protuberances; CON CANI: condylocanine length, from midpoint of mandibular condyle to anterior border of alveolus of lower canine; MOM1 COR: moment arm of temporal, length from middle of condyle to tip of coronoid process; MOM2 ANG: moment arm of masseter, length from middle of condyle to tip of angular process; and Dental Measurements — C^1–C^1: anterior palatal width, taken across the outer alveolar borders of the upper canines; M^3–M^3: posterior palatal width, taken across the outer alveolar borders of the third upper molars; UP MOL R: upper molariform row, length from PM^2 to M^3 (alveolar); MTR: maxillary toothrow, length from anterior alveolar border of canine to posterior alveolar border of M^3; UP CANIN: height upper canine, greatest length from point immediately dorsal to cingulum to end of tooth; M^3 WIDTH: width of M^3, greatest lateral-medial width of tooth; LOWER TR: lower toothrow, length from posterior alveolar border of m_3 to alveolar border of c_1; and LO CANIN: height lower canine, greatest length from point immediately ventral to cingulum to end of tooth.

Statistical Analyses

In order to study patterns of latitudinal and elevational gradients in *C. leucogaster*, the statistical procedures within the program STATISTICA AX (version 7.0) were utilized for all univariate and multivariate analyses. Firstly, *t*-test comparisons were employed to assess patterns of sexual dimorphism within and among OTUs. Subsequently, Analysis of Variance (ANOVA) was employed, using post-hoc Scheffé tests, to assess morphological differences among the OTUs. In the results section, for Scheffé tests only probability values are presented, whereas for one-way ANOVA the various statistical parameters are provided. Principal Component Analysis (PCA), with non-log-transformed data and a correlation matrix, was used to further differentiate populations (OTUs) in three-dimensional space. Finally, regression analyses were conducted that utilized the principal component scores from the different PCAs for each of the three different types of measurements (external, cranial, and dental) compared to different geographic variables (altitude and latitude). On the basis of an ecomorphological study of Malagasy bats (Ranivo and Goodman, 2007), cranial and dental variables do not always show clear patterns of covariance and herein these two variable types are presented separately.

Grandidier's Type Specimen of Chaerephon leucogaster

In Grandidier's (1869) description of *Nyctinomus leucogaster*, which is the nominate form of this taxon that was subsequently transferred to the genus *Chaerephon* Dobson, 1874, the type specimen was from the Menabe Region of western Madagascar; the lowland area centered around Morondava (Fig. 1). It was collected in 1869 at a site given as 'Mahab', which is most likely Mahabo (20°23'S, 44°40'E, about 50 m above sea level), 47 km east of Morondava. A footnote to the published species description (p. 337), 'Cette note est datée de Ménabé, 15 mai 1869', would indicate that the ms. had been sent from the field and the type specimen had not yet been deposited in a museum. We have extensively examined, to no avail, the collections of the MCZ and MNHN for this specific specimen, as both of these institutions hold Grandidier types of Malagasy bats (Rode, 1941; Helgen and McFadden, 2001).

The only specimen possibly fitting the details of the Grandidier type is MCZ 45094, which is in poor condition and the original specimen label bears the following information: 'Cote Ouest' [= west coast], '*Nyctinomus pumelus* [sic]', 'collection Grandidier', and '1901'. Based on numerous characters, including aspects of external, cranial, and dental measurements as well as pelage and soft part coloration, this animal is referable to *C. leucogaster*. However, the collection details are too ambiguous to clearly conclude that this specimen can be considered the type of *C. leucogaster* described by Grandidier (1869) and, further, the skull is considerably damaged.

In order to have a point of reference on what is a morphological representative of *C. leucogaster* from Madagascar, we designate here a neotype (FMNH 176137), which was collected in the Menabe Region of western coastal Madagascar, specifically at Belo sur Mer (20°44.139'S, 44°00.266'E) at 10 m above sea level on 11 November 2002 by Vola Razakarivony. This site is approximately 80 km southwest of Mahabo. The animal is an adult male, the body was preserved in formalin and subsequently transferred to 70% ethanol, the skull has been extracted, cleaned, and is in excellent condition. The designated neotype of *C. leucogaster* closely matches the diagnostic characters outlined in the original description of Grandidier (1869: 337), which include [our translation]: dorsal

pelage, throat, and chest dark brown; central portion of wing membranes white; lips, particularly upper, notably wrinkled and prominent; and the ears are large and attached at their base, as well as certain of his measurements, including total length of 80 mm; ear length of 14 mm, and tail length of 30 mm.

Molecular Study

Our objectives with the genetic analyses were to evaluate (i) the distinctiveness of *C. leucogaster* from other regional members of this genus; (ii) the phylogeographic relationships among different populations of this species on Madagascar; and (iii) the distinctiveness of recently discovered populations of *C. leucogaster* on Mayotte and Pemba. Genetic variation in *Chaerephon* spp. was investigated using mitochondrial cytochrome *b* ($n = 39$) and D-loop ($n = 71$) sequencing. Samples of *C. leucogaster* were obtained from numerous localities on Madagascar, as well as Mayotte and Pemba (Appendix I). Other samples, used as outgroups, included *Mops leucostigma*, *M. midas*, and *C. pumilus* from Madagascar (Appendix I).

Total genomic DNA was extracted from tissues preserved in 80% ethanol or EDTA using a DNeasy® DNA isolation kit (Qiagen, Cape Town) and stored in the elution buffer provided. The cytochrome *b* gene was amplified as two overlapping double stranded fragments (Saiki *et al.*, 1988). The 5' fragment was amplified with primers L 14723 (5'-ACCAATGCA ATGAAAAATCATCGTT-3') and H 15553 (5'-TAGGCAAA TAGGAAATATCATTCTGGT-3'), whilst the 3' fragment was amplified using L15146 (5'-CATGAGGACAAATATCATTCT GAG-3') and H15915 (5'-TCTCCATTTCTGGTTTACAA GAC-3') (Irwin *et al.*, 1991).

Polymerase Chain Reaction (PCR)-amplifications were performed in 25 µl reaction volumes each consisting of 9 µl genomic DNA solution (containing 30 ng DNA), 0.8 µl sterile water, 2.5 µl 10 x reaction buffer (Super-Therm), 4 µl $MgCl_2$ (25 mM) (Super-Therm), 0.5 µl dNTP mix (10 mM) (Roche Diagnostics), 0.2 µl Taq polymerase (5 U/µl) (Super-Therm) and 4 µl each of forward and reverse primer (6 µM). Thermal cycling parameters consisted of an initial denaturation step at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 60 s, annealing at 50°C for 90 s, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min.

The D-loop region was PCR-amplified as a single fragment using primers P (5'-TCCTACCATCAGCACCCAAAGC-3') and F (5'-GTTGCTGGTTTCACGGAGGTAG-3') (Wilkinson and Chapman, 1991). Where samples failed to amplify using this primer set, primer set P and E (5'-CCTGAAGTAGGAA CCAGATG-3') were used, as F is nested within E (Wilkinson and Chapman, 1991). PCR-amplifications were performed in 25 µl reaction volumes, in the manner described above. Thermal cycling parameters consisted of initial denaturation at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 90 s, extension 72°C for 2 minutes, and a final extension step at 72°C for 7 min.

For both cytochrome *b* and D-loop amplifications, target fragments were purified from excised gel bands using a QIAquick® Gel Extraction Kit (Qiagen Inc.). Purified DNA fragments were sequenced in the forward and reverse directions using the primers used for the initial amplifications. Sequencing was performed by Inqaba Biotechnical Industries Pty. Ltd., South Africa.

Electropherograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia). Sequence alignments were generated with BioEdit (Version 5.0.9 for Windows 95/98/NT) and its accessory CLUSTAL W alignment application (Thompson *et al.*, 1994) and corrected manually by visual inspection for alignment errors. Sequences were trimmed to a common length of 863 bp for the cytochrome *b* gene and 338 bp for the D-loop.

Haplotype analysis of both the cytochrome *b* and D-loop datasets was carried out using the program DnaSP4.10 (Rozas *et al.*, 2003). Modeltest (Posada and Crandall, 1998) was used to select the model of nucleotide substitution which best fits each sequence dataset. The model selected for cytochrome *b* dataset was the HKY+I+G model, whilst the GTR model was selected for the D-loop dataset. These models were used as appropriate in further distance, neighbor-joining, and Bayesian analyses.

Bayesian analysis was implemented in Mr Bayes version 3.0. Four Markov chains were run for 15 million generations each, and the first 500 000 trees discarded as burn-in. The priors for the five active parameters were: 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 branch lengths — branch lengths are unconstrained: exponential (10.0).

PAUP 4.0b10 for Macintosh (Swofford, 2002) was used to estimate nucleotide divergence and create neighbor-joining and maximum parsimony trees. For parsimony analysis, we used the random additions sequence option ($n = 100$) 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 and Kishino, 1993; Hillis and Bull, 1993). Statistical parsimony haplotype networks were calculated for both the cytochrome *b* and D-loop using TCS v.1.21 (Clement *et al.*, 2000).

Inferences of significant phylogeographic concordance within the Malagasy samples, based on D-loop sequences, were created using Nested Clade Phylogeographic Analysis implemented with the automated method of Panchal (2007). This utilizes the programs TCS (Clement *et al.*, 2000) for estimating gene genealogies by creating haplotype networks using the criteria of statistical parsimony and GeoDis (Posada *et al.*, 2000) for the cladistic nested analysis of the geographical distribution of the genetic haplotypes in the network. An exact permutational contingency test is performed for any clade at each nesting level. A chi-square statistic is calculated from the contingency tables in which rows are genetic clades and columns are geographical locations. Clades containing significant chi-square values are interpreted using Templeton's inference key (version: 11 November 2005). Loops in the haplotype network were resolved as suggested by Panchal (2007) by following the steps of Mardulyn (2001) using the criteria suggested by Crandall and Templeton (1993).

Ecological Niche Model Using MaxEnt

There are many problems inherent in using presence-only data (such as museum records) for estimating the predicted distributions of species, and alternative models have been developed and compared. The MaxEnt (Maximum Entropy) method (Phillips *et al.*, 2006) that employs a general machine learning algorithm, has been shown to perform well with presence-only

data, and to perform generally better than alternative 'climatic envelope' models such as GARP and BIOCLIM (see Elith *et al*., 2006 for a recent discussion). Based on 49 georeferenced distribution records (precision of 0.001 degrees) (excluding isolated records from Pemba Island, but including the Mayotte records) we used MaxEnt to estimate the predicted distribution of *C. leucogaster*. We used 10 continuous environmental variables including altitude as well as nine bioclimatic variables (WORLDCLIM version 1.4: http://biogeo.berkeley.edu/worldclim/ — Hijmans *et al*., 2005) reflecting means, extremes, and seasonal variation of temperature and precipitation: Bio1 (mean annual temperature), Bio4 (temperature seasonality), Bio5 (maximum temperature of warmest month), Bio6 (minimum temperature of coldest month), Bio7 (annual range of temperature), Bio12 (annual precipitation), Bio13 (precipitation of wettest month), Bio14 (precipitation of driest month), and Bio15 (precipitation seasonality). The environmental data were set to a spatial grid resolution of 2.5 arc minutes. The MaxEnt model was run with all distribution records (100% training), the regularization multiplier was set to 1.0, maximum number of iterations was set to 1000, convergence threshold was set to 1×10^{-5}, 'auto-features' was selected and output format was set to logistic. 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 of sensitivity versus 1-specificity]. Since MaxEnt produces a continuous probability (ranging from 0 to 1.0), we transformed the continuous model output to a map representing probabilities. 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

Sexual Dimorphism

The only external phenotypic feature that varied between the sexes was the postaural crest, which is distinctly less developed in adult females than in adult males. Comparison of the range of external, cranial, and dental measurements (Tables 1, 2, and 3)

Table 1. External measurements (in mm) of adult *C. leucogaster* from Madagascar, Mayotte, and Pemba. Measurements are presented as $\bar{x} \pm$ SD, and minimum–maximum, followed by the number of specimens (in parentheses). Sample sizes are not presented for fewer than three specimens. For each OTU the sexes are separated and there was no significant difference between the sexes. The hindfoot measurements of animals from Pemba include the claw and the other samples without the claw. Neotype specimen is FMNH 176137

Sex	Total length	Tail length	Hindfoot length	Ear length	Forearm length	Body mass
Madagascar - extreme south (OTU 1)						
♂♂	88.8 ± 3.55	31.7 ± 1.49	5.8 ± 0.75	15.7 ± 1.01	36.2 ± 1.17	9.0 ± 1.16
	83–93 (*11*)	30–34 (*11*)	5–7 (*11*)	14–18 (*11*)	34–38 (*11*)	7–11 (*11*)
♀♀	89.6 ± 2.37	32.3 ± 1.35	5.9 ± 0.72	15.8 ± 0.83	36.4 ± 1.09	9.1 ± 1.93
	83–92 (*16*)	30–35 (*16*)	5–7 (*16*)	15–18 (*16*)	34–38 (*16*)	7–11 (*4*)
Madagascar - southwest (OTU 2)						
♂ neotype	90	32	7	17	38	9.2
♀♀	89.6 ± 2.43	30.3 ± 1.36	7.3 ± 0.45	17.2 ± 0.39	36.3 ± 1.30	8.1 ± 0.53
	86–95 (*12*)	28–33 (*12*)	7–8 (*12*)	17–18 (*12*)	33–38 (*12*)	7.3–9.0 (*12*)
Madagascar - central west (OTU 3)						
♂♂	86.4 ± 2.73	30.7 ± 1.95	5.3 ± 0.53	15.7 ± 0.75	35.2 ± 1.05	7.5 ± 0.73
	80–93 (*50*)	26–37 (*50*)	5–7 (*50*)	14–17 (*16*)	33–37 (*50*)	5.6–10.0 (*50*)
♀♀	86.4 ± 2.22	30.7 ± 1.97	5.2 ± 0.50	15.7 ± 0.68	35.2 ± 1.03	7.6 ± 0.55
	82–92 (*79*)	27–36 (*79*)	5–7 (*79*)	13–17 (*79*)	33–37 (*79*)	6.5–9.0 (*33*)
Madagascar - northwest (OTU 4)						
♂♂	87.9 ± 2.67	32.1 ± 2.09	5.6 ± 0.53	16.4 ± 1.24	35.3 ± 2.18	9.1 ± 1.26
	85–92 (*9*)	29–35 (*9*)	5–6 (*9*)	15–18 (*9*)	31–38 (*9*)	7.8–11.5 (*9*)
♀♀	89.5 ± 1.93	31.5 ± 1.07	5.8 ± 0.46	16.9 ± 1.25	35.5 ± 1.07	9.2 ± 0.60
	86–92 (*8*)	29–32 (*8*)	5.6 (*8*)	15–18 (*8*)	34–37 (*8*)	8.2–10.0 (*7*)
Madagascar - east (OTU 5)						
♀♀	85	32	5	15.5	35	7.5
Pemba (OTU 6)						
♂♂	91.5 ± 2.64	31.6 ± 2.27	7.9 ± 0.32	16.2 ± 0.42	35.9 ± 0.88	9.2 ± 0.89
	88–98 (*10*)	28–36 (*10*)	7–8 (*10*)	16–17 (*10*)	35–37 (*10*)	7.4–10.0 (*10*)
♀♀	90, 90	28, 31	8, 9	15, 16	35, 35	8.6, 10.0
Mayotte (OTU 7)						
♂♂	91	31	6	18	38	8.8
♀♀	88	33	6	17	37	8.1

TABLE 2. Cranial measurements (in mm) of adult *C. leucogaster* of Madagascar, Mayotte, and Pemba. Measurements are presented as $\bar{x} \pm$ SD, and minimum–maximum, followed by the number of specimens (in parentheses). For each OTU the sexes are separated. n.s. = not-significant. Neotype specimen is FMNH 176137

Sex	GSL	CON INCI	ZYGO BR	POST ORB	MASTOID	PALATE	LACR WID	CON CANI	MOM1 COR	MOM2 ANG
Madagascar - south (OTU 1)										
♂♂	16.0 ± 0.28	15.1 ± 0.39	10.0 ± 0.24	3.7 ± 0.11	9.4 ± 0.23	6.5 ± 0.16	5.8 ± 0.26	10.4 ± 0.21	3.1 ± 0.12	3.1 ± 0.16
	15.4–16.4 (*12*)	14.3–15.5 (*12*)	9.6–10.3 (*12*)	3.5–3.8 (*12*)	8.9–9.7 (*12*)	6.2–6.7 (*12*)	5.2–6.1 (*12*)	10.1–10.7 (*12*)	2.8–3.2 (*12*)	2.9–3.4 (*12*)
♀♀	15.8 ± 0.49	14.8 ± 0.54	9.8 ± 0.27	3.7 ± 0.12	9.2 ± 0.23	6.3 ± 0.27	5.6 ± 0.22	10.2 ± 0.38	3.0 ± 0.17	3.0 ± 0.12
	15–16.4 (*16*)	13.8–15.3 (*16*)	9.2–10.1 (*16*)	3.5–3.9 (*16*)	8.8–9.6 (*16*)	5.8–6.7 (*16*)	5.3–6.0 (*16*)	9.3–10.6 (*16*)	2.6–3.2 (*16*)	2.8–3.2 (*16*)
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Madagascar - southwest (OTU 2)										
♂ neotype	16.0	15.1	10.1	9.3	10.5	6.4	5.8	10.5	2.9	3.3
♀♀	15.6 ± 0.32	14.6 ± 0.37	9.8 ± 0.19	3.6 ± 0.14	9.2 ± 0.22	6.2 ± 0.21	5.7 ± 0.19	10.2 ± 0.26	3.0 ± 0.1	2.9 ± 0.17
	15.2–16.3 (*12*)	14.2–15.4 (*12*)	9.6–10.2 (*12*)	3.5–3.9 (*12*)	8.9–9.6 (*12*)	6–6.7 (*12*)	5.5–6.0 (*12*)	9.9–10.7 (*12*)	2.8–3.2 (*12*)	2.8–3.1 (*12*)
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Madagascar - central west (OTU 3)										
♂♂	15.4 ± 0.38	14.5 ± 0.35	9.6 ± 0.23	3.6 ± 0.13	9.1 ± 0.19	6.2 ± 0.21	5.6 ± 0.22	10.0 ± 0.31	2.9 ± 0.13	2.9 ± 0.15
	14.3–16.2 (*50*)	13.5–15.2 (*50*)	9.0–10.1 (*50*)	3.3–3.9 (*50*)	8.6–9.4 (*50*)	5.8–6.7 (*50*)	5.8–6.7 (*50*)	9.1–10.7 (*50*)	2.5–3.2 (*50*)	2.6–3.2 (*50*)
♀♀	15.2 ± 0.35	14.2 ± 0.32	9.5 ± 0.20	3.5 ± 0.14	9.0 ± 0.19	6.0 ± 0.19	5.4 ± 0.24	9.8 ± 0.27	2.9 ± 0.11	2.8 ± 0.13
	14.1–15.8 (*77*)	13.4–14.9 (*77*)	8.9–9.9 (*76*)	3.3–3.8 (*77*)	8.5–9.4 (*77*)	5.6–6.5 (*77*)	4.8–6.0 (*77*)	9.2–10.3 (*75*)	2.6–3.1 (*75*)	2.5–3.2 (*75*)
	t = -2.93	t = -4.39	t = -3.51	t = -2.49	t = -3.06	t = -4.90	t = -2.98	t = -3.71	t = -2.44	t = -3.03
	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.01$	$P < 0.001$	$P < 0.01$	$P < 0.001$	$P < 0.05$	$P < 0.01$
Madagascar - northwest (OTU 4)										
♂♂	16.0 ± 0.29	15.0 ± 0.28	10.1 ± 0.14	3.7 ± 0.09	9.4 ± 0.26	6.4 ± 0.11	5.8 ± 0.22	10.5 ± 0.22	3.0 ± 0.07	3.2 ± 0.13
	15.5–16.3(*9*)	14.7–15.4 (*9*)	9.9–10.3 (*9*)	3.5–3.8 (*9*)	9.1–9.9 (*9*)	6.2–6.5 (*9*)	5.5–6 (*9*)	10.2–10.8 (*9*)	2.9–3.1 (*9*)	3.0–3.4 (*9*)
♀♀	15.4 ± 0.44	14.4 ± 0.37	9.8 ± 0.21	3.6 ± 0.12	9.2 ± 0.23	6.2 ± 0.22	5.6 ± 0.14	10.0 ± 0.25	2.9 ± 0.12	3.0 ± 0.15
	14.7–16.1 (*8*)	13.8–14.9 (*8*)	9.5–10.2 (*8*)	3.4–3.7 (*8*)	8.9–9.6 (*8*)	5.7–6.4 (*8*)	5.4–5.8 (*8*)	9.6–10.4 (*8*)	2.7–3.0 (*8*)	2.8–3.2 (*8*)
	t = -3.34	t = -3.60	t = -2.99	–	–	t = -2.91	t = -2.73	t = -4.03	t = -3.65	t = -2.29
	$P < 0.01$	$P < 0.01$	$P < 0.01$	n.s.	n.s.	$P < 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.05$
Madagascar - east (OTU 5)										
♀♀	15.4	14.3	9.5	3.4	9.3	6.1	5.4	9.7	2.9	2.6
Pemba (OTU 6)										
♂♂	16.3 ± 0.23	15.4 ± 0.20	10.3 ± 0.18	3.4 ± 0.07	9.5 ± 0.08	6.7 ± 0.10	5.9 ± 0.21	10.9 ± 0.15	3.3 ± 0.16	3.4 ± 0.16
	15.9–16.7 (*10*)	15.1–15.8 (*10*)	9.9–10.6 (*10*)	3.4–3.6 (*10*)	9.3–9.6 (*10*)	6.6–6.9 (*10*)	5.5–6.2 (*10*)	10.6–11.1 (*10*)	3.0–3.6 (*10*)	3.1–3.6 (*10*)
♀♀	16.1, 16.1	15.1, 15.3	10.1, 10.1	3.3, 3.4	9.4, 9.5	6.5, 6.8	5.5, 5.7	10.5, 10.6	3.9, 4.4	3.0, 3.2
	–	–	–	–	–	–	–	t = -3.15	–	–
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	$P < 0.05$	n.s.	n.s.
Mayotte (OTU 7)										
♂♂	16.2	15.0	9.9	3.5	9.4	6.6	5.8	10.5	3.3	3.1
♀♀	15.2	14.3	9.5	3.4	9.1	6.1	5.4	10	2.8	3.1

TABLE 3. Dental measurements (in mm) of adult *C. leucogaster* from Madagascar, Mayotte, and Pemba. Measurements are presented as $\bar{x} \pm$ SD, and minimum–maximum, followed by the number of specimens (in parentheses). For each OTU the sexes are separated. n.s. = not-significant. Neotype specimen is FMNH 176137

Sex	C^1–C^1	M^3–M^3	UP MOL R	MTR	UP CANIN	M^3WIDTH	LOWER TR	LO CANIN
Madagascar - south (OTU 1)								
♂♂	4.5 ± 0.21	7.1 ± 0.20	4.5 ± 0.06	6.0 ± 0.12	2.6 ± 0.22	1.8 ± 0.11	6.4 ± 0.16	2.2 ± 0.18
	4.2–4.9 (*12*)	6.8–7.5 (*12*)	4.4–4.6 (*12*)	5.8–6.2 (*12*)	2.1–2.9 (*12*)	1.6–2.0 (*12*)	6.1–6.7 (*12*)	2.0–2.5 (*12*)
♀♀	4.4 ± 0.23	7.1 ± 0.22	4.4 ± 0.11	5.9 ± 0.15	2.3 ± 0.20	1.8 ± 0.08	6.3 ± 0.18	2.1 ± 0.13
	3.9–4.8 (*16*)	6.7–7.4 (*16*)	4.2–4.6 (*16*)	5.7–6.1 (*16*)	1.9–2.7 (*16*)	1.6–1.9 (*16*)	5.9–6.6 (*16*)	1.9–2.4 (*16*)
	–	–	–	–	*t* = -3.24	–	–	*t* = -2.41
	–	–	–	–	*P* < 0.01	–	–	*P* < 0.05
Madagascar - southwest (OTU 2)								
♂ neotype	4.6	7.0	4.4	5.9	2.3	1.7	6.3	2.5
♀♀	4.3 ± 0.12	7.0 ± 0.20	4.4 ± 0.12	5.9 ± 0.16	2.3 ± 0.16	1.7 ± 0.08	6.2 ± 0.16	2.1 ± 0.09
	4.1–4.5 (*12*)	6.5–7.2 (*12*)	4.2–4.6 (*12*)	5.7–6.2 (*12*)	2.0–2.5 (*12*)	1.6–1.8 (*12*)	5.9–6.5 (*12*)	1.9–2.2 (*12*)
	t = -2.26	–	–	–	–	–	–	*t* = -4.78
	P = 0.05	–	–	–	–	–	–	*P* < 0.001
Madagascar - central west (OTU 3)								
♂♂	4.2 ± 0.18	6.8 ± 0.19	4.2 ± 0.14	5.7 ± 0.17	2.5 ± 0.14	1.7 ± 0.09	6.1 ± 0.21	2.2 ± 0.13
	3.8–4.7 (*49*)	6.4–7.2 (*49*)	4.0–4.5 (*49*)	5.4–6.1 (*49*)	2.0–2.8 (*49*)	1.4–1.8 (*49*)	5.6–6.6 (*49*)	2.0–2.5 (*49*)
♀♀	4.0 ± 0.16	6.8 ± 0.19	4.2 ± 0.11	5.7 ± 0.13	2.16 ± 0.12	1.7 ± 0.09	6.0 ± 0.18	1.9 ± 0.12
	3.6–4.4 (*71*)	6.4–7.5 (*71*)	5.3–5.9 (*71*)	5.3–5.9 (*71*)	1.9–2.5 (*71*)	1.5–1.9 (*71*)	5.6–6.5 (*71*)	1.5–2.2 (*71*)
	t = -7.42	–	–	*t* = -3.67	*t* = -13.63	–	*t* = -3.88	*t* = -10.62
	P < 0.001	n.s.	n.s.	*P* < 0.001	*P* < 0.001	n.s.	*P* < 0.001	*P* < 0.001
Madagascar - northwest (OTU 4)								
♂♂	4.5 ± 0.15	7.1 ± 0.15	4.4 ± 0.16	6.1 ± 0.19	2.6 ± 0.13	1.7 ± 0.06	6.5 ± 0.19	2.3 ± 0.08
	4.3–4.3 (*9*)	6.9–7.4 (*9*)	4.2–4.7 (*9*)	5.8–6.3 (*9*)	2.5–2.9 (*9*)	1.6–1.8 (*9*)	6.3–6.8 (*9*)	2.2–2.4 (*9*)
♀♀	4.3 ± 0.20	7.1 ± 0.11	4.3 ± 0.13	5.9 ± 0.12	2.3 ± 0.13	1.7 ± 0.05	6.2 ± 0.13	2.0 ± 0.10
	3.9–4.5 (*8*)	7.0–7.3 (*8*)	4.1–4.4 (*8*)	5.6–6.0 (*8*)	2.0–2.4 (*8*)	1.6–1.8 (*8*)	5.9–6.3 (*8*)	1.9–2.2 (*8*)
	t = -2.92	–	*t* = -2.40	*t* = -3.23	t= -5.60	–	*t* = -3.89	*t* = -6.48
	P < 0.05	n.s.	*P* < 0.05	*P* < 0.01	*P* < 0.001	n.s.	*P* < 0.01	*P* < 0.001
Madagascar - east (OTU 5)								
♀♀	4.0	6.7	4.1	5.5	2.3	1.6	5.9	2.1
Pemba (OTU 6)								
♂♂	4.7 ± 0.17	7.6 ± 0.17	4.7 ± 0.12	6.3 ± 0.14	2.8 ± 0.12	1.9 ± 0.05	6.7 ± 0.13	2.5 ± 0.10
	4.4–5.0 (*10*)	7.3–7.9 (*10*)	4.5–4.8 (*10*)	6.1–6.5 (*10*)	2.6–3.0 (*10*)	1.8–1.9 (*10*)	6.4–6.8 (*10*)	2.3–2.6 (*10*)
♀♀	4.0, 4.4	7.0, 7.6	4.4, 4.8	5.8, 6.3	2.3, 2.5	1.7, 1.9	6.3, 6.7	2.1, 2.2
	t = 3.49	–	–	–	*t* = 4.34	–	–	*t* = 4.23
	P < 0.01	n.s.	n.s.	n.s.	*P* < 0.01	n.s.	n.s.	*P* < 0.01
Mayotte (OTU 7)								
♂♂	4.3	6.9	4.4	5.9	2.5	1.7	6.3	2.1
♀♀	4.0	6.8	4.3	5.7	2.0	1.6	6.2	2.0

showed varied patterns among OTUs in sexually dimorphic variables. The number of individuals from the east region (OTU 5) and from Mayotte (OTU 7) is not sufficient for assessing patterns of sexual dimorphism. The only statistically significant variables for OTU 1 (extreme south) were height of upper canine and height of lower canine, for OTU 2 (southwest) were anterior palatal width and height of lower canine, and for OTU 6 (Pemba) were anterior palatal width and height of upper and lower canine (Table 3). In contrast, for OTUs 3 and 4, from the central west and northwest (respectively), there are notable differences between the sexes for numerous cranial and dental measurements, with most being statistically significant (Tables 2 and 3); for these two OTUs there is no external measurement displaying sexual dimorphism (Table 1). In some cases, such as Pemba, the number of available specimens for one sex is small and more samples may reveal patterns of sexual dimorphism for other variables. When animals from a given OTU show sexual dimorphism, it is the male that is the larger of the two sexes. As a result, in subsequent statistical analyses, males and females are treated separately.

Morphological Variation of C. leucogaster *on Madagascar*

External Measurements and Body Mass

On the basis of the ANOVA tests, males show some differences among the various OTUs. The number of male specimens for OTUs 2 and 5 are limited, and were excluded from the ANOVA analysis. OTU 1 appears largely identical to OTU 4, but differs statistically from OTU 3 in both hindfoot length ($F = 4.9$, $d.f. = 2$, $P < 0.05$) and body mass ($F = 22.2$, $d.f. = 2$, $P < 0.001$). Further, OTUs 3 and 4 showed differences in body mass ($F = 22.2$, $d.f. = 2$, $P < 0.001$), with individuals from the latter being larger or heavier than the former (Table 1).

In females, there were more differences in the external measurements between the various OTUs than amongst males. Only a single female specimen is available from OTU 5 and OTU 7 and these were omitted from the ANOVA comparisons. OTU 1 appears identical to OTU 4. There were dissimilarities in several of the variables between OTUs 1 and 2 and OTUs 1 and 3, and fewer distinctions between OTUs 3 and 4. For example, animals from OTU 3 are smaller in total length than those from OTU 1 ($P < 0.001$), OTU 2 ($P < 0.001$), and OTU 4 ($P < 0.05$; in all cases $F = 16.1$, $d.f. = 3$,). Tail length is longer amongst individuals from OTU 1 than OTU 2 ($P < 0.01$) and OTU 3 ($P < 0.01$; in both cases $F = 4.4$, $d.f. = 3$). In contrast, hindfoot length is longer for animals from OTU 2 than OTU 3 ($P < 0.001$) and OTU 4 ($P < 0.001$; in both cases $F = 54.6$, $d.f. = 3$). In the case of ear length, individuals from OTU 2 are longer as compared to OTU 1 ($P < 0.001$) and OTU 3 ($P < 0.001$; in both cases $F = 20.1$, $d.f. = 3$), and animals from OTU 3 are shorter than OTU 4 ($F = 20.1$, $d.f. = 3$, $P < 0.01$). These differences did not show a distinct north-south cline, as in some cases animals from the extreme south (OTU 1) were larger than the northwest (OTU 4) and for other variables the inverse.

Cranial Measurements

For males, there was not a single cranial variable that showed significant divergence between OTUs 1 and 4. In contrast, for OTUs 1 and 3 apart from two variables (postorbital width and moment arm of temporal) all of the others showed statistically significant differences at a level of $P < 0.05$, and, in all cases, the average measurement of the former OTU was larger than the latter OTU. Further, numerous differences were found between OTUs 3 and 4, with the former being smaller than the latter except for postorbital width and moment arm of temporal.

A principal component analysis of the cranial variables for males indicates that for the first axis, all of the variables have high loadings except for postorbital width and moment arm of temporal, which have lower loadings (Table 4). On the second axis, postorbital width has a high positive loading and moment arm of masseter a high loading. The first axis explains 67.4% of the variance, the second axis an additional 8.5%, and the third axis an additional 6.3% (total explained variation is 82.2%). These results indicate that size is the most important aspect for loadings on the first principal component axis. A projection of axes 1 and 2 of this analysis indicate partial overlap in the specimens from OTU 3 and those from OTUs 1, 2, and 4 (this analysis is not illustrated here but is similar in configuration to Fig. 2A, which also includes animals from Mayotte and Pemba).

The pattern of variation between the OTUs tends to be very similar for females as to that described above for males, but no difference was found between OTUs 1 and 2, and only condyloincisive length showed a significant difference ($F = 12.4$, $d.f. = 3$, $P < 0.05$) between OTUs 1 and 4. Accordingly, OTU 1 was notably different from OTU 3, with the exception of the postorbital width and moment arm of temporal measurements, and in all

TABLE 4. Factor loadings of principal component analyses of cranial and dental characters of male and female *C. leucogaster* from Madagascar. The values in bold indicate the variables that are notably correlated with a given factor (> 0.70)

Variable	Male			Female		
	PC 1	PC 2	PC 3	PC 1	PC 2	PC 3
Cranial						
GSL	**-0.944**	0.008	-0.195	**-0.915**	0.150	-0.260
CONINCI	**-0.950**	-0.022	-0.127	**-0.919**	0.202	-0.181
PALATE	**-0.800**	-0.125	-0.416	**-0.782**	0.224	-0.402
LACR WID	**-0.730**	0.176	0.248	-0.697	-0.458	0.023
POST ORB	-0.502	**0.807**	0.053	-0.586	-0.652	-0.281
ZYGO BR	**-0.877**	-0.084	0.209	**-0.896**	-0.020	0.238
MASTOID	**-0.856**	0.080	0.191	**-0.830**	-0.025	0.318
CON CANIN	**-0.935**	-0.099	-0.124	**-0.948**	0.095	-0.036
MOM1 COR	**-0.832**	-0.105	-0.139	**-0.743**	0.275	0.145
MOM2 ANG	-0.674	-0.350	0.469	**-0.738**	-0.072	0.429
Eigenvalue	6.740	0.854	0.625	6.617	0.839	0.705
% total variation explained	67.4	76.0	82.2	66.1	74.5	81.6
Dental						
C^1–C^1	**0.834**	-0.059	0.136	**-0.834**	-0.035	-0.077
M^3–M^3	**0.851**	-0.266	0.088	**-0.789**	0.276	-0.101
UP MOL	**0.867**	-0.195	0.042	**-0.820**	0.179	-0.186
MTR	**0.935**	-0.109	0.132	**-0.921**	0.104	-0.183
UP CANIN	**0.650**	0.508	0.047	**-0.710**	-0.468	0.296
M3 WIDTH	0.638	-0.248	**-0.716**	-0.660	0.358	0.630
LOWER TR	**0.907**	0.039	0.183	**-0.906**	0.078	-0.173
LO CANIN	0.440	**0.766**	-0.199	-0.588	-0.689	-0.005
Eigenvalue	4.892	1.030	0.633	4.943	0.948	0.599
% total variation explained	61.1	74.0	81.8	61.8	73.7	81.2

cases it was the former OTU that was, on average, larger than the latter ($P < 0.05$). The same general pattern held for OTUs 2 and 3, which showed significant differences ($P < 0.05$) for six of the variables (greatest skull length, condyloincisive length, greatest zygomatic breadth, breadth at mastoids, lacrimal width, and condylo-canine length), with the former being larger than the latter. The variable greatest zygomatic breadth showed differences between OTUs 3 and 4 ($F = 19.7$, $d.f. = 3$, $P < 0.01$).

In the principal component analysis of females, all ten cranial variables showed heavy factor loadings on the first axis with the exception of two variables, lacrimal width and postorbital width, showed (Table 4). On the second axis, no variable demonstrated a pronounced loading. The first axis explains 66.1% of the variance, the second axis an additional 8.4%, and the third axis an additional 7.1% (total explained variation is 81.6%). As with males, size variation associated with the first axis explains the vast majority of variation across OTUs. A projection of axes 1 and 2 of this analysis, indicates a largely overlapping distribution of specimens from OTU 3 and those from OTUs 1, 2, 4, and 5 (this analysis is not illustrated here but is similar in configuration to Fig. 2B, which also includes animals from Mayotte and Pemba), although animals from the extreme south (OTU 1) tend to be separated in many cases from those from the central west (OTU 3).

Dental Measurements

Amongst the male specimens, no difference was found in the dental measurements between individuals from OTUs 1 and 4. In contrast, animals from OTUs 1 and 3 showed statistically significant differences ($P < 0.001$) in six of the eight variables (anterior palatal width, posterior palatal width, maxillary toothrow, width of M^3, and lower toothrow), with the mean measurements from the former OTU being larger. Further, in five of the eight variables (anterior palatal width, posterior palatal width, posterior palatal width, maxillary toothrow, and lower toothrow), there were statistically significant differences ($P < 0.01$) between OTUs 3 and 4, and in all cases, the latter had a greater mean measurement than the former.

A principal component analysis of the dental variables for males indicates that for the first axis, all are positively correlated and most, with the exception of width of M^3 and height lower canine, show heavy loadings (Table 4). Notable loading

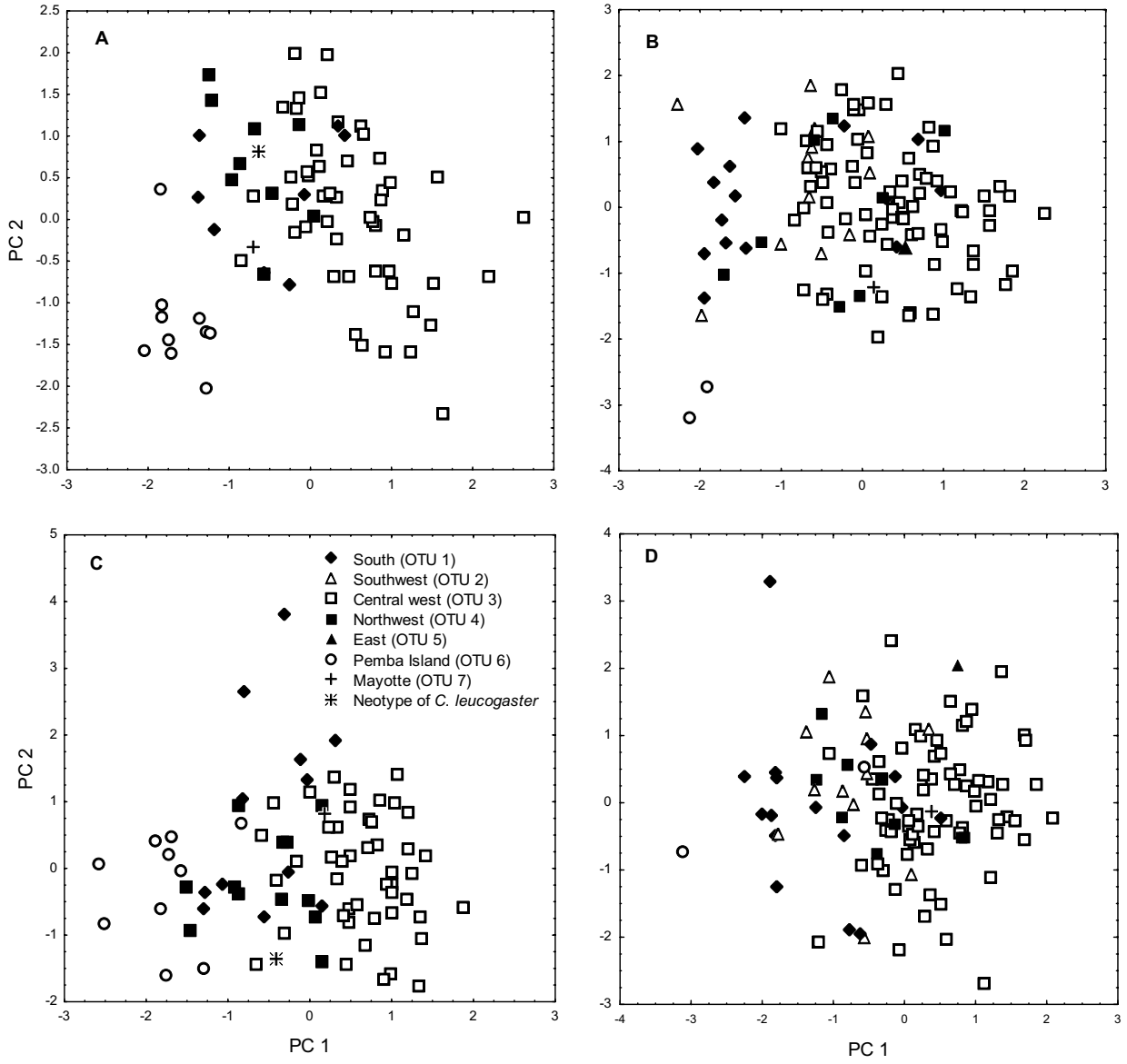

FIG. 2. Projection of principal components (PCs) 1 and 2 for *C. leucogaster* from different regions of Madagascar based on OTU designations and the designated neotype, as well as the islands of Mayotte and Pemba. A: cranial variables — males; B: cranial variables — females; C: dental variables — males; and D: dental variables — females

scores were exhibited by height lower canine on the second axis and width of M^3 on the third axis. The first axis explains 61.1% of the variance, the second axis an additional 12.8%, and the third axis an additional 7.9% (total explained variation is 81.8%). This would indicate that the size associated with the first axis is the most important aspect to explain patterns of variation across the OTUs. A projection of axes 1 and 2 of this analysis indicate notable overlap in the specimens from OTU 3 and those from OTUs 1, 2, and 4 (this analysis is not illustrated here but is similar in configuration to Fig. 2C, which also includes animals from Mayotte and Pemba). There was a tendency for males from OTUs 1 and 4 to be slightly separated from the other OTUs.

The pattern for female specimen from OTUs 1, 2, and 4 are very similar to those of males from these same zones. All of the eight variables show significant differences ($P < 0.05$) between OTU 1, always the largest mean measurement, and OTU 3. Three variables (anterior palatal width, posterior palatal width, maxillary toothrow) showed statistical differences ($P < 0.05$) in the mean measurements between OTUs 3 and 4, with the former being smaller than the latter, and four variables (anterior palatal width, posterior palatal width, maxillary toothrow, lower toothrow) showed statistical differences

($P < 0.01$) in the mean measurements between OTUs 2 and 3, with the former being larger than the latter.

In the principal component analysis of females, all of the eight dental variables showed negative loadings on the first axis and all but two variables, greatest lateral-medial width of tooth and height lower canine, showed heavy loadings (Table 4). On the second and third axes, no variable contributed significantly in accounting for the variation. The first axis explains 61.8% of the variance, the second axis an additional 11.9%, and the third axis an additional 7.5% (total explained variation is 81.2%). As with males, size variation associated with the first axis accounts for patterns of geographic variation across the different OTUs. A projection of axes 1 and 2 of this analysis indicate broad overlap in the specimens from the different Madagascar OTUs (this analysis is not illustrated here but is similar in configuration to Fig. 2D, which also includes animals from Mayotte and Pemba).

Latitudinal Variation in C. leucogaster *on Madagascar*

This species was found along the north-south trajectories from Belo sur Mer to Toliara and from Mahajanga to Antanimbary (Fig. 1); in the first zone the number of males obtained is insufficient for meaningful regression analyses. For males in the region from Mahajanga to Antanimbary, no relationship was found for latitude regressed against PC1 scores obtained from the PCA analysis for external variables, while the cranial and dental variables showed positive correlations ($R^2 = 0.03$, $F = 15.17$, $n = 46$, $P < 0.001$ and $R^2 = 0.19$, $F = 10.56$, $n = 46$, $P < 0.01$, respectively). In the case of females across the region from Mahajanga to Antanimbary, the PC1 scores were significant when regressed against latitude: external measurement ($R^2 = 0.07$, $F = 5.06$, $n = 74$, $P < 0.05$), cranial measurements ($R^2 = 0.43$, $F = 52.45$, $n = 71$, $P < 0.001$), and dental measurements ($R^2 = 0.33$, $F = 32.15$, $n = 68$, $P < 0.001$). Thus, for these three variable types there is an augmentation in size as a function of increasing latitude.

Elevational Variation in C. leucogaster *on Madagascar*

Based on our specimens, obtained across the elevational range from near sea level to 230 m, regression analyses of the PC1 scores of external measurements with altitude showed no statistically significant differences for either males or females. However, comparisons made for PC1 scores plotted for the cranial measurements against altitude found strong positive correlations for males ($R^2 = 0.41$, $F = 31.13$, $n = 46$, $P < 0.001$) and females ($R^2 = 0.21$, $F = 17.94$, $n = 71$, $P < 0.001$). The same general pattern, but at a lower level of significance, was found for PC1 scores of dental measurements plotted against altitude – males ($R^2 = 0.21$, $F = 11.60$, $n = 46$, $P < 0.01$) and females ($R^2 = 0.15$, $F = 11.39$, $n = 68$, $P < 0.01$). Hence, with increasing elevation there is a correlated increase in size.

Patterns of Geographic Variation in C. leucogaster *on Western Indian Ocean Islands*

Using separate comparisons for adult male and female specimens of *C. leucogaster*, a series of ANOVA analyses for the external, cranial, and dental variables show that the Pemba population (OTU 6) differed notably from Malagasy populations, with the Pemba animals being considerably larger. On the basis of male external measurements, total length is notably greater for animals from OTU 6 as compared to those from the central west of Madagascar (OTU 3) ($F = 9.9$, *d.f.* = 3, $P < 0.001$). The same pattern was found in females with individuals from Pemba being larger than those from Madagascar. For the cranial measurements of males and females, the majority of variables showed a slight difference between animals from OTU 6 and those from OTUs 3 and 4 ($P < 0.05$). For OTU 1 and OTU 6, five of the 10 variables showed statistical differences ($P < 0.01$) for males and one variable (moment arm of masseter) for females. In the case of the dental variables, males from OTU 6 were statistically different for all variables as compared to OTU 3 ($P < 0.001$) and for females, three of the eight variables (posterior palatal width, maxillary toothrow, lower toothrow) also showed a significant divergence ($P < 0.01$). A notable difference in size ($P < 0.05$) was apparent for males between OTU 6 and OTU 4 for five of the eight variables (posterior palatal width, maxillary toothrow, width of M^3, lower toothrow, and height lower canine). These differences are supported by the principal component analyses for both the cranial and dental measurements (Fig. 2), where there is a complete or nearly distinct separation between animals from Pemba and the other collection regions, and the animals from Mayotte fall within the range of those from Madagascar.

From a phenotypic aspect, the Pemba animals showed the same general pelage and soft part

coloration patterns as specimens from Madagascar and Mayotte. The most pronounced differences are that animals from Pemba have darker dorsal pelage coloration then those from Madagascar and Mayotte. Further, the white portion of the under side of the Pemba animals extends across most of the venter, while for those from Madagascar and Mayotte the white is limited to the mid-ventral posterior portion of the underside. Other than size, no difference was found in cranial or dental structure or morphology of the animals from Pemba, as compared to Madagascar and Mayotte (Fig. 3).

Ecological Niche Modeling

The MaxEnt algorithm converged after 1,200 iterations with a regularized training gain of 1.833. Model performance as assessed by the area under the curve (AUC) was very high (0.95), indicating efficient classification of suitable versus unsuitable habitats. The environmental variable with the highest explanatory power when used in isolation was bio_4 (temperature seasonality). The environmental variable that decreased the overall gain of the model most when omitted was bio_1 (annual mean temperature). The MaxEnt model (Fig. 4) revealed an apparent large disjunction caused by unsuitable habitats separating populations from the northwest and the south and southwest. Apart from a continuous large block of suitable habitat corresponding largely with xerophytic vegetation in the semi-arid south, suitable habitats are patchily distributed in the central west (deciduous forests), north-west (subhumid forests), and the east (humid forests). The large zone in the central west classified as 'unsuitable habitat' is an area of meteorological and ecological transition between the more 'suitable' northern and southern areas; this zone was not visited during the course of our recent field studies and we suggest that this conclusion may be an artifact of sampling. The southeastern record of *C. leucogaster* at Manakara occupies an area of unsuitable habitat

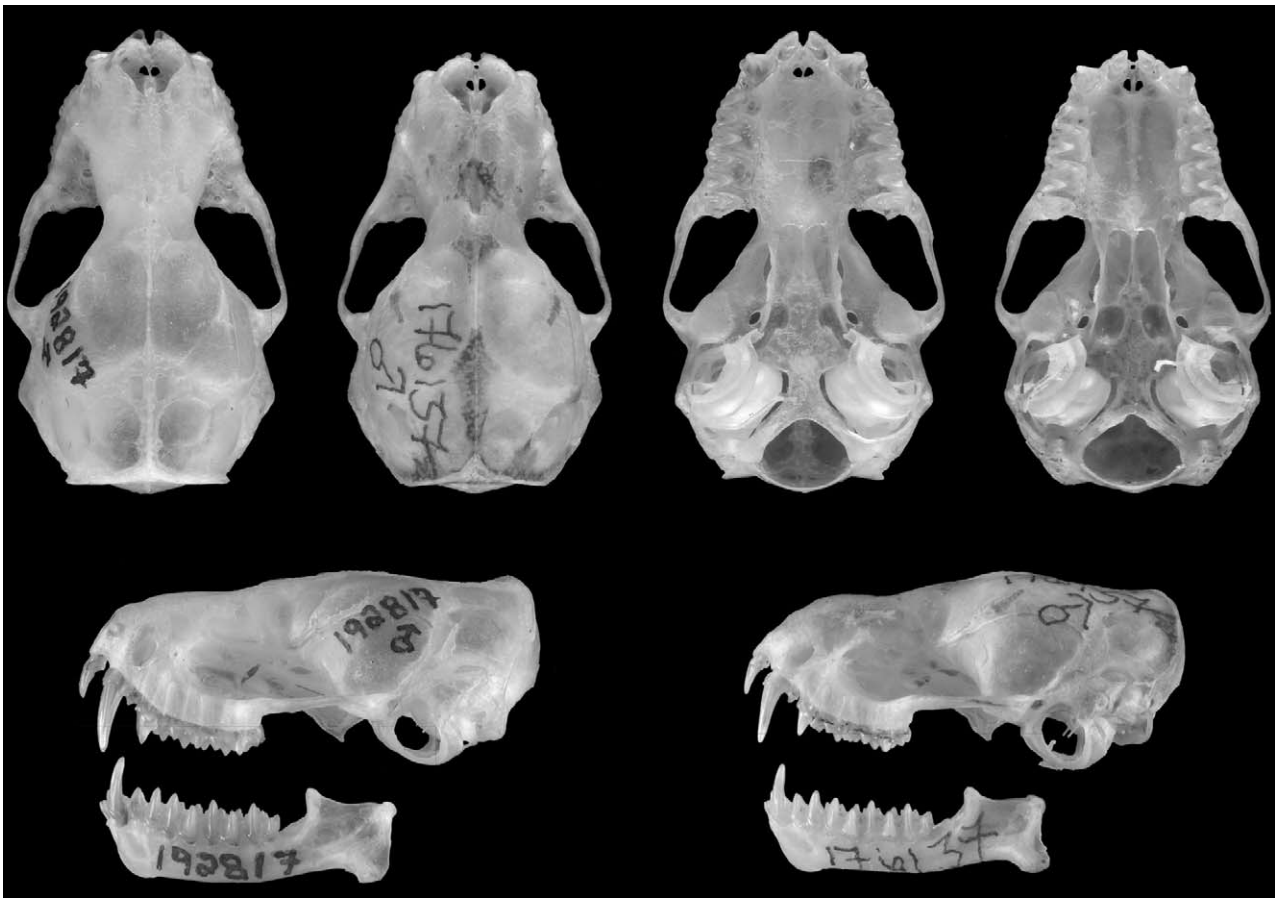

FIG. 3. Dorsal, ventral, and lateral views of adult male crania and mandibles of *C. leucogaster* from Pemba Island, Tanzania (left — FMNH 192817) and neotype from Bel sur Mer, Madagascar (right — FMNH 176137). (Photograph taken by John Weinstein, FMNH image No. Z94472d.)

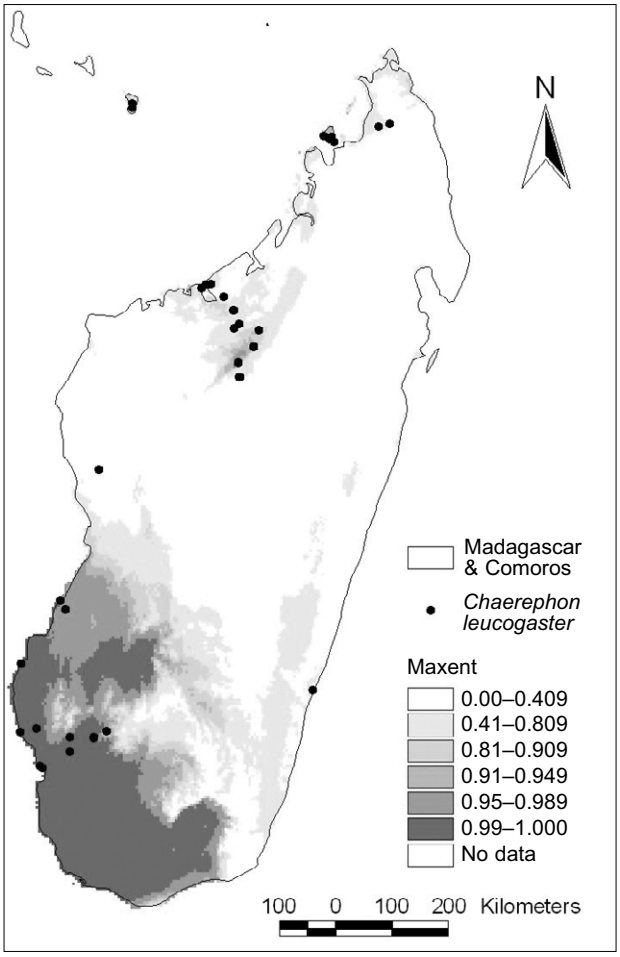

FIG. 4. Modeled potential distribution (MaxEnt) of *C. leucogaster* based on 103 known records of occurrence from Madagascar and Mayotte. Localities based on museum collecting records shown as dots. Shading represents ranges of probability of occurrence (i.e. habitat suitability)

in terms of the modeled bioclimatic optimum for the species as a whole.

Phylogeny of Malagasy and Western Indian Ocean Island Chaerephon *spp.*

Bayesian, maximum parsimony, and neighbor-joining analysis of cytochrome *b* sequences (863 nucleotides) (Fig. 5) showed that all *Chaerephon* taxa formed a strongly supported monophyletic group (bootstrap 100%) with respect to the outgroups, *M. leucostigma* and *M. midas*. Further, *C. leucogaster* from Madagascar, Mayotte, and Pemba form a well-supported monophyletic group distinct from the sister species *C. pumilus* from Madagascar. The *leucogaster* group is supported by congruent results from the following analyses: Bayesian (posterior probability 1.00), maximum parsimony (bootstrap 99%), and neighbor-joining (bootstrap 100%). There is no significant support for any substructure within the *C. leucogaster* group, which comprises six haplotypes (see section on phylogeography, below).

The structure of phylogenetic trees based on 338 nucleotides of the D-loop (Fig. 6) was congruent with the cytochrome *b* based trees. Support for a monophyletic *C. leucogaster* group, sister to *C. pumilus* from Madagascar was good (maximum parsimony bootstrap 98%, Bayesian posterior probability 0.95). Neighbor-joining analysis is not included owing to the presence of a large number of undefined distances. There is moderate support for the distinctness of the haplotype 11 samples (from Pemba and Mayotte) from the Malagasy haplotypes (1–10).

Phylogeography of C. leucogaster on *Western Indian Ocean Islands*

Cytochrome b *Haplotype Analysis*

Analysis of 863 nucleotides of the cytochrome *b* gene for 39 *C. leucogaster* samples yielded six haplotypes based on four variable sites (Appendix II). The haplotype diversity was 0.718 (variance 0.00214), whilst the nucleotide diversity per site was 0.00111 (variance << 0.00001). The average number of nucleotide differences between samples was 0.95. The six *C. leucogaster* cytochrome *b* haplotypes are separated by genetic distances of between 0.00116 and 0.00349 (Table 5). Genetic distances between *C. leucogaster* and *C. pumilus* (Madagascar) range from 0.01771 to 0.02619, whilst those obtained between *Chaerephon* taxa and the *Mops* outgroups range from 0.11903 to 0.12198. A statistical parsimony haplotype network, showing mutational relationships between haplotypes, with 95% confidence, is presented in Fig. 7. The six cytochrome *b* haplotypes are separated by between one and three mutational steps, whilst the closest *C. pumilus* (Madagascar) sample is 15 mutational steps different from any of the *C. leucogaster* haplotypes.

D-loop Haplotype Analysis

Analysis of 338 nucleotides of the D-loop for 71 *C. leucogaster* samples yielded 11 haplotypes based on nine variable sites (Appendix III). The haplotype diversity was 0.870 (variance 0.00052), whilst the nucleotide diversity per site was 0.00737 (variance 0.00002). The average number of nucleotide differences between samples was 2.00. Genetic distances of between 0.00398 and 0.03307 separate

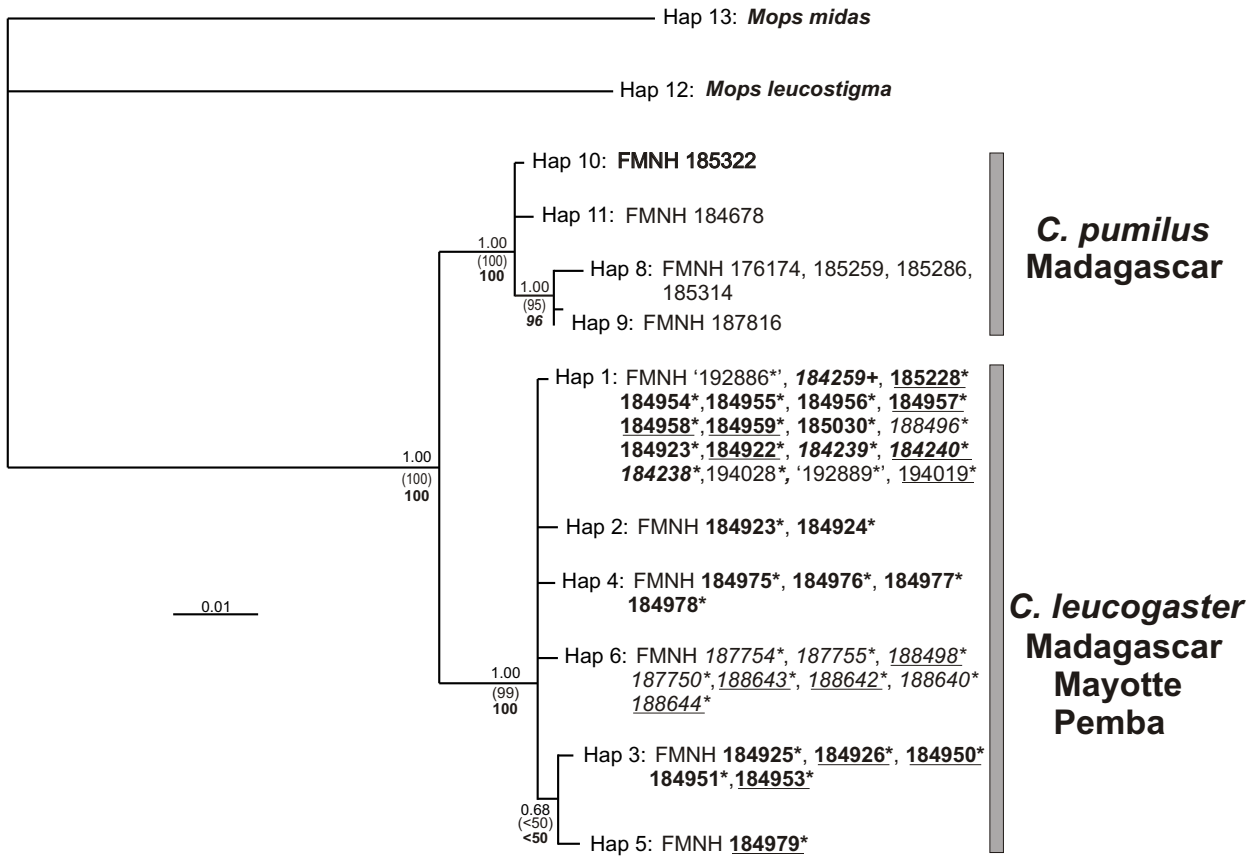

FIG. 5. Dendrogram (Bayesian, maximum parsimony, and neighbor-joining) based on 863 bp of the cytochrome *b* gene, showing relationships between 39 samples of *C. leucogaster* with reference to the outgroups, *C. pumilus* (eastern Madagascar), *Mops leucostigma,* and *M. midas.* Nodal support is indicated as follows: Bayesian posterior probabilities (normal font, top), bootstrap values for maximum parsimony (brackets, middle) and neighbor-joining (bold, bottom) analysis. Information is encoded in the text as follows: ALTITUDE – * 0–100 m, + 101–500 m. GENDER – male (not underlined), female (underlined). LATITUDE – 10°S [Pemba] normal font within quotation marks, 12°S [Mayotte] normal font, 13°S [Madagascar] *italicized font*, 15-17°S [Madagascar] **bold font**, 22-23°S [Madagascar] ***bold italicized font***

the 11 D-loop haplotypes. From the haplotype network (Fig. 8), it can be seen that most of the 11 *C. leucogaster* haplotypes are separated by one mutational step, the only exception being haplotype 11 (containing one sample each from Pemba and Mayotte), which is separated from the other haplotypes by a minimum of six mutational steps. The closest Malagasy *C. pumilus* sample is 24 mutational steps different from any of the *C. leucogaster* haplotypes.

Both Fu and Li's D* test statistic (1.47561) and Fu and Li's F* test statistic (1.15366) were non-significant ($P > 0.10$) in accordance with the assumption of an expanding population (Table 6). The expansion coefficient (S/d) was relatively high (4.80), confirming an expanding population. The distribution of pairwise distances followed an essentially unimodal distribution (Fig. 9) [the raggedness, $r = 0.077$ was non-significant ($P > 0.05$) (Rogers and Harpending, 1992)]. On the other hand, the non-significant value for Fu's Fs statistic (-1.730, $P > 0.05$) does not indicate an expanding population. Taking the weight of evidence to indicate an expanding population, following Rogers and Harpending (1992), we used the formula $\tau = 2ut$, to obtain an estimated time since expansion: where τ (tau) is the mutation rate in generational units (1.303 in our study), u is the product of mutation rate (μ) per generation [two rates for the D-loop were used from Rogers and Harpending (1992): (i) 17.3% divergence per million years or $\mu = 1.73 \times 10^{-7}$ mutations per site per generation and (ii) 33% divergence or 3.3 mutations $\times 10^{-7}$) multiplied by the sequence length (338 bp), and t is the time (in generations) since expansion (generation time taken as two years — Russell *et al*., 2005). Applying this formula, we obtain an estimated time since expansion of between

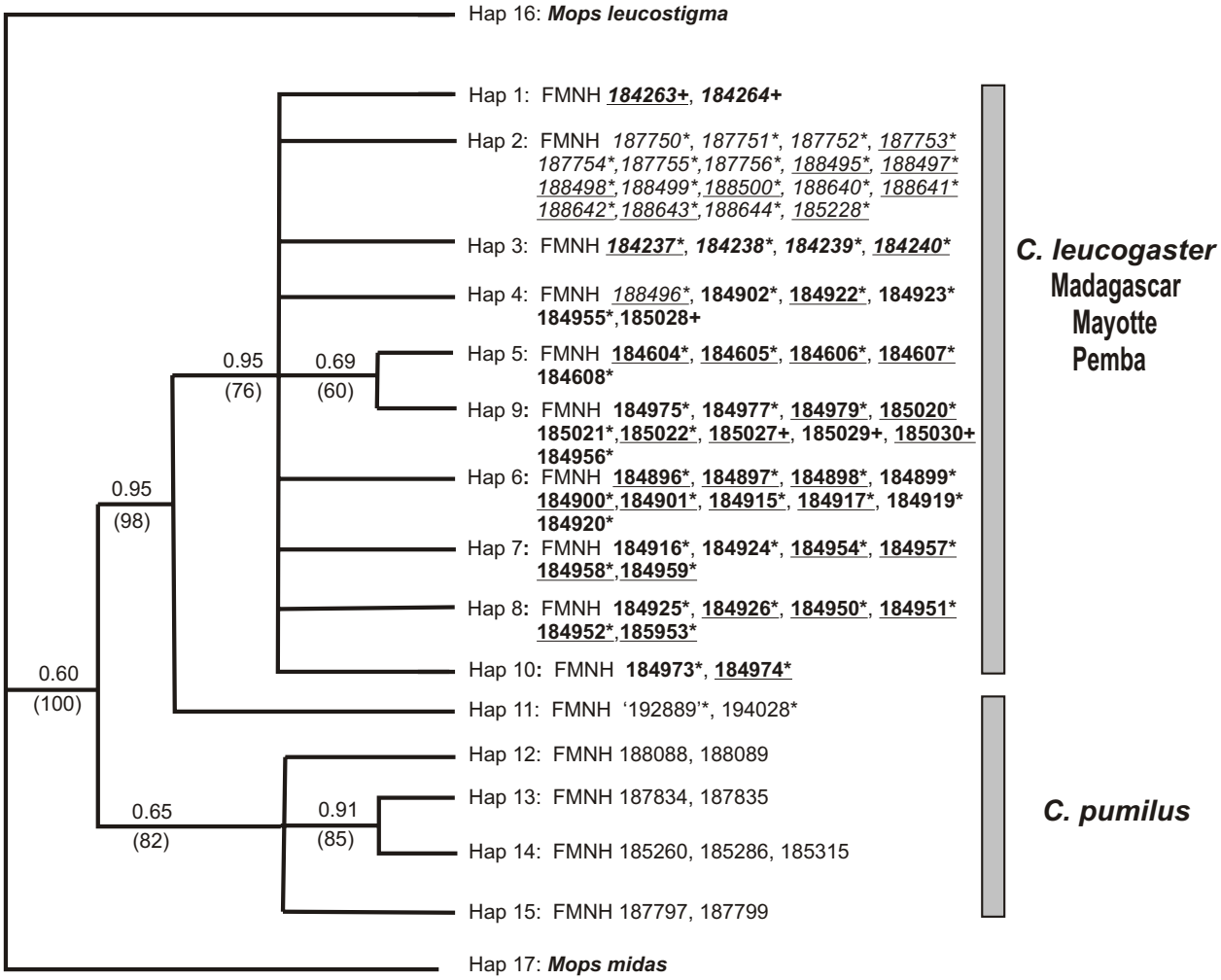

FIG. 6. Dendrogram (Bayesian and maximum parsimony) based on 338 bp of the D-loop, showing relationships between 71 samples of *C. leucogaster* with reference to the outgroups, *C. pumilus* (eastern Madagascar), *M. leucostigma* and *M. midas*. Nodal support is indicated as follows: Bayesian posterior probabilities (normal font, top), bootstrap values for maximum parsimony (brackets, bottom). Information is encoded in the text as follows: ALTITUDE – * 0–100 m, + 101–500 m. GENDER – male (not underlined), female (underlined). LATITUDE – 10°S [Pemba] normal font within quotation marks, 12°S [Mayotte] normal font, 13°S [Madagascar] *italicized font*, 15–17°S [Madagascar] bold font, 22–23°S [Madagascar] ***bold italicized font***

5,842 (33% divergence rate) and 11,143 years (17% divergence).

Comparisons of Various Parameters to Haplotypic Variation

Latitudinal Variation

The Malagasy samples comprise five distinct cytochrome *b* haplotypes (Fig. 7) and 10 distinct D-loop haplotypes (Fig. 8). As described below, both cytochrome *b* and D-loop show haplotypes exclusive to the north and south of the range of *C. leucogaster* on Madagascar. Cytochrome *b* haplotype 6 and D-loop haplotype 2 are exclusive to the northwest of the range, corresponding to 13°S latitude and including the near shore islands of Nosy Be and Nosy Komba. These haplotypes are not reciprocally monophyletic with the rest of the network, however, as one sample from this region exhibits both cytochrome *b* and D-loop haplotypes found in the middle latitude band (15º to 17ºS). Samples from the extreme south region (Sakaraha and Toliara) (22º to 23ºS) are haplotype 1, the most common cytochrome *b* haplotype (Fig. 7). In the case of the D-loop, however, samples from the extreme south region form two distinct exclusive haplotypes, 1 and 3, which are separated from each other and the rest of the haplotype network by one mutational step (Fig. 8).

The permutational contingency test based on the nested cladogram (Fig. 10) was significant for

TABLE 5. Genetic distance (×100), based on 863 bp of the cytochrome *b* gene, between six *C. leucogaster* haplotypes, *C. pumilus*, and the outgroups (*M. leucostigma* and *M. midas*)

Species	Hap	1	2	3	4	5	6	7	8	9	10	11
C. leucogaster	1	–										
C. leucogaster	2	0.116	–									
C. leucogaster	3	0.116	0.232	–								
C. leucogaster	4	0.116	0.232	0.232	–							
C. leucogaster	5	0.232	0.349	0.116	0.116	–						
C. leucogaster	6	0.116	0.232	0.232	0.232	0.349	–					
C. pumilus	7	2.375	2.497	2.497	2.497	2.619	2.497	–				
C. pumilus	8	2.132	2.253	2.253	2.253	2.375	2.253	0.232	–			
C. pumilus	9	1.769	1.890	1.890	1.890	2.011	1.890	0.583	0.349	–		
C. pumilus	10	1.890	2.011	2.011	2.011	2.132	2.011	0.701	0.466	0.116	–	
M. leucostigma	11	11.914	11.917	12.071	11.751	11.907	12.071	11.896	11.577	11.258	11.259	–
M. midas	12	11.918	12.065	12.065	11.771	11.917	12.065	12.495	12.198	11.757	11.903	12.682

clades 1.2 ($P < 0.001$), 1.3 ($P < 0.01$), 2.1 ($P < 0.001$), 2.2 ($P < 0.05$), and the Total Cladogram ($P < 0.001$) (see Table 7). This analysis, based on D-loop sequences, gives statistical confidence to the qualitative findings, reported above, of distinct northern and southern haplotypes of *C. leucogaster*. There is significant phylogeographic association ($P < 0.05$) within clades 1.3 and 2.2, supporting the distinctness of haplotypes from the northern latitude band, within clades 1.2 and 2.1, sustaining the distinctness of haplotypes from the southern latitude band and within the total cladogram (2.1 versus 2.2), following an overall segregation of haplotypes between northern and southern latitudes.

Altitude

Most of the genetic samples came from altitudes of less than 100 m; there was no apparent association of haplotypes with altitude for either cytochrome *b* or the D-loop (Figs. 7 and 8).

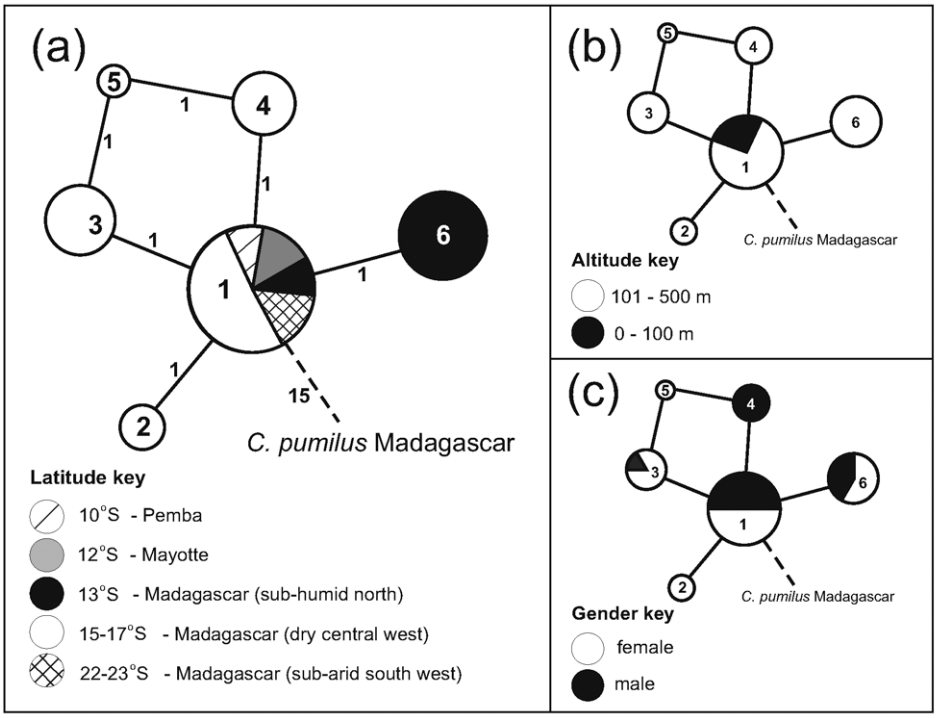

FIG. 7. Networks showing mutational relationships between six cytochrome *b* (863 bp) haplotypes of *C. leucogaster* obtained from 39 samples with reference to the outgroup, *C. pumilus* (east Madagascar): (a) overlay of latitudes samples taken, (b) overlay of altitudes samples taken, and (c) overlay of sample sex. Numbers within circles are haplotype numbers. Numbers adjacent to connecting lines are mutational steps

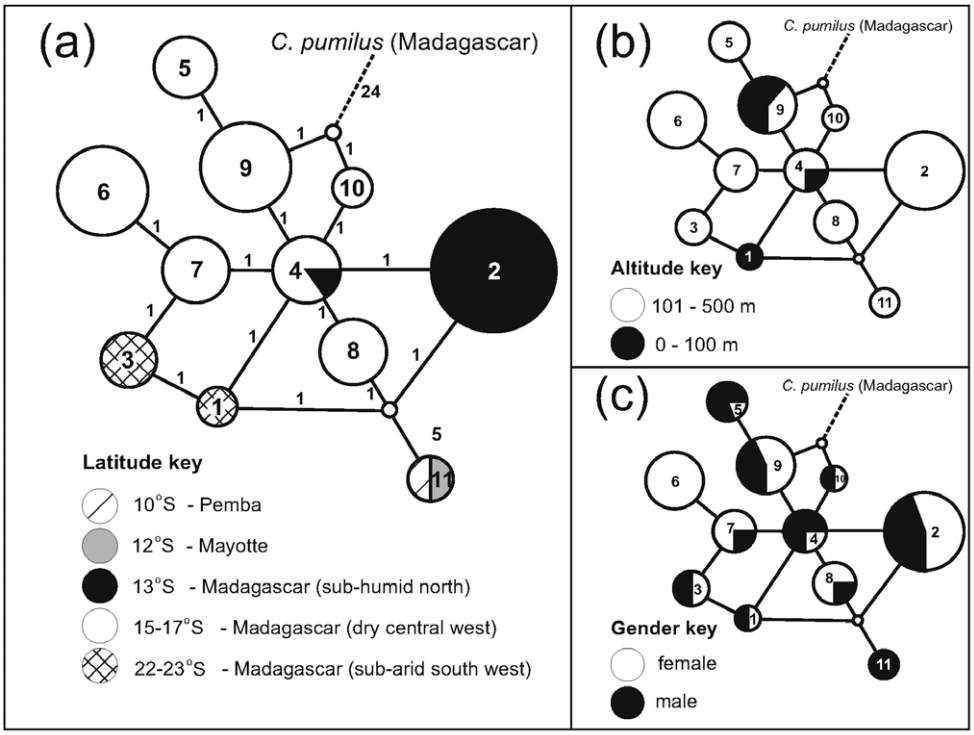

FIG. 8. Networks showing mutational relationships between 11 D-loop (338 bp) haplotypes of *M. leucostigma* obtained from 71 samples with reference to the outgroup, *C. pumilus* (eastern Madagascar): (a) overlay of latitudes of samples taken, (b) overlay of altitudes of samples taken, and (c) overlay of sample gender. Numbers within circles are haplotype numbers. Numbers adjacent to connecting lines are mutational steps

Sex

There is no apparent association of haplotypes with sex for either cytochrome *b* or the D-loop (Figs. 7 and 8).

TABLE 6. Diversity and neutrality statistics based on 370 nucleotides of the D-loop

Parameter	D-loop	Expectation#
Nucleotide diversity (π)	0.00934	Low
Haplotype diversity (*h*)	0.891	High
Expansion coefficient (S/d)	4.800	High
Fu and Li's (1993) F*	1.15366	Not significant
Fu and Li's (1993) D*	1.47561	Not significant
Fu's (1997) Fs	-1.730	Significant
Raggedness (rg)	0.0768	Not significant
Mismatch distribution	Unimodal	Unimodal
Tau (τ)	1.30322	–
Time since expansion (yr BP)	5,842 – 11,143 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 $\mu = 1.73 \times 10\text{-}7$ mutations per site per generation, and 33.0% divergence or 3.3 mutations × 10-7) multiplied by sequence length (290 bp) and t was the time (in generations) since expansion (generation time taken as two years)

DISCUSSION

On the basis of new specimen material of *C. leucogaster* obtained on Madagascar, Mayotte (Comoros Archipelago), and Pemba Island (Tanzania), we make the following conclusions. (i) Certain populations demonstrate notable measurement differences between the sexes and sexual dimorphism shows geographical variation. (ii) Certain populations display noteworthy morphological (but not genetic) differences between those occurring along the western side of Madagascar and across portions of the western Indian Ocean. (iii) Genetic distances across the sample range are very low and are consistent with population-level differences. (iv) D-loop data from Malagasy samples reveal shallow but significant phylogeographic structuring into three latitudinal groups, which correlate with regions of suitable habitat predicted by MaxEnt ecological niche modeling and with Cornet's bioclimatic zones (OTUs 1, 3, and 4). The Pemba and Mayotte island samples form a distinct phylogeographic group separated from the Malagasy samples by a low genetic distance. (v) The notably morphologically larger population from Pemba is genetically similar to smaller animals

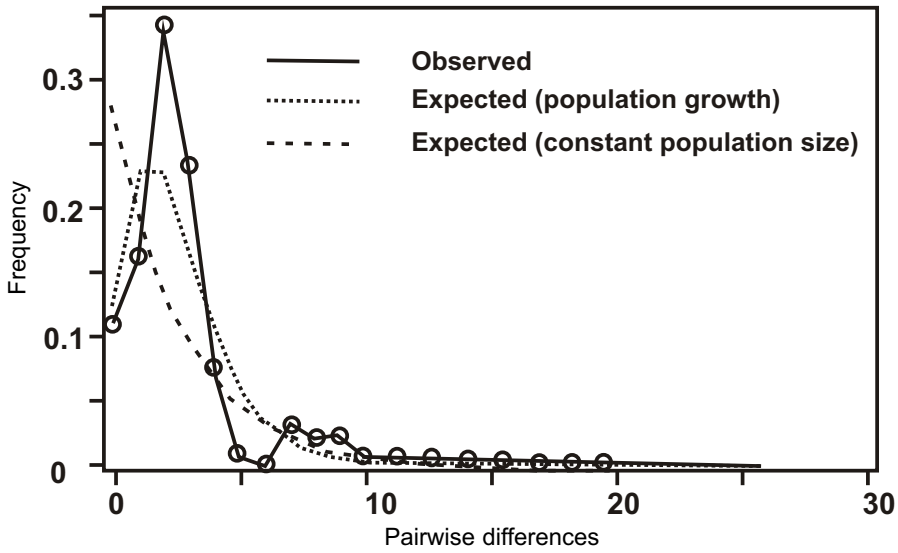

FIG. 9. Distribution of pairwise nucleotide differences for D-loop data of 71 *C. leucogaster* samples from Madagascar, Pemba, and Mayotte

from Mayotte (D-loop) and Madagascar (D-loop and cytochrome *b*).

Aspects of the Natural History of C. leucogaster *on Madagascar and New Information on their Distribution in the Western Indian Ocean Islands*

On the basis of information published before the recent wave of chiropterological surveys on Madagascar (post-1970), the distribution of *C. leucogaster* included much of the western portion of the island, from the near shore island of Nosy Be south to Toliara (Peterson *et al*., 1995; Eger and Mitchell, 2003; Fig. 1). More recent surveys have found this species at a number of other sites (Russ *et al*., 2003; Goodman and Cardiff, 2004; Goodman *et al*., 2005; Rakotonandrasana and Goodman, 2007; Kofoky *et al*., 2007; Fig. 1). Its known distribution includes most of the western length of the island. It is a species of lower elevations, although a specimen from the Zombitse Forest, to the east of Sakaraha, was obtained at 870 m. The prediction of the MaxEnt analysis that portions of the central west and extreme northwest comprise unsuitable habitat for *C. leucogaster* is almost certainly an artifact of sampling; these are zones not visited in the context of the current project.

We have one recent record from the eastern lowland part of Madagascar — at Manakara (Fig. 1); this is one of the few reports from the eastern portion of the island. The previous record of this species on the eastern near shore island of Isle Sainte Marie (Peterson *et al*., 1995) is in error and the locality of this specimen was Sainte Marie de Marovoay, southeast of Mahajanga (Kaudern, 1915). An additional older eastern record is a specimen obtained in the late 1800s and labeled 'Fort Dauphin' (Tolagnaro) (BMNH 94.2.3.2).

TABLE 7. Table showing inferences from Nested Clade Analysis in *C. leucogaster*. Clade refers to a group containing significant chi-square value(s), chain of inference is the path through the set of questions comprising Templeton's key, interpretation is according to the chain of inference in Templeton's key

Clade	Chain of inference	Interpretation
1.2	1-2-3-5-6-13-14 NO	Long-distance colonization and/or past fragmentation
1.3	1-2-3-5-6-13 YES	Long-distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion
2.1	1-2-3-5-6*-7-8 YES	Restricted gene flow/dispersal but with some long-distance dispersal over intermediate areas not occupied by the species; or past gene flow followed by extinction of intermediate populations
2.2	1-2-11-12-13-14 NO	Long-distance colonization and/or past fragmentation (not necessarily mutually exclusive)
Total Cladogram	1-19 NO	Allopatric fragmentation

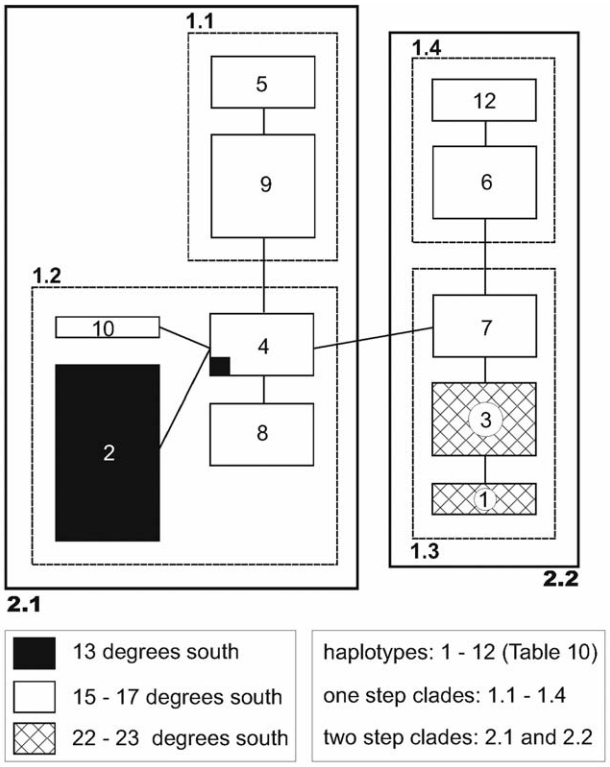

FIG. 10. Nested cladogram based on a haplotype network generated using the program TCS (Clement *et al*., 2000) based on 69 D-loop sequences of *C. leucogaster* samples from Madagascar

Specimens referable to *C. leucogaster*, based on aspects of cranial size and pelage coloration, have recently been collected on Mayotte in the Comoros Archipelago and on the offshore Tanzanian island of Pemba (see Appendix I); these records expand the known distribution of this taxon in the western Indian Ocean (e.g., Louette, 2004). *Chaerephon* specimens historically reported from Pemba (and Unguja-Zanzibar) have been identified as *C. pumilus* (Swynnerton and Hayman, 1951; Pakenham, 1984); without associated voucher numbers to verify identifications, it is possible that these animals are referable to *C. leucogaster*. Swynnerton and Hayman (1951) list records of *C. pumilus* from mainland coastal sites such as Bagamoyo and Kitaya, and these identifications also beg confirmation.

As with most other species of Molossidae bats on Madagascar, the vast majority of known day roost sites of *C. leucogaster* are in synanthropic settings (Peterson *et al*., 1995; Eger and Mitchell, 2003; Russ *et al*., 2003; Goodman *et al*., 2005). It has been found only once in recent years in a natural day roost; this case in 2002 under the bark of a dead and standing tree in the Parc National de Kirindy-Mite (Goodman and Cardiff, 2004). This is compared to 34 synanthropic settings discovered by FHR during her transect surveys and 26 other synanthropic sites found by others researchers (Peterson *et al*., 1995; Russ *et al*., 2003; Goodman and Cardiff, 2004; Goodman *et al*., 2005; Rakotoson Ranivo, 2007; Rakotonandrasana, 2008). Hence, only 1.4% of the known day roost sites are in natural settings. To our knowledge, this species has not been documented roosting in caves. This begs the question as to the original natural day roost sites this species used before the wave of anthropogenic change of the island and the construction of buildings.

In the majority of cases, the physical settings of synanthropic day roosts include under roofing and eaves or in the attics of older buildings. In a few cases, they can be found in recently constructed buildings (2–5 years old), which generally have the architectural style of older one-story colonial style civic buildings (e.g., schools and hospitals), with metal roofs and false ceilings made of wooden slats. Further, most of the buildings with day roost sites of *C. leucogaster* have air circulation holes in the gable and are buildings less than 6 m in height (ground to the roof ridge). In many cases this species can be found in the same buildings as *C. pumilus*, *M. midas*, and *M. leucostigma*, but not necessarily cohabiting the same day roost space. In one case, *C. leucogaster* was found occurring in the same day roost building as *Mormopterus jugularis* (Peters, 1865). The individuals of *C. leucogaster* from Pemba used in this study were taken in a similar setting — in the attic of a single-story hospital with a false ceiling and co-occurring with a recently described species of *Mops* (Stanley, 2008).

Although we have inventoried numerous stations for bats in portions of Madagascar above 900 m, no evidence of *C. leucogaster* has been found in the more upland portions of the island. Given that the same architectural style of buildings where this species was found in lowland areas between Mahajanga and Antanimbary (see below) also occur at higher elevations and are occupied by other synanthropic bat species (e.g., Ratrimomanarivo *et al*., 2008), it would appear that the lack of day roost sites is not a limiting factor in this species' distribution and that the upper elevation limit of this species is controlled by other biotic or abiotic factors.

Geographic Differentiation

Based on the morphological analyses, *C. leucogaster* shows modest geographic variation in size

in portions of western Madagascar. Using the bioclimatic zones of Cornet (1974), which was used herein to classify the OTUs, animals from the sub-arid area of the island in the extreme south and southwest (OTUs 1 and 2) and the sub-humid region of Nosy Be (OTU 4), are larger in certain measurements than those of the dry deciduous forest zones of the central west (OTU 3). These patterns of geographical variation are not in agreement with simple clines in certain meteorological data, although they are concordant with D-loop data (see below).

Although a large number of villages were surveyed in the eastern portion of Madagascar for synanthropic bats, we found across the vast area of OTU 5 only a single individual of *C. leucogaster*; this is an adult female at Manakara (FMNH 185228). Based on PCA analyses for cranial and dental measurements, this individual falls within the spread of points of animals obtained in the western portion of the island. This is supported by molecular data, as both cytochrome *b* and D-loop analyses place this sample with the most common western haplotypes (1 and 2, respectively). Whether the Manakara animal represents some rare or periodic movement between western or eastern portions of Madagascar or a recently colonizing population in the east is unknown. However, the morphological and genetic analyses indicate that this individual cannot be distinguished from those obtained in the western portion of the island.

The population of *C. leucogaster* on the island of Mayotte, about 300 km from the coast of Madagascar at the level of Mahajanga, is not distinguishable in morphological or genetic aspects from animals obtained in the western portion of Madagascar (see Fig. 2). In contrast, the population of *C. leucogaster* on Pemba, 1,000 km direct flight distance from the northwestern (Nosy Be) coast of Madagascar, shows notable morphological divergence from the Malagasy populations of this taxon (Fig. 2). However, genetically they are very similar (see discussion below).

Genetic Variation in C. leucogaster

Analysis of cytochrome *b* sequence data provides strong support for the monophyly of *C. leucogaster* across a region including Madagascar, Mayotte, and Pemba. This monophyly is also supported by D-loop data, which shows more variation than cytochrome *b*. Genetic variability within the *C. leucogaster* group is low: cytochrome *b* sequences from 39 individuals yielded only six haplotypes, each separated by no more than one mutational step. Samples from Pemba and Mayotte shared a cytochrome *b* haplotype with Malagasy samples, highlighting their lack of genetic distinctness. The maximum genetic distance between *C. leucogaster* haplotypes was 0.35%, which is considerably lower than the mean inter-population distance of 1.7% reported for bats by Baker and Bradley (2006). This supports the idea that there may be a single, genetically rather uniform *C. leucogaster* group across the study region, and that large bodies of water, such as the Mozambique Channel, are not significant barriers to dispersal and gene flow.

The outgroup, *C. pumilus* from the eastern side of Madagascar, forms a well-supported reciprocally-monophyletic group, sister to *C. leucogaster* and separated from it by cytochrome *b* genetic (GTR-corrected) distances ranging from 1.8 to 2.7%. These distances appear to be more representative of the intraspecific (mean 1.6%, range 0.6–2.3%, $n = 10$) or even interpopulation (mean 1.7%, range 1.4–1.9%, $n = 2$) values reported for bats by Baker and Bradley (2006) than the values reported for sister species (mean 8.3%, range 3.3–14.7%, $n = 10$), although it should be noted that no molossid bats are included in their sample. The maximum intraspecific genetic distance between 49 *M. leucostigma* samples from across Madagascar is 0.4% whilst the distance (HKY85-corrected) between *M. leucostigma* and its morphologically well-differentiated sister species, *M. condylurus*, is 2.5% (Ratrimomanarivo *et al*., 2008). Similarly, the maximum cytochrome *b* distance between 22 *M. midas* samples from Madagascar and South Africa was 0.1% (Ratrimomanarivo *et al*., 2007), whilst the distance (GTR-corrected) between sister species of *Otomops martiensseni* (Matschie, 1897) and *O. madagascariensis* Dorst, 1953, from Africa and Madagascar is 4.4% (Lamb *et al*., 2008). Thus, the *leucogaster*/*pumilus* (Madagascar) distance values of 1.8% to 2.7%, combined with the well-supported reciprocal monophyly of each group, are consistent with the designation of these taxa as separate species.

Genetic Variability in Molossids

Genetic variability within equivalent bat taxa appears to vary greatly (4.45-fold at the species level — Baker and Bradley, 2006) and it appears that variability in *C. leucogaster* is lower than that seen in the non-molossid taxa analyzed by these authors. Further, there is a range of genetic variability within molossids, and *C. leucogaster* appears to fall

somewhere in the middle of this. Analysis of both cytochrome *b* and D-loop sequence data shows moderate variation within *C. leucogaster* relative to other molossid species groups. Haplotype diversity for both cytochrome *b* and the D-loop (0.718, 0.870) is higher than that found for *M. leucostigma* (0.367, 0.758) (authors' unpublished data) and *M. midas* (0.608, 0.468), which has been reported to be very conservative (Ratrimomanarivo *et al*., 2007). It is low, however, compared to values for comparable populations such as: *Otomops madagascariensis* (0.945, 0.968), *O. martiensseni* (0.876, 0.952) (authors' unpublished data), and *Tadarida brasiliensis* (0.987, 0.998) (Russell *et al*., 2005). Nucleotide diversity per site in *C. leucogaster* (0.0011, 0.0073) follows a similar trend, and is generally higher than that for the conservative *M. leucostigma* (0.0005, 0.0009) and *M. midas* (0.0008, 0.0035), but low compared to values for comparable populations of *O. madagascariensis* (0.0072, 0.0196) and *O. martiensseni* (0.0036, 0.0302) (authors' unpublished data).

Genetic Variation of C. leucogaster *Samples from Mayotte and Pemba*

Chaerephon leucogaster samples from Pemba and Mayotte, islands located 1,300 and 320 km (respectively) from the nearest coast of Madagascar, are identical to each other and not distinct from mainland Malagasy animals in their cytochrome *b* sequences. As expected, the faster-evolving D-loop provides greater resolution and shows a degree of differentiation, as the identical Pemba and Mayotte samples are a minimum of six mutational steps (1.4%) different from the mainland Madagascar samples. The genetic similarity of animals from Pemba, Mayotte, and Madagascar might be an indication of relatively frequent movement of *C. leucogaster* between these islands. The size difference between the notably larger animals from Pemba and the smaller animals from Mayotte and Madagascar is not reflected in the cytochrome *b* and D-loop sequences generated in this study.

Genetic Variation within Malagasy C. leucogaster *Samples*

Distinctiveness of Animals from the 13° Latitude Band

This sub-humid region, to the north of Madagascar, includes the adjacent near shore islands of Nosy Be and Nosy Komba, the former approximately 12 km distant from the Malagasy mainland. It is separated from the mid-latitude dry deciduous forest zone (15º to 17ºS) (Cornet's Bioclimatic Zone 3) by a region of habitat classified by the MaxEnt ecological niche modeling as 'unsuitable' for *C. leucogaster* (Fig. 4) and corresponds to Cornet's Bioclimatic Zone 4.

Eight out of nine sample animals from the 13ºS zone exhibit a unique cytochrome *b* haplotype. This pattern is repeated with the D-loop, where 17 out of 18 samples show a unique haplotype. Thus, samples from this region appear to show a level of phylogeographic concordance. Although the genetic distances separating the 13ºS sample group from the nearest haplotype in the network are low (one mutational step — 0.1% and 0.3% for cytochrome *b* and D-loop, respectively) they are significant, as is demonstrated by the Nested Clade Phylogeographic Analysis of the D-loop data (Fig. 10, Table 7), which shows significant associations within clades 1.2, 2.1, and the total cladogram.

The 12 km distance between Nosy Be and the mainland does not appear to be a barrier to gene flow, given the genetic identity of the island and mainland samples within the 13°S region. This is not surprising, as samples from Mayotte and Pemba, which are separated by a far greater distance (980 km), have common cytochrome *b* and D-loop haplotypes.

Distinctiveness of Animals from the 22° to 23°S Latitudinal Band

This region, in the sub-arid southwest of Madagascar, includes samples from Toliara and Sakaraha. It is separated from the mid-latitude band containing dry deciduous forest (15º to 17ºS) (Cornet's Bioclimatic Zone 3) by a region classified by the Maxent ecological niche modeling as 'unsuitable' for *C. leucogaster* (Fig. 4) and corresponds to Cornet's Bioclimatic Zones 1 and 2.

Cytochrome *b* data shows no difference between samples from this region and those from areas to the north. However, analysis of the more variable D-loop data shows that samples from this region form two exclusive haplotypes, one mutational step distinct from each other and the rest of the network (Fig. 8). Samples from this region are thus genetically distinct, although the difference is shallow. This distinction is supported by the results of the Nested Clade Phylogeographic Analysis (Fig. 10, Table 7), which show significant associations within clades 1.3, 2.2, and the total cladogram. These may be attributed to the exclusive location of certain D-loop haplotypes in the sub-arid 22º to 23ºS latitude zone.

Concordance of Morphological and Genetic Variation of C. leucogaster *within Madagascar*

As mentioned previously, *C. leucogaster* shows geographic variation in size in portions of western Madagascar. Animals from the sub-arid area of the island in the extreme south and southwest (OTUs 1 and 2) and the northerly sub-humid region at 13º (OTU 4), are larger in certain measurements than those of the dry deciduous forest zones of the central west (OTU 3). These patterns of geographical variation are concordant with genetic data, which indicate that samples from OTUs 1 and 2 and OTU 4 exhibit unique D-loop haplotypes, even though the genetic distance separating them from the rest of the network is low (one mutational step in each case). The D-loop data are also concordant with the results of the MaxEnt modeling (Fig. 4).

Nested Clade Analysis gives several interpretations, which might explain these concordances. In the case of the genetically distinct samples from OTUs 1 and 2 and OTU 4, these include apparent long distance colonization (across the regions of 'unsuitable habitat') (Table 7). Other explanations include restricted gene flow with some dispersal over intermediate areas not occupied by the species or past gene flow followed by extinction of intermediate populations. Another explanation is that there is some clinal genetic variation between the southwestern and northwestern regions, and the lack of samples from this intermediate zone has yielded a certain artificial separation of populations from these different regions.

Expansion of C. leucogaster *populations*

Analysis of diversity and neutrality statistics for *C. leucogaster* from Madagascar, Mayotte, and Pemba indicate that it is likely that this population has been expanding for between 5,842 and 11,143 years. These values, in the absence of suitable chiropteran D-loop substitution rates in the literature, were based on rates reported by Rogers and Harpending (1992) for primates, given the similarity of chiropteran and primate cytochrome *b* substitution rates (Nabholz *et al*., 2008). Allowing for this assumption, it is still likely that the *C. leucogaster* population expansion began long before human colonization of the island, dated at about 2,300 BP (Burney *et al*., 2004), and the subsequent construction of building architectural styles primarily used over the past century by this species for day roosting. *Chaerephon leucogaster* populations have thus been expanding roughly since the last glacial maximum, although whether this could have caused a population bottleneck, perhaps due to the prevalence of cooler, drier conditions, vegetation changes, and consequent loss of suitable habitat for roosting remains a matter for speculation, but certain aspects can be addressed.

Few precise details are available on changes in vegetational types in lowland areas of the western half of the island, the region this bat species is found, but, in general, the late Pleistocene and Holocene of Madagascar saw a shift to drier climates and more arid natural vegetational types (Burney, 1997, 1999). These changes were most notable in the extreme southwest portion of the island, the zone the MaxEnt analysis indicates as the most suitable habitat for this species; a result that is contradictive with regards to Holocene climatic changes creating population genetic bottlenecks. At the beginning of this projected period of population expansion (5,842 and 11,143 years ago), *C. leucogaster* occupied natural day roost sites. The apparent recent shift from natural sites to synanthropic sites within the past century may have allowed for a massive expansion of the effective population size of *C. leucogaster* as well as presumed expansion of its geographic distribution. However, based on our calculations, as the expansion appears to have begun before synanthropic sites were available, this begs the question as to the nature of the original day roost sites, and the reason for their abandonment in favor of synanthropic sites. It is important to mention that over the past few hundred years forest cover in the lowland portions of the island has been massively reduced associated with anthropogenic actions (Green and Sussman, 1990; Smith, 1997; Harper *et al*., 2007) and, as this species is known to roost in trees of native forest, these changes may have had a considerable impact on population structure.

Recently Peterson and Nyári (2008) found using an overlay of ecological niche model reconstructions of Pleistocene late glacial maxima on previously hypothesized molecular phylogroups, that in a genus of South American bird there is considerable concordance between patterns of speciation and Late Pleistocene geography associated with climatic changes. This study highlights that signals of interpreted population expansion dates to the late glacial maximum may not be 'population bottlenecks' but rather actual speciation events.

In many ways the patterns of morphological and genetic variation in *C. leucogaster* across the islands

in the western Indian Ocean shows parallels with those found in *M. leucostigma* (Ratrimomanarivo *et al*., 2008) on Madagascar. This species shows notable morphological differences between the eastern and western slopes of the island, under very different bioclimatic regimes. However, these different populations, as well as on two islands in the Comoros (Mohéli and Anjouan), show remarkably little haplotypic or genetic distinctiveness. Hence, as in *C. leucogaster*, an adaptive aspect associated with meteorological, dietary or other factors seems the best means to explain the morphological patterns in these taxa. We are currently conducting parallel studies with the other synanthropic molossids on Madagascar to determine if this is a common pattern amongst them.

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Appendix I

Material utilized during the course of this study. Specimen with museum numbers in normal script were used in the morphological study; those in italics were used in the extraction of cytochrome *b*; those in bold and italics for cytochrome *b* and D-loop; and those in bold, italics, and underlined for D-loop

Chaerephon leucogaster

MADAGASCAR: Province d'Antsiranana, Ambatozavavy (Nosy Be), 13°22.012'S, 48°18.927'E, 13 Mar. 2006 (FMNH 187753, 187757); Ambilobe, 13°11.5'S, 49°03.5'E, 23 May 2003 (FMNH 176332, 176333, 176373); Betsiaka, 13°09.421'S, 49°14.190'E, 8 Feb. 2004 (FMNH 179381); Dzamadzar (Nosy Be), 13°21.095'S, 48°11.307'E, 2 Jan. 2006 (FMNH ***188497***, ***188498***, ***188499***, ***188500***); near Hell-ville (Nosy Be), 13°24.308'S, 48°18.201'E, 30 Dec. 2005, 13 Mar. 2006 (FMNH ***187750***, ***187751***-***187753***, ***187754***-***187755***, ***187756***, ***188495***, ***188496***); Nosy Komba, 13°26.562'S, 48°20.874'E, 15 Feb. 2006 (FMNH 188626-188628, 188639, ***188640***, ***188641***, ***188642***-***188644***, 188646). Province de Fianarantsoa, Manakara, 22°09.418'S, 48°01.009'E, 16 May 2005 (FMNH ***185228***). Province de Mahajanga, Ambalanjanakomby, 16°42.062'S, 46°04.304'E, 10–12 Mar. 2005 (FMNH 184921, ***184922***–***184926***, 184927, 184928, 184931–184934, 184937–184944, 184946–184949); Ambondromamy, 16º26.173'S, 47º09.329'E, 14–15 Mar. 2005 (FMNH ***184950***–***184954***); Andranofasika, 16°20.229'S, 46°50.794'E, 16–17 Mar. 2005 (FMNH ***184955***–***184959***, 184960–184962, 184968, 184970); Ankazomborona, 16º06.961'S, 46º45.400'E, 18, 20, 22 Mar. 2005 (FMNH ***184975***–***184979***, 184982, 184983, 184986, 184988, 184993–184995); Ankijabe, 16°24.807'S, 46°45.876'E, 18 Mar. 2005 (FMNH ***184973***–***184974***); Antanimbary, 17º11.104'S, 46º51.306'E, 3 Mar. 2005 (FMNH 184891–184895, ***184896***–***184902***, 184903–184907); Antsalova, 18.40°S, 44.37°E, 20 Feb. 2001 (FMNH 169671, 169696); Berivotra, 15°54.245'S, 46°35.873'E, 25 Mar. 2005 (FMNH 185016–185019, ***185020***–***185022***, 185023–185026, ***185027***–***185029***, ***185030***, 185031, 185033, 185034); Katsepy, 15°45.805'S, 46°14.695'E, 20 Oct. 2002 (FMNH 175889–175893); Maevatanana, 16°57.452'S, 46°49.433'E, 7–8 Mar. 2005 (FMNH 184910, 184911, 184914, ***184915***–***184917***,

APPENDIX I. Continued

184918, ***184919–184920***); Mahajanga, 15°42.778'S, 46°18.752'E, 3–6 Dec. 2004, 24 Mar. 2005 (FMNH ***184604–184608***, 184609, 184611–184619, 184621–184624, 184627, 184630–184636, 184638–184643, 184646, 184648–184650, 185006, 185010–185013). Province de Toliara, Andranovory, 23°08.481'S, 44°08.769'E, 14 Oct. 2004 (FMNH 184476, 184477); Ankililoaka, 22°46.548'S, 43°36.889'E, 17 Feb. 2003 (FMNH 176186, 176189); Belo sur Mer, 20°44.139'S, 44°00.266'E, 11 Nov. 2002 (FMNH 176130–176136, 176137 [neotype], 176138–176141); Forêt de Zombitse, 22°49.07'S, 44°44.01'E, 17 Apr. 1993 (FMNH 151946); Kirindy–Mite, 20°53.2'S, 44°04.8'E, 16 Nov. 2002 (FMNH 176111); Morombe, 21°44.417'S, 43°22.333'E, 17 Mar. 2003 (FMNH 176175–176177, 176181, 176185); Sakaraha, 22°54.546'S, 44°31.574'E, 15, 18, 19 Oct. 2004 (FMNH 184250, 184252–184254, 184256–184258, ***184259***, 184260, 184262, ***184263–184264***); Toliara, 23°23.704'S, 43°43.219'E, 7, 11 Oct. 2004 (FMNH ***184237***, ***184238–184240***, 184241, 184244–184247); Tsifota, 22°49.445'S, 43°21.913'E, 22 Feb. 2003 (FMNH 176190); ARCHIPEL DES COMORES: Mayotte, Coconi, 12°49.923'S, 45°08.215'E, 27 Feb. 2007 (FMNH *194019*); Mayotte, Poroani, 12°53.609'S, 45°08.550'E, 27 Feb. 2007 (FMNH ***194028***); TANZANIA: Pemba Island, Kaskazani, 4.96487°S, 39.71456°E, 5 Aug. 2006 (FMNH 192817–192821, *192886*, 192887, 192888, ***192889***, 192891– 192893).

Chaerephon pumilus

MADAGASCAR: Province de Fianarantsoa, Ranomafana (Ifanadiana), 21°15.456'S, 47°27.355'E, 28 Dec. 2005 (FMNH ***188088–188089***); Ifanadiana, 21°18.394'S, 47°38.144'E, 25 May 2005 (FMNH *185322*); Farafangana, 22°49.275'S, 47°49.860'S, 26 Apr. 2005 (FMNH *185259,* ***185260***); Vohipeno, 22°21.997'S, 47°50.206'E, 8 May 2005 (FMNH ***185286***); Manakara, 22°09.418'S, 48°01.009'E, 17 May 2005 (FMNH *185314,* ***185315***); Vangaindrano, 23°21.300'S, 47°35.763'E, 23 Apr. 2005 (FMNH *185230*). Province de Toamasina, Ranomafana–Antsinanana, 18°57.636'S, 48°50.845'E, 11 Nov. 2005 (FMNH ***187834–187835***); Toamasina, 18°08.441'S, 49°22.670'E, 21, 25 Oct. 2005 (FMNH ***187797***, ***187799***, *187816*); Anjiro, 18°52.945'S, 47°58.245'E, 7 Feb. 2005 (FMNH *184678*).

Mops leucostigma

MADAGASCAR: Province de Mahajanga, Ankazomborona, 16°06.961'S, 46°45.400'E, 18 Mar. 2005 (FMNH ***185098***); ARCHIPEL DES COMORES: Mohéli Island, Fomboni, 12°16.882'S, 43°44.272'E, 29 Nov. 2006 (FMNH *194508*).

Mops midas

MADAGASCAR: Province de Toliara, Sakaraha, 22°54.429'S, 44°31.793'E, 20 Oct. 2004 (FMNH ***184306***).

APPENDIX II

Cytochrome *b* haplotype data for different taxa used in the phylogeographic analysis

Taxon	Hap	*N*	Sample codes
C. leucogaster	1	19	FMNH 192886, 184259, 184954, 184955, 184956, 184957, 184958, 184959, 185030, 188496, 184923, 184922, 184239, 184240, 184238, 194028, 192889, 194019, 185228
	2	2	FMNH 184923, 184924
	3	5	FMNH 184925, 184926, 184950, 184951, 184953
	4	4	FMNH 184975, 184976, 184977, 184978
	5	1	FMNH 184979
	6	8	FMNH 187754, 187755, 188498, 187750, 188643, 188642, 188640, 188644
C. pumilus	8	4	FMNH 185230, 185259, 185286, 185314
	9	1	FMNH 187816
	10	1	FMNH 185322
	11	1	FMNH 184678
M. leucostigma	12	1	FMNH 194508
M. midas	13	1	FMNH 184306

APPENDIX III

D-loop haplotype data for different taxa used in the phylogeographic analysis

Taxon	Hap	*N*	Sample codes
C. leucogaster	1	2	FMNH 184263, 184264
	2	18	FMNH 187750, 187751, 187752, 187753, 187754, 187755, 187756, 188495, 188497, 188498, 188499, 188500, 188640, 188641, 188642, 188643, 188644, 185228.
	3	4	FMNH 184237, 184238, 184239, 184240

APPENDIX III. Continued

Taxon	Hap	*N*	Sample codes
	4	6	FMNH 188496, 184902, 184922, 184923, 184955, 185028
	5	5	FMNH 184604, 184605, 184606, 184607, 184608
	6	10	FMNH 184896, 184897, 184898, 184899, 184900, 184901,184915, 184917, 184919, 184920
	7	6	FMNH 184916, 184924, 184954, 184957, 184958, 184959
	8	6	FMNH 184925, 184926, 184950, 184951, 184952, 184953
C. leucogaster	9	10	FMNH 184975, 184977, 184979, 185020, 185021, 185022,185027, 185029, 185030, 184956
	10	2	FMNH 184973, 184974
	11	2	FMNH 192889, 194028
C. pumilus	12	2	FMNH 188088, 188089
	13	2	FMNH 187834, 187835
	14	3	FMNH 185260, 185286, 185315
	15	2	FMNH 187797, 187799
M. leucostigma	16	1	FMNH 185098
M. midas	17	1	FMNH 184306

Cryptic lineages of little free-tailed bats, *Chaerephon pumilus* (Chiroptera: Molossidae) from southern Africa and the western Indian Ocean islands

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We investigate mitochondrial DNA and craniometric variation in southern African and Malagasy populations of the small and morphologically variable, house-roosting molossid bat, *Chaerephon pumilus* in relation to Malagasy populations of the related, smaller-sized species, *C. leucogaster*. Both cytochrome *b* and D-loop sequences show *C. leucogaster* to be nested within *C. pumilus sensu lato*, with Malagasy *C. pumilus* forming a sister group to African *C. pumilus* and Malagasy *C. leucogaster*. Four distinct D-loop clades are found in southern African populations, all of which occur sympatrically in the greater Durban area of KwaZulu-Natal Province, whilst two of the Durban clades also characterize 1) northern KwaZulu-Natal and low-lying (<600 m) areas of Swaziland, and 2) 'inland' populations comprising the Kruger National Park and higher-lying (>600 m) areas of Swaziland. Clades from low-lying areas show evidence of historical demographic expansion around 3300–13 000 years ago (KwaZulu-Natal coastal clade, Clade A1) to 14 700–60 000 years ago (Durban clade, Clade B1), whilst the inland clade (Clade B2a) was demographically more stable. The origin of these clades can be explained by sea level and vegetation changes hypothesized to follow the Last Glacial Maximum (LGM) after 18 000 years ago. Sympatric clades are shown to differ significantly in the proportional width of the braincase, and ongoing work will test evidence for acoustic and other morphological differences between them.

Key words: **Chiroptera, mitochondrial, DNA D-loop, cytochrome *b*, phylogeography, population genetics, morphometrics, taxonomy.**

INTRODUCTION

The little free-tailed bat *Chaerephon pumilus* (Cretzschmar, 1830–31), a small, common, house-roosting molossid bat (forearm length *c.* 37 mm; mass *c.* 10 g), is characterized by extreme phenotypic variation throughout its broad distribution (confined mostly to altitudes <600 m: Fig. 1) in Africa and Madagascar (Peterson *et al.* 1995; Taylor 1999a; Simmons 2005). The species was described from Massawe, Eritrea, and currently includes nine synonyms (Simmons 2005). The often recognized form *limbata* from central and east Africa is noticeably white-winged (usually also with greater extent of white ventral body markings) compared with the dark-winged forms found elsewhere across this species' range. Jacobs *et al.* (2004) showed that cytochrome *b* haplotypes from dark-winged southern African and white-winged east and central African (Tanzania and Zambia) forms of *pumilus* exhibited 0.9% divergence. Distinct but genetically similar haplotypes also characterized the southern (several localities in Durban and surrounds) and northern (a single locality, Hell's Gate, bordering Lake St Lucia) regions of KwaZulu-Natal Province of South Africa (Jacobs *et al.* 2004).

Taylor (1999a) demonstrated that populations of *C. pumilus* from eastern South Africa and Swaziland revealed polymorphism in diagnostic characters such as body size, pelage coloration, the degree of development of the male aural crest and

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the extent of palatal emargination, thus rendering current identification keys as unreliable. Aspetsberger *et al.* (2003) reported significantly lower ultrasonic frequencies (19–23 kHz, with a peak at 21.0 kHz) from a population in Tanzania, compared with recordings made in South Africa; although Taylor *et al.* (2005) showed very similar calls from Kenya and South Africa (peak frequency 25.6 kHz). Taylor (1999b) and Fenton *et al.* (2004) both revealed two distinct sonotypes of animals ascribed to *C. pumilus* from the Durban region, having peak frequencies of 23.9 and 16.3 kHz. Further, more recent acoustic recordings from this same region reveal two divergent sonotypes of *C. pumilus* having peak frequencies of *c.* 25 kHz and *c.* 30 kHz (C. Schoeman, pers. comm.).

Observations of captive bats in the rehabilitation programme of the Bat Interest Group of KwaZulu-Natal (Bats KZN) suggest that at least two behaviourally distinct morphotypes occur in eastern South Africa, a larger browner type, which is behaviourally shy and rests in a 'flatter' posture and a smaller blacker type, which is less reserved and rests in a more upright posture. This variation is not correlated with age or sex of the individual.

Given these multiple lines of evidence for possible distinct lineages of *C. pumilus* within eastern southern Africa, we revisit the study of Jacobs *et al.* (2004), which showed distinct but genetically similar cytochrome *b* haplotypes (sequences of 604 bp) from southern and northern KwaZulu-Natal. In addition to more complete sampling of the cytochrome *b* gene (845 bp), and more comprehensive geographic sampling across eastern South Africa and Swaziland, we included sequences of 34 individuals from the faster-evolving 5′ hypervariable region (HV1) of the D-loop (314 bp). We also conducted morphometric analysis of skulls from museum specimens that were used in the molecular study. The primary objective was to investigate the possibility of genetically distinct lineages within animals currently referred as *C. pumilus* in eastern South Africa and neighbouring Swaziland, and to investigate possible cranial morphological correlates. A separate study will examine the possible correlation between genetic lineages, above-mentioned acoustic sonotypes and above-mentioned morph-behavioural types.

Phylogeographic and population genetic analysis of mtDNA sequences of the related small molossid bat species, *C. leucogaster* from Madagascar and smaller Indian Ocean islands (Pemba and Mayotte) revealed shallow geographic structuring of haplotypes (Fst = 0.79; Ratrimomanarivo *et al.*, in press (a,b)). On the other hand, populations of other larger-sized Afro-Malagasy molossid species show lower levels of geographic structuring: *Mops midas* from South Africa and Madagascar (Fst = 0.14; Ratrimomanarivo *et al.* 2007), *M. leucostigma* from Madagascar and the Comoros Islands (Fst = 0.20; Ratrimomanarivo *et al.*, in press (a)) and *Otomops madagascariensis* from Madagascar (Fst = 0.05; Lamb *et al.* 2008). Based on its similar size, life history and roosting habitats (in natural rock and tree crevices and often in attics of buildings), we expected to find a similar genetic profile (with significant geographic structuring) in African *C. pumilus* compared with *C. leucogaster* from Madagascar. A second aim of this study was to test this prediction.

MATERIALS & METHODS

Material analysed

Our study focused on *Chaerephon pumilus* from the eastern parts of South Africa (KwaZulu-Natal province) and Swaziland; two GenBank D-loop sequences from the Kruger National Park were also incorporated (Fig. 1, Table 1). Specifically, our molecular sample included individuals from the following localities: Durban (numerous specific records from the broader metropolitan region), Lake St Lucia (Hell's Gate and Charter's Creek; both on the western shores), uMkhuze Game Reserve, Kruger National Park, Mlawula (Swaziland), Rosecraft (Swaziland) and Wylesdale (Swaziland). Whilst all localities fall within the Savannah Biome (Rutherford & Westphal 1986), two are located at slightly higher elevations (*c.* 600 m) in the 'highveld' of Swaziland (Rosecraft and Wylesdale), whilst all others are at elevations <600 m. The KwaZulu-Natal and Mlawula localities are all in 'lowveld' (<600 m), within 60 km of the Indian Ocean coastline. However, east–west and south–north gradients of decreasing rainfall result in uMkhuze, Mlawula and Kruger National Park receiving <700 mm annual rainfall compared with Hell's Gate, Charter's Creek and Durban which receive >900 mm. The two Swaziland highland localities receive >1000 mm. The uMkhuze and Mlawula localities border the Lebombo Mountains, which run roughly south–north parallel to the ocean. Both these localities are separated from the seashore by a broad coastal plain. The Durban localities border the escarpment, which, at this latitude, is closer to the coast than it is further

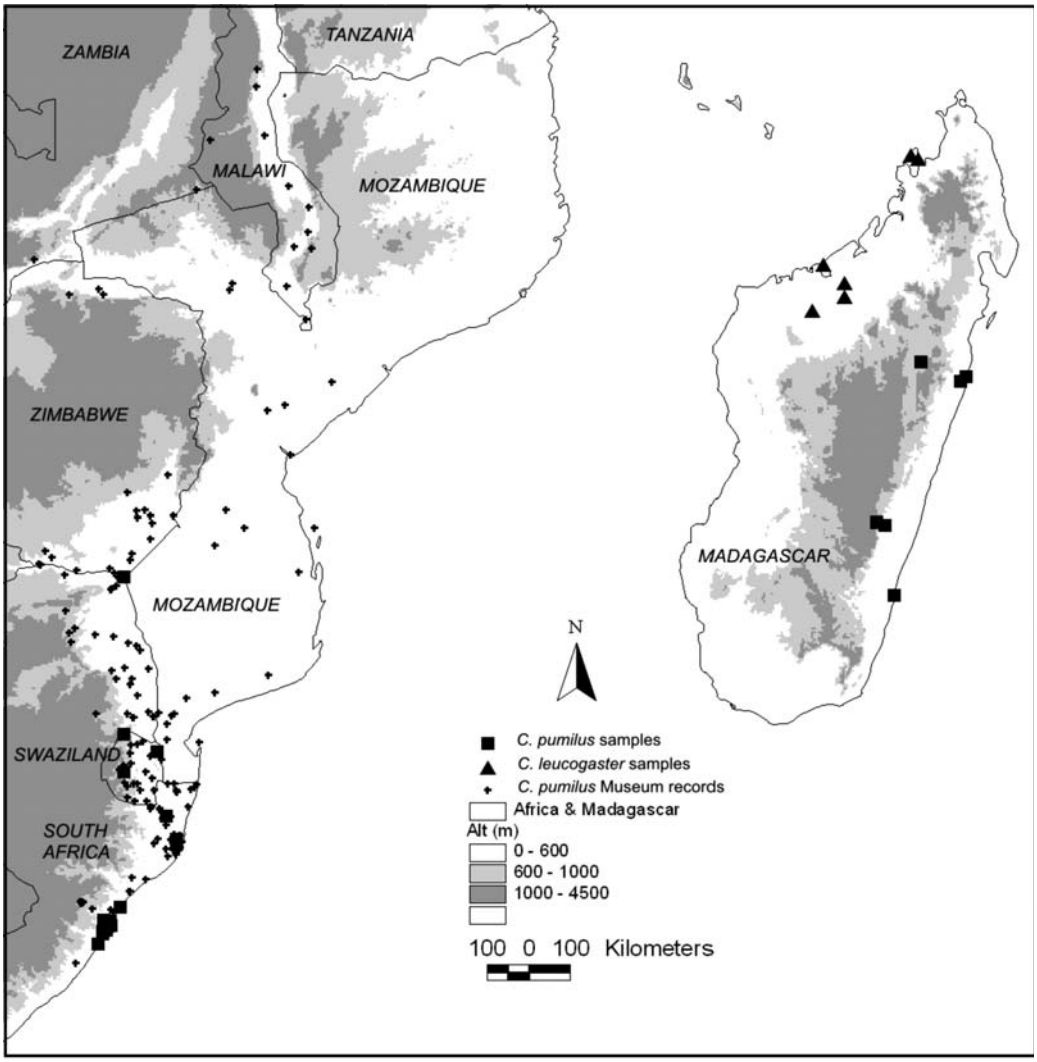

Fig. 1. Map of southeastern Africa and Madagascar showing distribution of samples of *Chaerephon* used in the study (closed squares for *C. pumilus* and closed triangles for *C. leucogaster*) in relation to specimen records (crosses) obtained from museum collections (Monadjem *et al.*, in press; Goodman & Ratrimomanarivo, unpubl. data).

north. In the case of Hell's Gate and Charter's Creek of Lake St Lucia, there are no bordering hills or escarpments, separating these localities from the Indian Ocean (Fig. 1, Table 2).

Samples of muscle or liver were collected from *C. pumilus* individuals for DNA sequencing (D-loop: $n = 34$; cytochrome *b*: $n = 11$). When available, associated voucher specimens were deposited in the Durban Natural Science Museum (Table 1). For comparison, and to contextualize local genetic differences, we also included three samples each of *C. pumilus* and *C. leucogaster* from Madagascar (Table 1). As an outgroup, we used a single sample of *Mops midas* from the study of Ratrimomanarivo *et al.* (2007) (GenBank Accession number: EF 474034).

Molecular analyses

The genetic variation of *Chaerephon* was investigated using DNA sequences of mitochondrial D-loop ($n = 34$) and cytochrome *b* ($n = 11$). DNA was isolated from liver, heart, kidney, or muscle tissues preserved in 80% ethanol or EDTA using a DNeasy® DNA isolation kit (QIAGEN).

The cytochrome *b* gene was amplified as two overlapping double-stranded fragments (Saiki

Table 1. Details of specimens included in study of D-loop and cytochrome *b* sequences. Specimens with asterisks indicate specimens whose skulls were used for craniometric analysis. Apart from Kruger National Park, or where otherwise noted (Swaziland [= SZ] and Madagascar [= MD]), localities fall within the KwaZulu Natal Province of South Africa. Specimens are from the Durban Natural Science Museum (DM) and Field Museum of Natural History (FMNH). CROW = Centre for Rehabilitation of Wildlife.

Museum no.	Lab code	Locality	Genbank no. D-loop	Cyt-*b*	Lat. & long. (decimal degrees)	Clade	D-loop	Cyt-*b*	Sex
Chaerephon pumilus									
DM 7363	D1	Durban Int. Airport	FJ415824	FJ415813	29.967S, 30.942E	A1	X	X	F
*DM 7367	D2	Hell's Gate (Lake St Lucia)	FJ415826	FJ415814	28.067S, 32.421E	A1	X	X	F
*DM 7368	D3	Hell's Gate	FJ415825	–	28.067S, 32.421E	A1	X		M
*DM 7369	D4	Hell's Gate	FJ415837	–	28.067S, 32.421E	A1	X		F
*DM 7370	D5	Hell's Gate	FJ415838	–	28.067S, 32.421E	A1	X		F
*DM 7371	D6	Hell's Gate	FJ415839	–	28.067S, 32.421E	A1	X		F
*DM 7372	D7	Hell's Gate	FJ415827	–	28.067S, 32.421E	A1	X		M
*DM 7373	D8	uMkhuze Game Reserve	FJ415828	FJ415815	27.583S, 32.217E	A1	X	X	F
*DM 7374	D9	uMkhuze Game Reserve	FJ415829	FJ415816	27.583S, 32.217E	A1	X	X	M
*DM 7378	D11	Durban: 13 Bunting Place, Amanzimtoti	FJ415830	–	30.05 S, 30.833E	A1	X		M
*DM 7381	D14	Hell's Gate (Captive born to DM 7382)	FJ415841	–	28.067S, 32.421E	A1	X		F
*DM 7382	D15	Hell's Gate	FJ415831	–	28.067S, 32.421E	A1	X		F
DM 7384	D17	Hell's Gate	FJ415832	–	28.067S, 32.421E	A1	X		M
No number	D42	Hell's Gate	FJ415835	–	28.067S, 32.421E	A1	X		?
No number	D39	Durban	FJ415842	–	29.867S, 31.00E	A1	X		?
*DM 7385	D18	Durban: Bluff	FJ415836	–	29.933S, 31.017E	A1	X		F
*DM 7387	D20	Durban: Bluff (Captive born to DM 7384)	FJ415840	–	29.933S, 31.017E	A1	X		M
DM 7401	D22	Durban: Athlone Park, Amanzimtoti	FJ415843	–	30.05 S, 30.883E	A1	X		?
DM 7913	D30	Durban: Illovo	FJ415833	–	30.1 S, 30.833E	A1	X		F
DM 7525	D23	Charters Creek (Lake St Lucia)	–	FJ415819	28.2 S, 32.417E	A1		X	M
DM 7922	D31	SZ: Mlawula	–	FJ415820	26.192S, 32.005E	A1		X	?
DM 8036	D35	SZ: Mlawula	FJ415834	FJ415821	26.192S, 32.005E	A1	X	X	M
DM 7851	D26	Durban: Umbilo	FJ415844	–	29.833S, 31.00E	A2	X		?
No number	D40	Durban: Yellowwood Park	FJ415845	–	29.917S, 30.933E	A2	X		?
*DM 7377	D10	Durban: Kissen Lane, Amanzimtoti	FJ415846	–	30.05 S, 30.883E	B1	X		F
*DM 7379	D12	Durban: Morningside	FJ415848	FJ415817	29.833S, 31.00E	B1	X	X	F
*DM 7380	D13	From CROW rehab. centre, Durban	FJ415849	FJ415818	Unknown	B1	X	X	F
*DM 7383	D16	From CROW rehab. centre, Durban	FJ415850	–	Unknown	B1	X		M
*DM 7386	D19	Ballito (captive born)	FJ415847	–	29.533S, 31.217E	B1	X		M
*DM 7907	D28	Durban: Carrington Hts	FJ415852	–	29.883S, 30.967E	B1	X		M
*DM 7910	D29	Pinetown, Underwood Rd	FJ415853	–	29.817S, 30.85E	B1	X		F
*DM 8030	D34	Park Rynie, Ocean View Farm	FJ415854	–	30.317S30.733E	B1	X		M
DM 7905	D27	Durban: Athlone Park	FJ415851	–	30.016S, 30.917E	B1	X		?
DM 8348	D37	Durban City Hall	FJ415855	–	29.858S, 31.025E	B1	X		M

Continued on p. 59

Table 1 (*continued*)

Museum no.	Lab code	Locality	Genbank no. D-loop	Cyt-*b*	Lat. & long. (decimal degrees)	Clade	D-loop	Cyt-*b*	Sex
DM 8437	D38	SZ: Rosecraft	–	FJ415823	26.632S, 31.293E	B2a		X	?
*DM 8042	D36	SZ: Wylesdale	FJ415856	FJ415822	25.819S, 31.292E	B2a	X	X	F
No number	D43	Durban	FJ415857	–	Not available	B2a	X		?
No number		Kruger NP	AY347954	–	22.417S, 31.3E	B2a	X		?
No number		Kruger NP	AY347955	–	22.417S, 31.3E	B2a	X		?
FMNH 187816		MD: Fanandrana	–	–	18.252 S, 49.268E	–		X	F
FMNH 185322		MD: Ifanadiana	–	–	21.307 S, 47.636E	–		X	F
FMNH 184656		MD: Ambatondrazaka	–	–	17.830 S, 48.419E	–		X	F
FMNH 185260		MD: Farafangana	–	–	22.821 S, 47.831E	–	X		M
FMNH 187799		MD: Toamasina	–	–	18.141 S, 49.378E	–	X		M
FMNH 188088		MD: Ranomafana	–	–	21.258 S, 47.456E	–	X		F
Chaerephon leucogaster									
FMNH 184924		MD: Ambalanjanakomby	–	–	16.701 S, 46.072E	–		X	M
FMNH 184979		MD: Ankazomborona	–	–	16.116 S, 46.757E	–		X	F
FMNH 188644		MD: Nosy Komba	–	–	13.443 S, 48.348E	–		X	F
FMNH 184974		MD: Ankijabe	–	–	16.414 S, 46.765E	–	X		F
FMNH 188500		MD: Dzamadzar	–	–	13.353 S, 48.192E	–	X		F
FMNH 184608		MD: Mahajanga	–	–	15.713 S, 46.313E	–	X		M
Mops midas									
FMNH 184306		MD: Sakaraha	EF 474034	–	22.907S, 44.530E	–	X	X	?

et al. 1988). The 5′ fragment was amplified with primers L14723 (5′-ACCAATGCAATGAAAAAT CATCGTT-3′) and H15553 (5′-TAGGCAAATAGG AAATATCATTCTGGT-3′), whilst the 3′ fragment was amplified using L15146 (5′-CATGAGGAC AAATATCATTCTGAG-3′) and H15915 (5′-TCT CCATTTCTGGTTTACAAGAC-3′) (Irwin *et al.* 1991).

Polymerase Chain Reaction (PCR)-amplifications were performed in 25 μl reaction volumes each consisting of 9 μl genomic DNA solution (containing 30 ngμ DNA), 0.8 μl sterile water, 2.5 μl 10 × reaction buffer (Super-Therm), 4 μl $MgCl_2$ (25 mM) (Super-Therm), 0.5 μl dNTP mix (10 mM) (Roche Diagnostics), 0.2 μl *Taq* polymerase (5 U/μl) (Super-Therm) and 4 μl each of forward and reverse primer (6 μM). Thermal cycling parameters consisted of an initial denaturation step at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 60 s, annealing at 50°C for 90 s, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min.

Table 2. Summary of topographic, biome and climatic features of sampled localities.

Locality	Altitude	Annual rainfall	Topography	Biome
Durban	<600 m	>900 mm	Close to escarpment edge	Savanna
uMkhuze Game Reserve	<600 m	<700 mm	Foothills of Lebombo Mts	Savanna
Lake St Lucia, W Shores (Hell's Gate, Charter's Creek)	<600 m	>900 mm	No topography	Savanna
Swaziland 'Highveld' (Rosecraft, Wylsdale)	>600 m	>1000 mm	Highlands	Savanna
Swaziland 'Lowveld' (Mlawula)	<600 m	<700 mm	Foothills of Lebombo Mts	Savanna
Kruger National Park	<600 m	<700 mm	No notable topography	Savanna

The 5′ hypervariable region of the D-loop (HV1) was PCR-amplified as a single fragment using primers P (5′-TCCTACCATCAGCACCCAAAGC 3′) and F (5′-GTTGCTGGTTTCACGGAGGTAG 3′) (Wilkinson & Chapman 1991). Where samples failed to amplify using this primer set, primer set P andE (5′- CCTGAAGTAGGAACCAGATG -3′) were used, as F is nested within E (Wilkinson & Chapman 1991). PCR-amplifications were performed in 25 ml reaction volumes, in the manner described above. Thermal cycling parameters consisted of initial denaturation at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 90 s, extension 72°C for 2 min, and a final extension step at 72°C for 7 min. Negative controls (lacking in DNA template) were used to ensure that contaminating DNA was not being amplified.

For both cytochrome *b* and D-loop amplifications, target fragments were purified from excised gel bands using a QIAquick® Gel Extraction Kit (QIAGEN Inc.). Purified DNA fragments were sequenced in the forward and reverse directions using the primers used for the initial amplifications. Sequencing was performed by Inqaba Biotechnical Industries, South Africa.

Sequences were aligned using the CLUSTAL W option (Thompson *et al.* 1994) in BioEdit version 5.0.9 (Hall 1999) modified by eye. Aligned sequences were cropped to a common length of 314 base pairs for D-loop and 845 base pairs for cytochrome *b.*

Modeltest 3.7 (Posada & Crandall 1998) was used to select (under the AIC criterion) the model of nucleotide substitution which best fit the cytochrome *b* and the D-loop sequence datasets. The optimal model selected as appropriate for both datasets was the HKY+I model.

Bayesian analysis of cytochrome *b* and D-loop data was implemented in Mr Bayes version 3.0 (Ronquist & Huelsenbeck 2003). Four Markov chains were run for 15 million generations each, and the first 500 000 trees were discarded as burn-in. The burn-in value was determined by inspection, and exceeded the number of generations needed to achieve stationarity. The default values for the four incrementally-heated Markov chains were used. The priors for the five active parameters were: 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 branch lengths = branch lengths are unconstrained: exponential (10.0). The resultant phylograms were 50% majority-rule consensus trees.

In some inter-specific analyses, a hierarchical tree format may be inappropriate for representing relationships among haplotypes because the period over which the samples have evolved is so short that historical dispersal and/or incomplete lineage sorting manifests in shared haplotypes (Posada & Crandall 2001; Kratysberg *et al.* 2004). In such instances, a haplotype network is more appropriate to show relationships among the sampled haplotypes by using multiple pathways to illustrate possible recombination, homoplasy or reverse mutations. We constructed statistical parsimony haplotype networks from D-loop sequence data using TCS 1.21 (Clement *et al.* 2000). During computer runs, the connection limit was set at 40 steps in order to ensure the joining of sub-networks. Gaps were treated as the fifth state.

In order to test for significant geographic molecular variance structure, the D-loop dataset excluding gaps (n =314 bp) was analysed by hierarchical Analysis of Molecular Variance (AMOVA) using the program Arlequin 3.01 (Excoffier *et al.* 2006). Data were grouped into six geographic localities: Durban, Lake St Lucia (Hell's Gate), uMkhuze Game Reserve, Kruger National Park, Mlawula (Swaziland) and Wylesdale (Swaziland).

Fixation indices were calculated (for individuals and populations) 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 and populations. After each permutation round, all statistics were recomputed to obtain their null distributions.

The D-loop dataset was also used for population genetic and demographic analyses performed separately for each of three major genetically-defined southern African clades (Clades A1, B1 and B2a). Following Rogers & Harpending (1992), Petit *et al.* (1999) and Russell *et al.* (2005), we used haplotype (h) and nucleotide (π) diversity values, neutrality tests (F_s, Fu 1997, and D* and F*, Fu & Li 1993), 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 4.10 (Rozas *et al.* 2003); to be conservative, we did not consider gaps (i.e. indels). Based on the distribution of pairwise nucleotide differences, the time since expansion, tau (τ), could be calculated in mutational units. Data from captive bats (E.J. Richardson and W. White, pers. comm.) indicate an estimated average generation time of approximately two years for the species. Given this generation time and using minimum and maximum estimates of D-loop mutation rates calculated for noctule bats (*Nyctalus noctula*; which are similar in life history and flight capabilities to molossid bats) based on divergence rates per million years of 6.3% ($\mu = 0.63 \times 10^{-7}$ per site generation) and 25% ($\mu = 2.5 \times 10^{-7}$ per site per generation) (Petit *et al.* 1999), 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 (314 bp), and t was the time (in generations) since expansion.

Morphometric analysis

Using a dial callipers with 0.01 mm precision, 12 craniometric variables were measured on 22 adult skulls (individuals with fused basioccipital sutures, fully erupted dentition and discernible molar wear) of *C. pumilus* in the Durban Natural Science Museum collection of known D-loop sequence, belonging to Clades A1 (n =13), B2a (n =1) and B1 (n =8) defined by the molecular study (see Results). To correct for the possibility of different growth rates due to captivity, three adult individuals born in captivity (two of the A1 group and one of the B1 group) were excluded from the morphological analysis. The 12 craniometric variables (defined in detail by Freeman 1981) included: 1) greatest length of skull (GLS), 2) condylobasal skull length (CBL), 3) palatal length (PAL), 4) zygomatic width (ZYG), 5) mastoid breadth (MAST), 6) braincase width, measured at posterior root of zygomatic arch (BCW), 7), height of braincase (HBC), 8) rostral width (ROSW), 9) inter-orbital width (IOW), 10) maxillary width between outer crowns of upper M3s (M3M3), 11) upper toothrow length from anterior surface of canine alveolus to posterior alveolus of M3 (CM3), and 12) mandible length (MDL). To correct for size differences between variables, natural logarithm-transformed variables were used for multivariate analyses. We first tested for sexual dimorphism by subjecting the largest group sample (Clade A1) to t-tests for each of the 12 craniometric variables and to discriminant function analysis of all variables with samples grouped by sex. In order to test for significant craniometric divergence between the A (A1) and B (combining B1 and B2a) molecular clades, we used a forward step-wise discriminant function analysis using the programme XLSTAT (Addinsoft 2007).

RESULTS

Molecular analysis

Phylogenetic (Bayesian) analysis of both cytochrome *b* and D-loop sequences revealed paraphyly in *Chaerephon pumilus* and *C. leucogaster* as currently defined in the taxonomic sense (Peterson *et al.* 1995; Taylor 1999a; Simmons 2005) (Fig. 2). In both cases, Malagasy samples identified as *C. pumilus* formed a sister clade to a clade comprising *C. leucogaster* from Madagascar and all southern African *C. pumilus* samples. While the former clade was well supported (posterior probability 1.00) by both analyses, the latter clade was better supported by the cytochrome *b* data (probability 0.94) than by the D-loop data (probability 0.70). The second major clade could be subdivided into two clades: 1) a well supported Clade A (probability 0.94 and 0.99 for cytochrome *b* and D-loop, respectively), which comprised mostly lower-lying, northeastern KwaZulu-Natal and eastern Swaziland localities, as well as a few Durban localities, and 2) a poorly supported Clade B (probabilities 0.70, 0.63 for cytochrome *b* and D-loop, respectively), which included both *C. leucogaster* and *C. pumilus* from the southern African localities of Durban and 'inland' (Kruger National Park and 'highveld' localities in western Swaziland). D-loop sequences permitted more detailed phylogenetic analysis, which revealed poorly to well supported subclades within A (A1 and A2) (probabilities 0.99, 1.00) and B (B1, B2a and B2b [probabilities 0.89, 0.99, 0.98]; where B2b comprises *C. leucogaster*) (Fig. 2b). Clades A and B are separated by an uncorrected cytochrome *b* genetic distance of 0.9%. Clade B2b (*C. leucogaster*) is separated from Clade B2a by a distance of 0.6% and from Clade A1 by a distance of 0.7%.

Cytochrome *b* sequences comprised four southern African haplotypes of which two included all northern KwaZulu-Natal specimens (Hell's Gate,

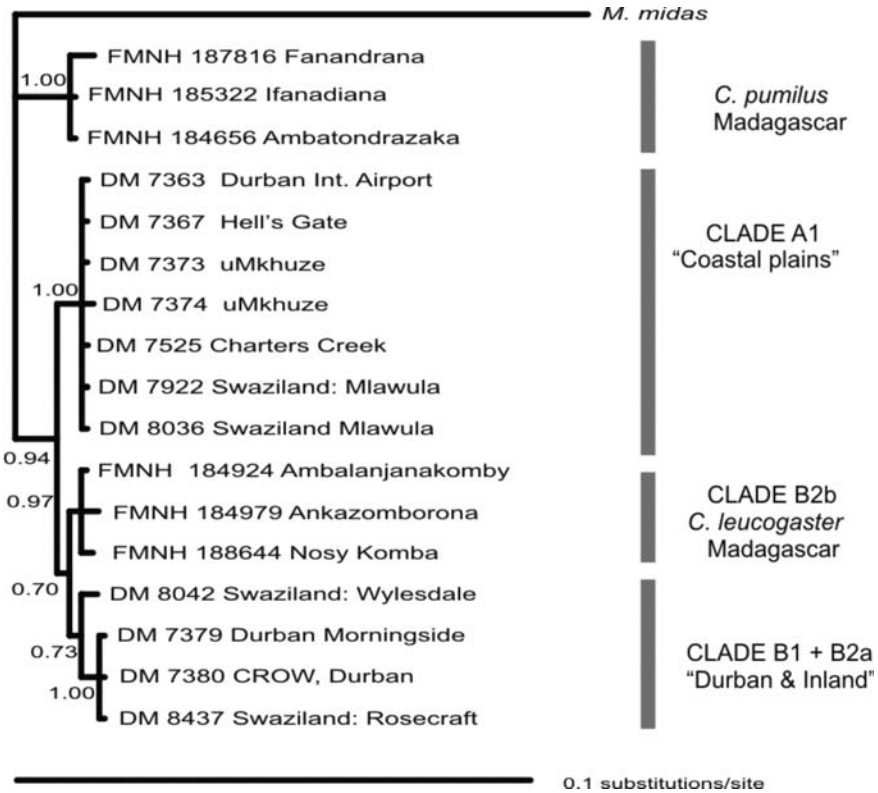

Fig. 2. **a**, Bayesian phylogram of African *Chaerephon pumilus* (*n* = 11) in relation to Malagasy *C. leucogaster* (*n* = 3) and *C. pumilus* (*n* = 3) based on 845 nucleotides of the mitochondrial cytochrome *b* region (ngen = 5000000, burnin = 10000). CROW = Centre for Rehabilitation of Wildlife, Durban, South Africa. DM = Durban Natural Science Museum. FMNH = Field Museum of Natural History, Chicago. Branch lengths are proportional to the amount of evolutionary change along each lineage. Values at nodes represent posterior probabilities. *Continued on p. 63*.

uMkhuze and Charter's Creek), a few Durban specimens and the easterly 'lowveld' (lower elevation) Swaziland locality of Mlawula, whilst the other two were restricted to southern KwaZulu-Natal (Durban metropolitan region) and two westerly 'highveld' (higher elevation) localities of Rosecraft and Wylesdale in Swaziland (Fig. 3a).

D-loop sequences comprised 13 haplotypes (Table 3), calculated using DnaSP (i.e. excluding indels), which could be categorized into five groups coinciding with the major clades and subclades defined above (A1, A2, B1, B2a, B2b). Significantly, clades are distinguished by a relatively high number of mutational steps (10-33), whereas haplotype differences within clades are typically distinguished by only single steps. In the clades with the largest number of sequenced individuals, A1 and B1, haplotypes form star-like configurations with one common, central haplotype from which rarer haplotypes usually separated by 1 or 2 steps. Divergent A2, B1 and B2a clades are present sympatrically within the greater Durban metropolitan area (Fig. 3b).

Results of AMOVA from D-loop data indicated significant geographic variation (Fst = 0.432, $P < 0.001$). Thus, 43.2% of the molecular variance was explained by differences among localities whilst 56.8% was explained by within-population variation.

Results of molecular diversity and neutrality tests for the three major southern African clades (A1, B1 and B2a) are shown in Table 4. Clades A1 and B1, but not Clade B2a, show evidence (e.g. high S/d, significant Fs and a unimodal mismatch distribution; Fig. 4, Table 4) of an historical population expansion, dated at some 3300–13 000 years ago for Clade A1 and 14 700–60 000 years ago for Clade B1.

Morphometric analysis

Since the results were non-significant ($P > 0.05$) for sexual dimorphism in *C. pumilus* for all

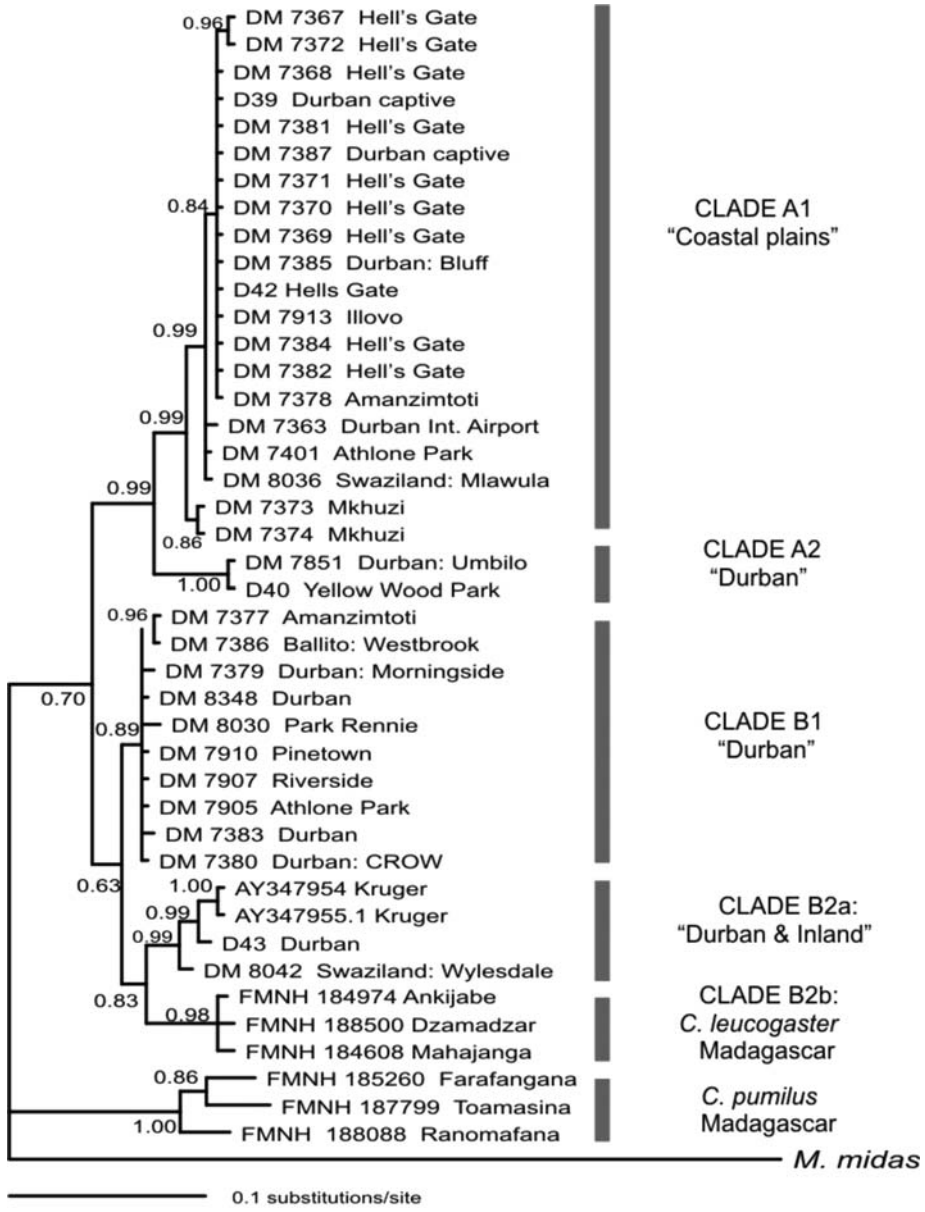

Fig. 2 (*continued*)**. b**, Bayesian phylogram of African *Chaerephon pumilus* ($n = 36$) in relation to Malagasy *C. leucogaster* ($n = 3$) and *C. pumilus* ($n = 3$) based on 314 nucleotides of the mitochondrial D-loop region (ngen = 5 000 000 , burnin = 10 000). CROW = Centre for Rehabilitation of Wildlife, Durban, South Africa. DM = Durban Natural Science Museum. FMNH = Field Museum of Natural History, Chicago. D = Laboratory number assigned to specimens which have not yet been accessioned. AY = Genbank accession numbers. Branch lengths are proportional to the amount of evolutionary change along each lineage. Values at nodes represent posterior probabilities.

univariate and multivariate tests, we combined sexes for all samples in the subsequent analyses. Forward stepwise discriminant functions analysis (DFA) of 12 ln-transformed variables (Fig. 5) selected just two variables (braincase width, BCW and upper maxillary tooth row, CM3), which collectively explained significant craniometric differentiation of Clades B and A (Wilks Lambda = 0.525; $P = 0.002$; 82% of individuals assigned to correct clade). A *t*-test revealed that mean braincase width was significantly greater ($t = 3.25$, $P < 0.01$) in Clade A (8.6 mm) than Clade B (8.4 mm). In

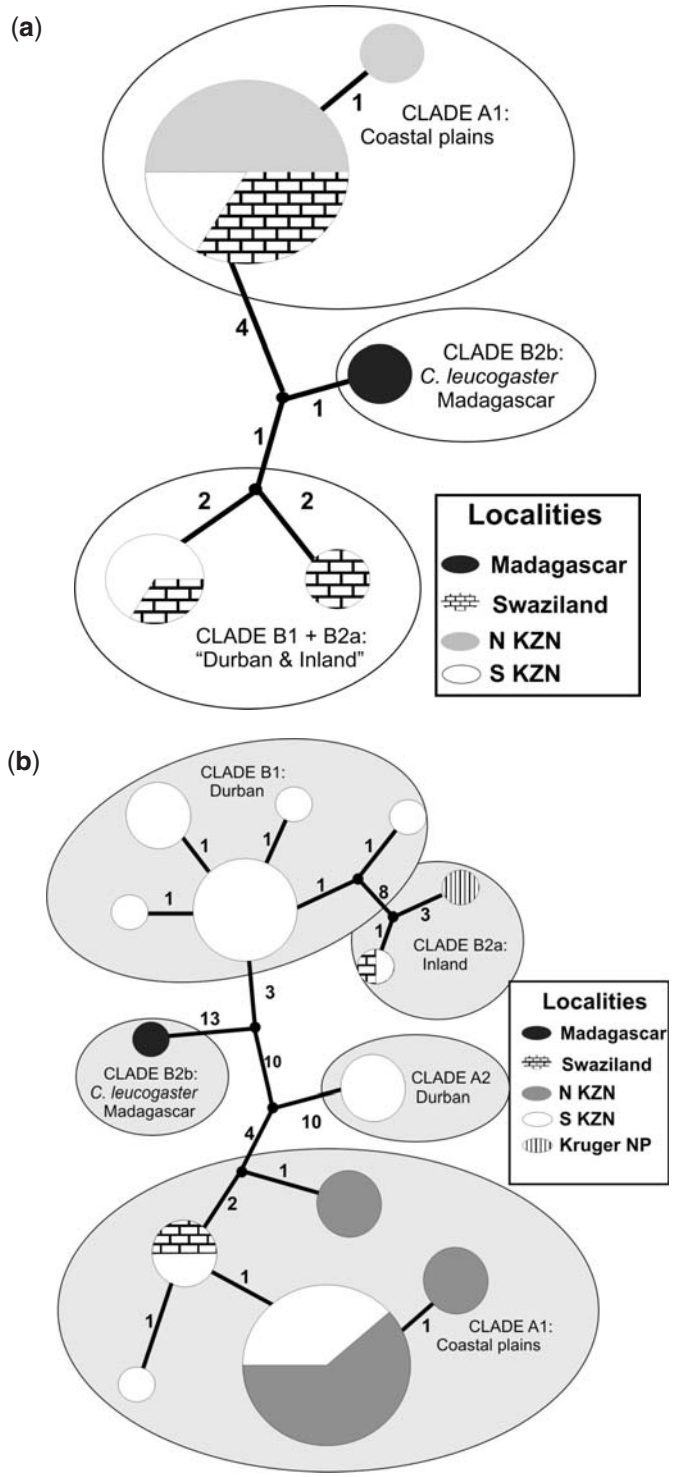

Fig. 3. **a,** Statistical parsimony network of cytochrome *b* data for African *Chaerephon pumilus* and Malagasy *C. leucogaster.* Circles are proportional to the number of specimens. **b**, Statistical parsimony network of D-loop data for African *Chaerephon pumilus* and Malagasy *C. leucogaster.* Circles are proportional to the number of specimens.

Table 3. Variable sites of 13 D-loop haplotypes, based on analysis of 312 base pairs (excluding indels) (total length with indels = 403), derived from 34 samples of *Chaerephon pumilus* from southern Africa (Abbreviations: Hap, haplotype number, *N* = number of samples). Presentation of locality information is not consistent with regards to localities, province, etc.

Taxon & Clade	Hap	*N*	Variable sites (out of 274)	Localities
C. pumilus Clade B2a	1	2	ATAAGACCCTGAATATTGCGTGTCTTCTTTTGAATTACAA	Kruger NP
	11	1	........T...G...C....A.................G	Swaziland (Wylesdale)
	12	1	............C..C....A..................	KZN (Ballito)
Clade B1	2	8	........T.A.....CATA.A.TA..C....G..C...G	Durban
	4	1	........T.A.....CATA.A.TA..C....G..C.A.G	Durban
	10	1	........T.A.....CATA.A.TA..C.......C...G	Durban:
Clade A1	3	11	.CGGC.TTT.AGG.GC.AT.CAC.ACTC....G.AC....	Hell's Gate, Durban
	5	1	GCGGC.TTT.AGG.GC.AT.CAC.ACTC....G.AC....	Hell's Gate
	6	1	.CGGC.TTT.AGG.GC.A..CAC.A.TC....G.AC.T..	Durban
	7	2	.CGGC.TTT.AGG.GC.AT.CAC.A.TC....G.AC....	Durban, Swaziland (Mlawula)
	9	2	CCGGC.TTT.AGG.GC.AT.CAC.ACTC....G.AC....	Hell's Gate
	13	2	CCGGC.TTT.AGG.GC.AT.CAC.ACTC....G.AC....	Mkhuze
Clade A2	11	2	CCGGC.TTT.AGG.GC.AT.CAC.ACTC....G.AC....	Durban

order to exclude the possibility that observed differences were not due to ontogenetic (age-related) variation, and assuming that skull size is a good predictor of age (Morris 1972) we regressed BCW against skull size (CBL) for both Clade A and Clade B samples; the resultant plots (Fig. 6) show: 1) that braincase width is significantly explained ($r^2 = 0.59$, $P < 0.01$) by skull size in Clade B but not Clade A ($r^2 = 0.14$, $P > 0.05$); and 2) at a given size (age) braincase width is proportionately greater in Clade A than Clade B.

DISCUSSION

Phylogeny and phylogeography of southern African *Chaerephon*

Ongoing research based on mitochondrial DNA sequences suggests that *C. leucogaster* and *C. pumilus* may be paraphyletic taxa with *C. leucogaster* nested within populations currently attributed to *C. pumilus* (Ratrimomanarivo *et al.*, in press (a,b)). In order to establish the phylogenetic relationships of our study sample of southeastern African *C. pumilus,*

Table 4. Neutrality statistics for three defined major clades of southern African *Chaerephon* based on D-loop sequences.

	Clade A1 ($n = 20$)	Clade B1 ($n = 10$)	Clade B2a ($n = 4$)	Expectation[#]
Nucleotide diversity (Pi)	0.00399	0.00368	0.01062	Low
Haplotype diversity (*h*)	0.574	0.756	0.833	High
Expansion coefficient (*S/k*)	4.789	4.325	1.800	High
Fu & Li's (1993) F*	0.18541	–1.52186	0.17272	Not significant
Fu & Li's (1993) D*	0.54727	–1.34803	0.17969	Not significant
Fu & Li's (1993) Fs	–0.540	–1.896	0.888	Significant
Raggedness (*r*)	0.0913	0.1151	0.3056	Not significant
Mismatch distribution	Unimodal	Unimodal	Multimodal	Unimodal
Tau (τ)	0.252	1.156	2.968	–
Time since expansion (yr BP[†])	3 360–12 956[†]	14 726–59 434[†]	–	–

[#]Expected trends for a model of demographic population expansion (Hull & Girman 2005)

[†]Values obtained from formula $\tau = 2ut$, following Rogers & Harpending (1992) and Petit *et al.* (1999), where u was the product of mutation rate (μ) per generation (D-loop mutation rates taken from Petit *et al.* (1999) for the bat *Nyctalus noctula*: 6.3% to 20% divergence per million years, or $\mu = 0.63 \times 10^{-7}$ to 2.0×10^{-7} mutations per site per generation) multiplied by sequence length (314 bp) and t was the time (in generations) since expansion (generation time taken as two years).

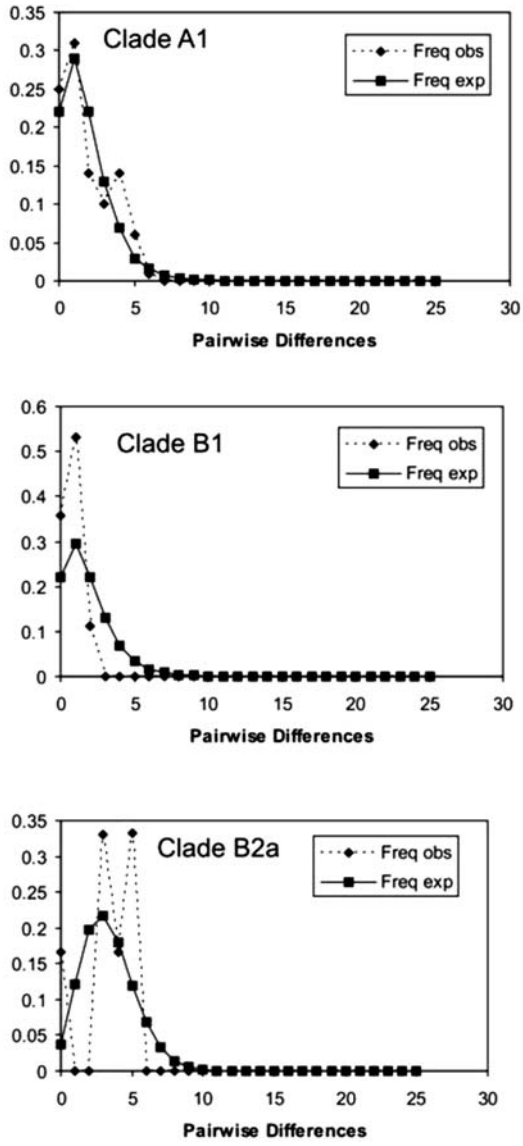

Fig. 4. Mismatch coefficients for Clades A1 and B1 and B2a of *Chaerephon pumilus* from southern Africa.

we thus included material of *C. leucogaster* and *C. pumilus* from Madagascar. However, detailed taxonomic revision of the Malagasy Region *C. leucogaster* and *C. pumilus* is beyond the scope of this study and is covered by separate ongoing studies (Ratrimomanarivo *et al.*, in press (b), and unpubl.). Within the context of the present study, broader geographic and taxonomic sampling allowed us to gauge the evolutionary and taxonomic importance of observed differences between clades identified from African populations.

Our data show that at least three D-loop clades occur in the greater Durban area (Clades A1, B1 and B2a), whilst only one (Clade A1) is found in the coastal plains and adjacent lowlands of northern KwaZulu-Natal and eastern Swaziland, and one (Clade B2a) occurs in the inland regions of South Africa (Kruger National Park and highlands of Swaziland). Thus, Clade A1 is shared between northeastern lowland localities and the Durban region, whilst Clade B2a is shared between inland localities and the Durban region. Malagasy *C. leucogaster* comprise a subclade (B2b) of Clade B. These data suggest either a) *leucogaster* is conspecific with *pumilus* or b) Clades A1, B1 and B2a are cryptic species. Since *C. leucogaster* is well characterized morphologically (Ratrimomanarivo *et al.*, in press (b)), the latter hypothesis may be preferred; however, much wider genomic and geographic sampling, and consideration of relevant type material, is necessary to resolve the taxonomic status of these clades.

Although the genetic distances between clades is small (0.6–0.9% cytochrome *b* uncorrected divergence; i.e. within the range of values reported to separate east and southern African cytochrome *b* haplotypes of *C. pumilus*: Jacobs *et al.* 2004), it should be noted that clades are separated by a large number (10-33) of mutational steps relative to the much lower within-clade average number of 0.7 (Clade B1), 1.8 (Clade A1) or 3.3 (Clade B2a) pairwise nucleotide differences. Previous studies (Ratrimomanarivo *et al.* 2007; Lamb *et al.* 2006, 2008) on *Mops midas* and *Otomops* spp. also suggest that the mtDNA mutation rate (for both cytochrome *b* and D-loop) is relatively low in molossids; thus good species may be separated at lower cytochrome *b* genetic distances than the average mammalian values suggested by Bradley & Baker (2006). In any event, caution should be exercised in applying the genetic species concept of Bradley & Baker (2006), especially since mammalian mtDNA substitutions rates vary by orders of magnitude between and even within orders (Nabholz *et al.* 2008).

Population genetics and historical demography

As expected, we found significant geographic structuring of *C. pumilus* populations from southern Africa (Fst = 0.445, $P < 0.001$), as also reported in the similar-sized, related molossid species, *C. leucogaster* (Fst = 0.792, $P < 0.001$; Ratrimomanarivo *et al.*, submitted) but not in the larger (and presum-

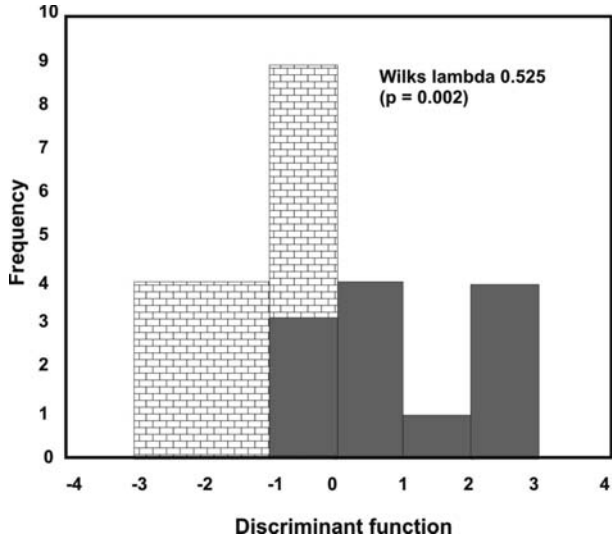

Fig. 5. Frequency histogram of scores from two-group discriminant functions analysis of 10 craniometric measurements taken from museum voucher skulls of southern African *Chaerephon* specimens known to belong to Clades A1 and B1. Brick fill = Clade A1. Shaded fill = Clade B1.

ably more vagile) molossids, *M. midas* (Fst = 0.14; Ratrimomanarivo *et al.* 2007), *M. leucostigma* (Fst = 0.2; Ratrimomanarivo *et al.* 2008) or *Otomops madagascariensis* (Fst = 0.05, Lamb *et al.* 2008).

Neutrality statistics and mismatch distributions provide evidence for an historical expansion in Clades A1 (northern lowland; *c.* 3300–13 000 years BP) and B1 (Durban; *c.* 15 000–60 000 years BP) but not in Clade B2a (predominantly inland). These dates hinge on the accuracy of our mutation rate estimates, which were based on values calculated for noctule bats of similar body size, life history and flight capabilities (Petit *et al.* 1999). Given the high rate of saturation in D-loop at higher taxonomic levels, and the scarcity of recent molossid fossils (McKenna & Bell 1997; Arroyo-Cabrales *et al.* 2002; Jones *et al.* 2005), we could not use our data to independently calculate a realistic

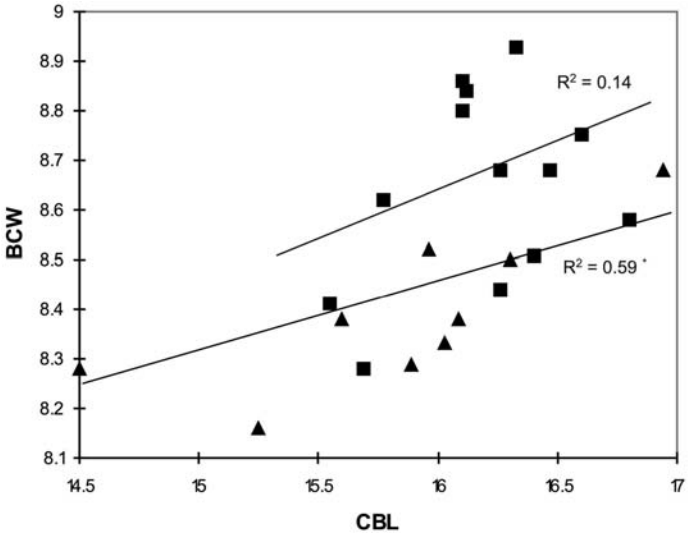

Fig. 6. Scatterplot of braincase width (BCW) *versus* condylobasal skull length (CBL) for specimens of southern African *Chaerephon* known to belong to Clades A1 (squares) and B1 (triangles) based on genetic analyses. Regression lines shown for each clade.

empirical estimate of the D-loop divergence rate.

Based on community ecological analysis of forest-specialist mammalian and bird communities from southeastern Africa, 'scarp forests' (occurring along the eastern seaboard at elevations of 300–500 m) may have acted as major refugia during the Last Glacial Maximum (LGM) around 18 000 years BP, from which recolonisation occurred succeeding this event (Lawes *et al.* 2007). Community patterns further suggested a southward expansion of tropical faunas following the LGM, particularly after the expansion of Indian Ocean coastal forests into the region from 8000 years BP, and subsequent secondary contact between tropical southward-expanding fauna and temperate fauna occupying scarp forest relicts. In terms of chronology, such a scenario is consistent with the estimated dates for historical expansion of *C. pumilus* Clade A1 (coastal populations) between about 3000 and 13 000 years BP, but not with the dates for the expansion of the B1 clade (Durban), which seem to coincide with or just pre-date the LGM (15 000–60 000 years BP). Possibly, these postulated expansion events occurred independently just after the LGM (from nearby scarp forests onto the coast at Durban, where Clade B1 is restricted), and then as a much later (Clade A1) expansion as a component of the tropical fauna expanding southwards from eastern Africa.

As a component of the forest mammal fauna of the region, it is reasonable to assume that climatic events, which shaped community structure, may also have impacted species of forest-associated bats at a population level. Although in southern Africa, *C. pumilus* is currently typically associated with synanthropic roosts, it is also known to roost in natural crevices in trees and exposed rocky formations (Taylor 2000). Prehistorically, the species' distribution would have been dependent on the availability of such natural roosts. The escarpment of eastern southern Africa would have provided natural crevices in exposed rock formations, as well as natural holes in mature trees associated with scarp forests and savannas. Assuming that prior to the LGM the southern limit of the distribution of *C. pumilus* was similar to its present range, i.e. just south of Durban, the effect of the LGM and sea level changes, which submerged much of the low-lying Indian Ocean plain, would have resulted in extinction of lowland populations and survival of populations in relict scarp forest. It is then plausible that *C. pumilus* comprised an element in the tropical mammalian fauna, which followed the southward expansion of Indian Ocean forests from about 8000 years, as Clade A1. Although early Stone Age humans occupied the southeastern coast of Africa for some 1.5 Ma, they were hunter gatherers who dwelt in caves and natural shelters. Iron Age people entered the region between 1500 and 2000 years ago (Laband 1995). Although these people occupied large villages it is unlikely that the architectural style of their huts would have been suitable as roosting sites for *C. pumilus.* Only with the arrival of European settlers in the early nineteenth century were modern dwellings constructed, which may have provided suitable day-roost sites for molossid bats. Thus, it is unlikely that the post LGM expansion of *C. pumilus* was linked with humans.

Our D-loop data show that the predominantly inland Clade B2a of *C. pumilus* lacked evidence for historical expansion, showing instead a multimodal mismatch distribution more typical of stable populations. Thus populations from inland, for example from Kruger National Park and the highlands of Swaziland, may have originated from stable populations, which survived the LGM in relic scarp forests; any populations occupying lower-lying savannas and coastal forest would have been largely extirpated by climatic or sea level changes (Lawes *et al.* 2007). On the other hand, populations comprising Clade A1 could have originated from southward-expanding populations associated with expansion of Indian Ocean coastal forests and their fauna from tropical eastern African refugia, starting about 8000 years BP (Lawes *et al.* 2007). The genetic distinctiveness of Clades A1 and B1 could de due to separate invasions, with the latter occurring either before or just after to the LGM.

The above scenario explains how three divergent clades can occur sympatrically in the Durban region. The Indian Ocean coastal plain is very narrow at Durban, where the escarpment occurs close to the coast and in fact is included in the metropolitan region near the Pinetown suburb, some 14 km west of the coast. North of Durban, the width of the coastal plain escarpment expands dramatically, with the escarpment moving further west from the coast. Thus it is not surprising that Durban should comprise a 'melting pot' which combined populations derived from both inland and coastal clades. Fragmentation of the ancestral population at the LGM (18 000 years BP) would have resulted in allopatric differentiation or extinction in isolated relic populations, and subsequent

secondary contact following the later recolonisation event(s). Whether some 10 000–60 000 years of geographical isolation would result in speciation depends to some extent on the acquisition of phenotypic characters (e.g. in morphology, karyotype or echolocation call), which would result in positive assortment on secondary contact. If Durban is a zone of secondary contact as here suggested, nuclear genetic markers would be very useful to determine the evolutionary fate (e.g. the degree of hybridization) of the lineages. Our dates for historical expansion of Clade A1 correspond closely with those obtained for the related molossid, *C. leucogaster* from the west coast of Madagascar (Ratrimomanarivo *et al.*, in press (a,b)), where morphological characters allow recognition of this clade as a good species.

Morphological evidence for evolutionary lineages

Our preliminary morphological analyses revealed a significant difference in proportional braincase size between the Clades A and B, which suggests that geographical isolation may have been accompanied by adaptive morphological divergence. Further studies in progress will examine cranial variation in our sample of vouchered specimens within the context of much broader geographical samples from the range of *C. pumilus* as well as with reference to the comparison of type and topotypic material from Massawe, Eritrea. Hayman & Hill (1971) summarize the taxonomic complexities inherent in this large assemblage of forms which invites a multidisciplinary and geographically comprehensive taxonomic review and is almost certainly a composite of multiple cryptic species (see for example, recent resurrection of *C. pusillus* from the western Seychelles; Goodman & Ratrimomanarivo 2007).

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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 log-normal 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%.

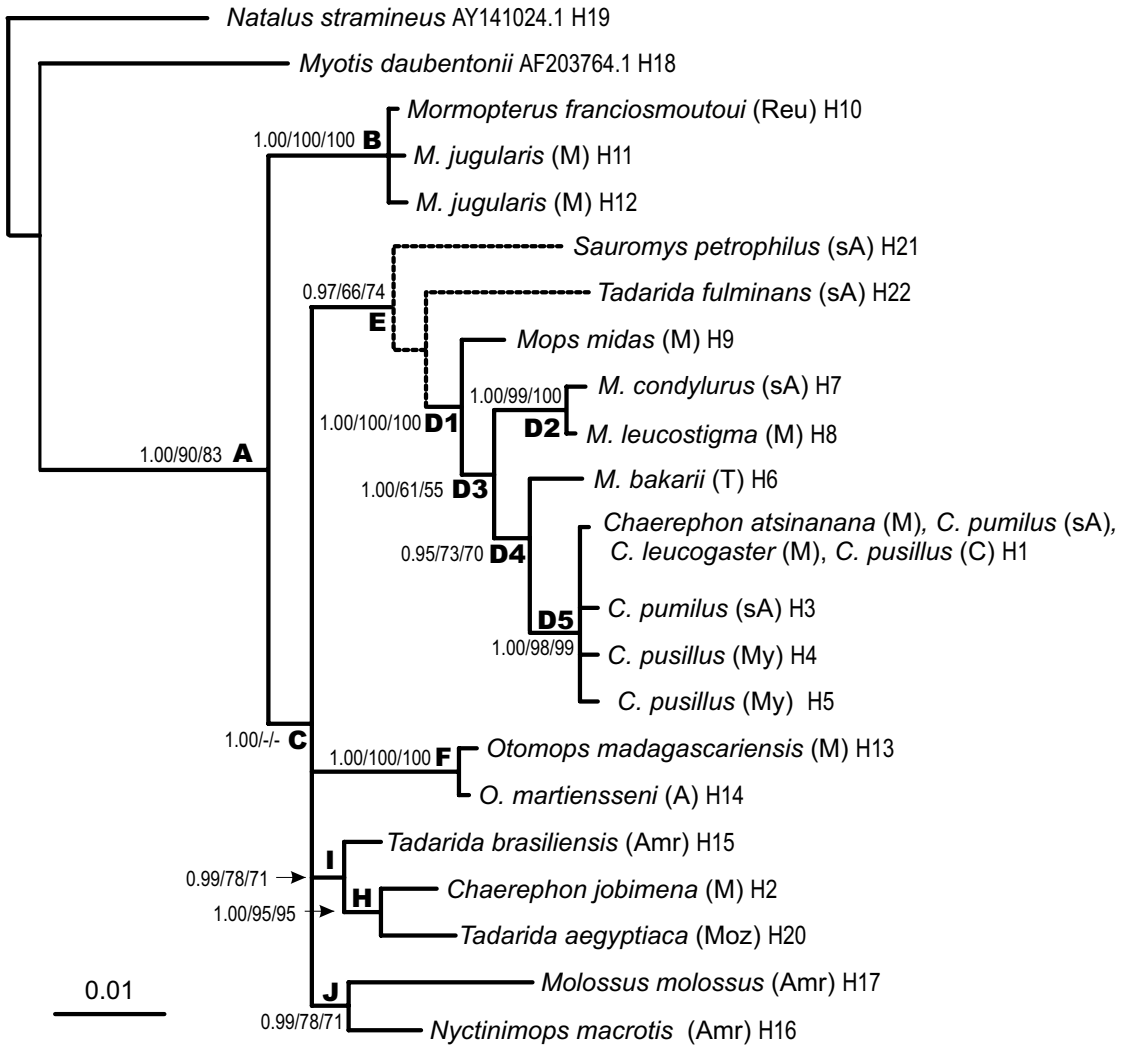

FIG. 1. Bayesian inference tree based on analysis of 1262 nucleotides of the nuclear Rag2 gene illustrating evolutionary relationships between molossid bat haplotypes and non-molossid outgroups. Nodal support values are represented as (Bayesian posterior probability / maximum parsimony bootstrap percent / 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

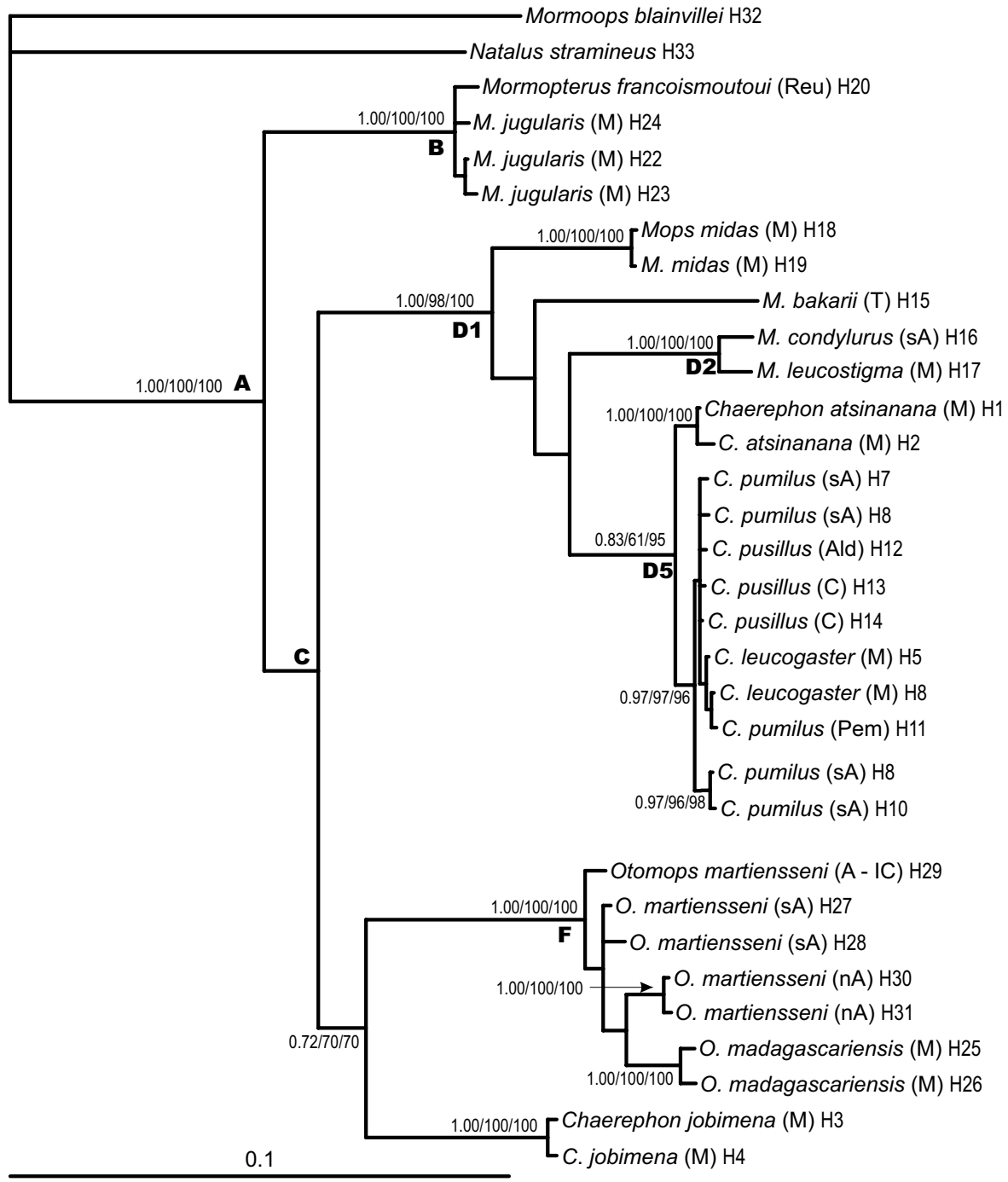

FIG. 2. Bayesian inference tree based on analysis of a concatenated Rag2-cytochrome *b* sequence (2031 nucleotides) illustrating evolutionary relationships between molossid bat haplotypes and non-molossid outgroups. Nodal support values are represented as (Bayesian posterior probability / maximum parsimony 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*

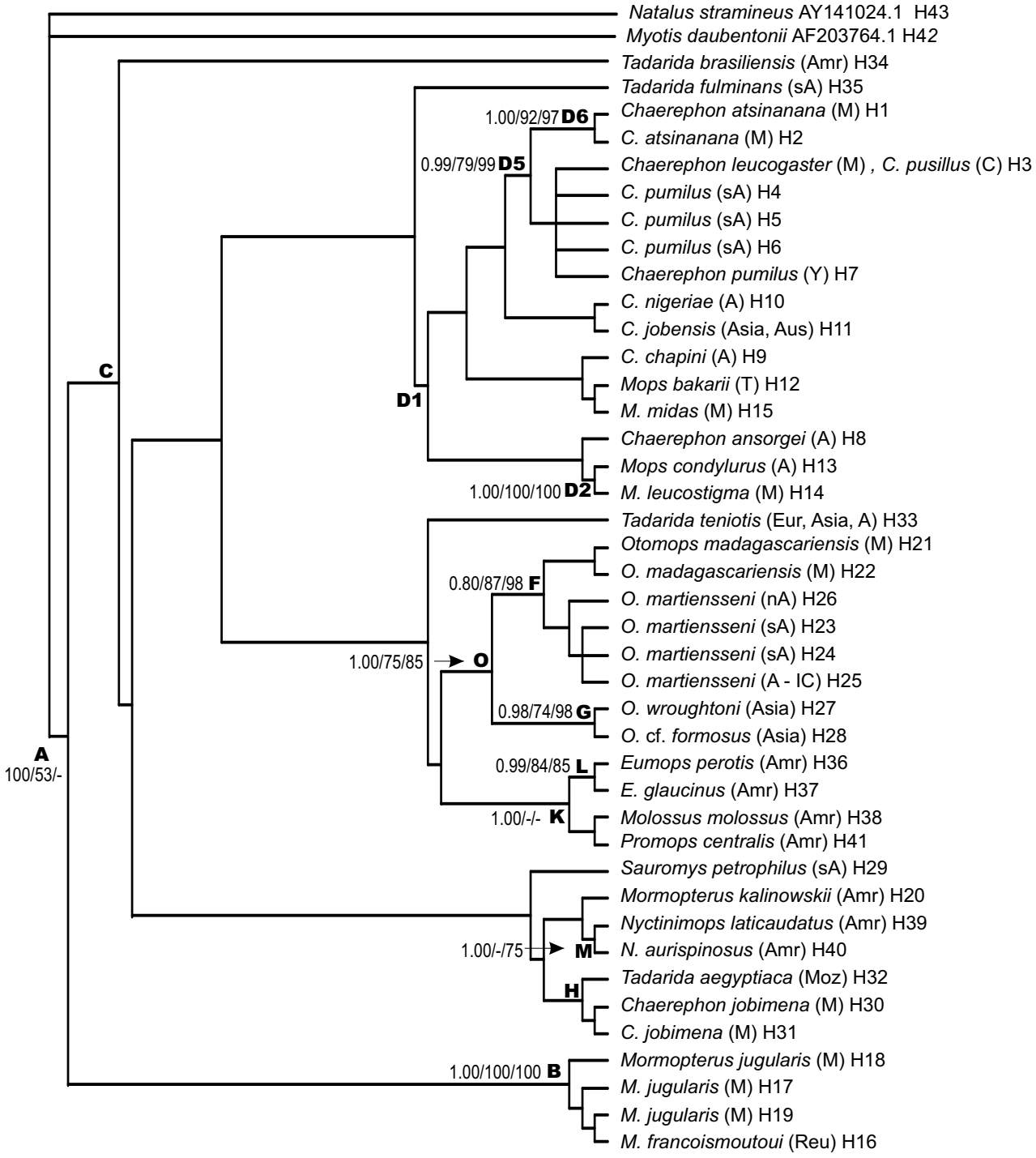

FIG. 3. One of two most likely trees derived from maximum likelihood analysis of 325 nucleotides of the mitochondrial cytochrome *b* gene illustrating evolutionary relationships between molossid bat haplotypes and non-molossid outgroups. Nodal support values are represented as (Bayesian posterior probability / 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.

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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*	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

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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

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Abstract

The species delimitations of African, Arabian Peninsula, and western Indian Ocean island members of the Molossidae bat species complex *Chaerephon pumilus* remain largely unresolved. Based on genetic analyses this group is paraphyletic, with *C. leucogaster* nested within *C. pumilus* sensu lato, and the latter is composed of several distinct clades. DNA was isolated from a specimen of *C. p. pumilus* obtained at the type locality (Massawa, Eritrea). Although incomplete, this sequence allowed us to clearly define which clade is referable to nominate *pumilus*, a critical step in resolving the systematics of this species complex. Using morphological and molecular genetic (cytochrome *b* and D-loop sequences) characters, we establish that *C. leucogaster* and *C.* '*pumilus*' on Madagascar represent two different lineages and that the Malagasy population referred to *C.* '*pumilus*' is specifically distinct from those on Africa, the

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Arabian Peninsula, the Comoros Archipelago, and the western Seychelles (Aldabra). The Madagascar population is here described as a new species, *Chaerephon atsinanana* **sp. nov.** This taxon is common in the eastern portion of Madagascar, particularly in synanthropic settings, across an elevational range from near sea level to 1100 m, and there are no immediate conservation concerns. Members of the *C. pumilus* species complex from the western Seychelles are referable to *C. pusillus*, to which populations from the Comoros (Mayotte, Anjouan, Mohéli, and Grande Comore) are also assigned.

Résumé

Les délimitations du complexe d'espèces *Chaerephon pumilus*, membre des chauves-souris Molossidae de l'Afrique, de la Péninsule Arabique et de l'Océan Indien occidental, sont largement non résolues. D'après les données génétiques, ce groupe est paraphylétique, avec *C. leucogaster* inclus dans *C. pumilus* sensu lato, ce dernier taxon étant composé de plusieurs clades distincts. L'ADN a été isolé à partir d'un spécimen de *C. p. pumilus* obtenu dans la localité type (Massawa, en Erythrée). Bien qu'incomplète, la séquence obtenue nous a permis de définir clairement le clade de référence pour la nomination de *pumilus*, une étape cruciale dans la résolution de la systématique de ce complexe d'espèces. L'utilisation des caractères morphologiques et moléculaire (séquences de cytochrome *b* et de D-loop) nous a permis d'établir que *C. leucogaster* et *C.* '*pumilus*' à Madagascar correspondent à deux lignées différentes et que la population malgache désignée comme *C.* '*pumilus*' est spécifiquement distincte de celles de l'Afrique, de la péninsule arabique, de l'Archipel des Comores et des Seychelles occidentales (Aldabra). Nous nommons cette nouvelle espèce, *Chaerephon atsinanana* **sp. nov**. Cette dernière est commune dans la partie orientale de Madagascar, en particulier comme espèce synanthropique, et présente une distribution altitudinale allant du niveau de la mer jusqu'à 1100 m d'altitude. A l'heure actuelle, l'espèce ne semble pas nécessiter une attention particulière en termes de conservation. Les membres du complexe *C. pumilus* des Seychelles occidentales sont désignés sous le nom de *C. pusillus*, ainsi que les populations des Comores (Mayotte, Anjouan, Mohéli et Grande Comore).

Key words: taxonomy, morphology, molecular genetics, *Chaerephon*, Madagascar, Africa, Comoros, Aldabra

Introduction

As currently configured, the small Molossidae bat, *Chaerephon pumilus* (Cretzschmar 1830-1831), has a broad distribution across much of sub-Saharan Africa and on offshore islands of both the Atlantic and Indian Ocean coasts of this continent, East to the Arabian Peninsula, and on islands in the western Indian Ocean (Bouchard 1998; Harrison & Bates 1991; Simmons 2005). The nominate form was described based on specimens collected at Massawa, Eritrea. On the Arabian Peninsula, this taxon appears limited to Yemen, specifically the portion directly across the Red Sea from Eritrea (Harrison & Bates 1991). Animals currently assigned to *C. pumilus* sensu lato show considerable phenotypic variation with bat taxonomists recognizing 12 different forms (Koopman 1994). In contrast, given the considerable levels of dichromatism in certain populations, particularly associated with wing and pelage coloration, it has been suggested that "attempts at subspecific separation based mainly on colour are profitless" (Hayman & Hill 1971, p. 64).

Seven forms of *C. pumilus* sensu lato have been named from the eastern and southern portions of the African continent and western Indian Ocean islands: *limbata* Peters 1852 (type locality: Mozambique Island, Mozambique, but see Turni & Kock 2008); *naivashae* Hollister 1916 (type locality: Naivasha Station, Kenya); *hindei* (Thomas 1904) (type locality: Fort Hall, Kenya); *elphicki* Roberts 1926 (type locality: southeastern Transvaal, South Africa); *langi* Roberts 1932 (type locality: northern Botswana); *leucogaster* (Grandidier 1869) (type locality: Menabe Region, western Madagascar, neotype locality: Belo sur Mer [Ratrimomanarivo *et al*. 2009a]); and *pusillus* (Miller 1902) (type locality: Aldabra Island, western Seychelles). On the basis of phenetic analyses associated with skull morphology, Peterson *et al*. (1995) proposed to elevate three forms (*hindei*, *limbata*, and *naivashae*) to full species. While this suggestion may indeed be valid, the lack of a phylogenetic context and diagnoses to separate these forms renders their taxonomic conclusion difficult to interpret in light of other potential factors to explain the patterns of morphological variation in this species complex (e.g., Ratrimomanarivo *et al*. 2008). Given apparent high levels of morphological polymorphism in *C. pumilus* sensu lato, overlaid on clinal and geographic variation, it has been rather problematic to sort out the systematics of this putative species complex.

On Madagascar, three *Chaerephon* are currently recognized, *C. leucogaster*, *C. pumilus* sensu lato, and *C. jobimena* - the last-mentioned of these was recently named and is morphologically distinct (Goodman & Cardiff 2004; Ratrimomanarivo *et al.* 2009a; Simmons 2005). In general, *C. leucogaster* is a species occurring in the dry western portion of the island and on western near-shore islands, while *C. pumilus* occurs in more mesic areas of the east (Figure 1). The form *pusillus*, applied to animals from Aldabra, was recently elevated to a full species based on morphological comparisons (Goodman & Ratrimomanarivo 2007). The specific identity of the animals occurring in the Comoros Archipelago (Mayotte, Anjouan, Mohéli, and Grande Comore) remains uncertain, although they have been assigned to *C. pumilus* (Louette 2004).

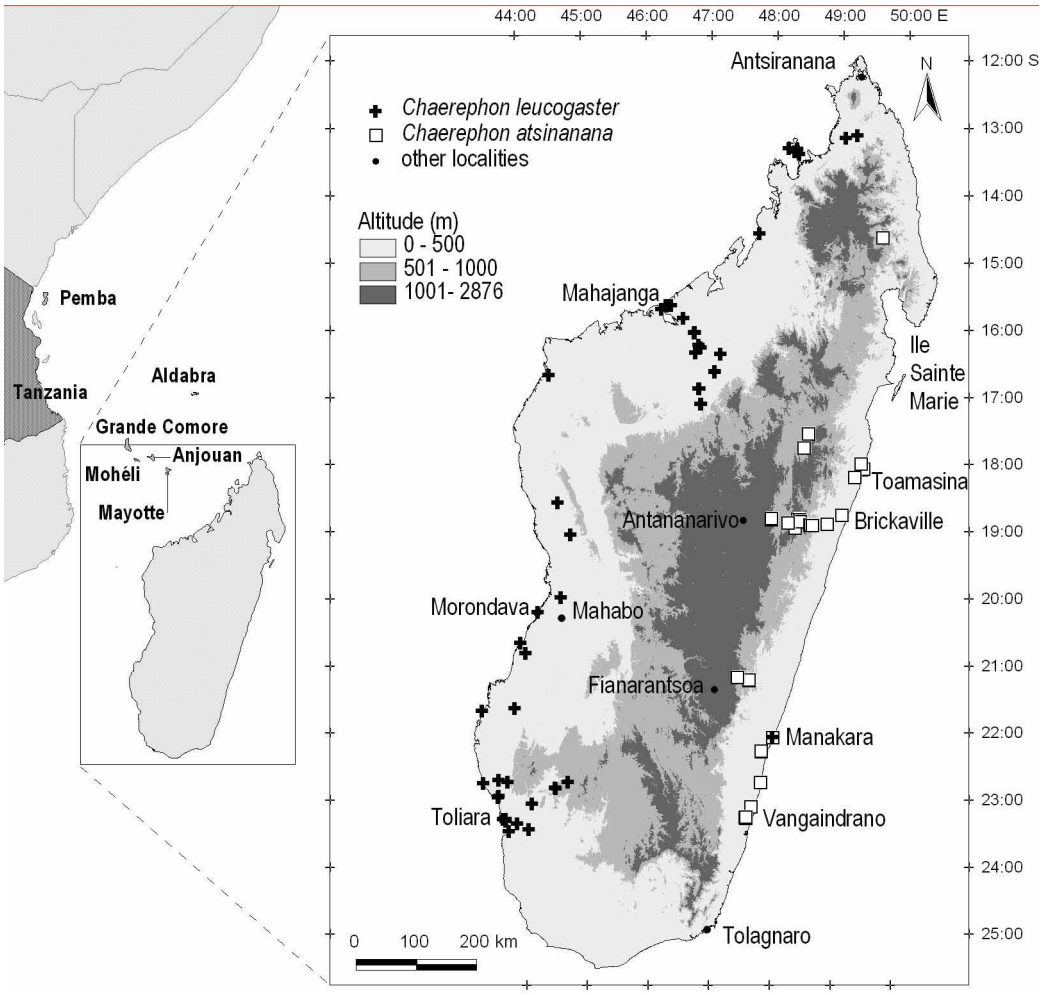

FIGURE 1. Distribution of *Chaerephon* '*pumilus*' (described herein as *C. atsinanana* **sp. nov.).** Map to the left is of the eastern portion of Africa, the Comoros Archipelago, Aldabra, and Madagascar. The larger map to the right of Madagascar illustrates known collection localities of specimens of *C. atsinanana* (eastern Madagascar) and *C. leucogaster*. The two species occur in sympatry at Manakara.

Genetic markers have proven an important means to unravel phylogenetic and phylogeographic patterns in species complexes similar to *C. pumilus* (Bickford *et al.* 2006; Pfenninger & Schwenk 2007), setting the framework for systematic revisions incorporating morphological characters. This step-wise technique has been successfully applied to other taxonomically complicated bat groups occurring on western Indian Ocean islands (e.g., Goodman *et al.* 2009; Ratrimomanarivo *et al.* 2008, 2009a; Weyeneth *et al.* 2008). A few molecular studies have been conducted on the *C. pumilus* species complex. Taylor *et al.* (2009) investigated patterns in mitochondrial DNA (cytochrome *b* and D-loop) variation in animals from southern Africa and Madagascar, including individuals from the latter locality identified as *C. leucogaster* and *C. pumilus*. They found four distinct D-loop clades in southern African populations, all of which are sympatric in the greater

Durban area of the KwaZulu-Natal Province, indicating possible incipient speciation or cryptic species. This study also found *C. leucogaster* to be nested within *C. pumilus* sensu lato, and Malagasy *C. pumilus* forming a sister group to a complex of clades of African *C. pumilus* and Malagasy *C. leucogaster.* Jacobs *et al.* (2004) showed that cytochrome *b* haplotypes from dark-winged southern African (classically assigned to nominate *pumilus*) and white-winged east and central African forms (classically assigned to the form *limbatus*) diverged by 0.9%. Further, seemingly fixed differences are known in the echolocation calls of African animals assigned to *C. pumilus* (Aspetsberger *et al.* 2003; Fenton *et al.* 2004; Taylor 1999a), further indicating possible differentiation associated with sympatric cryptic species. These studies demonstrate certain levels of differentiation across the range of *C. pumilus* sensu lato and supports Simmons (2005) prediction that "This complex probably includes more than one species".

Our point of departure for resolving the evolutionary history of the *C. pumilus* species complex is an explicit phylogeny based on molecular genetic data. With the established clades, we then apply morphological and morphometric data to diagnose and delineate the taxonomy of these animals. In order to resolve fully the evolutionary history of the species complex, various types of data are needed, such as sequence information from holotypes, lectotypes or topotypes, karyology, and vocalizations, as well as broad geographical sampling. Hence, this is a step-wise procedure and herein we commence the first of a series of systematic studies to establish: 1) which clade represents the nominate form of *pumilus*, 2) whether Malagasy populations of *C. leucogaster* and *C.* '*pumilus*' are specifically distinct from one another, 3) patterns of morphological and molecular variation in western Indian Ocean island populations (Madagascar, Comoros, and Aldabra), and 4) the specific status of the Madagascar population, which is described here as a new species.

Materials and methods

Specimens

In a taxonomic revision as presented herein, specimens, particularly those with associated tissues, form the critical point of comparison for the resolution of phylogenetic and phylogeographic patterns. Recent inventory work in parts of Africa and offshore islands, the Arabian Peninsula, as well as western Indian Ocean islands have provided such critical material. Specimens used in this study are housed in the Durban Natural Science Museum (DM), Durban; Field Museum of Natural History (FMNH), Chicago; Département de Biologie Animale, Université d'Antananarivo (UADBA), Antananarivo; Muséum national d'Histoire naturelle (MNHN), Paris; Museum of Comparative Zoology (MCZ), Harvard University, Cambridge; Royal Ontario Museum (ROM), Toronto; Senkenberg Museum Forschunginstitut (SMF), Frankfurt; The Natural History Museum (formerly British Museum (Natural History) [BMNH]), London; The National Museum of Natural History (formerly United States National Museum [USNM]), Washington, D.C.; and Museum für Naturkunde, Humboldt Universität zu Berlin (formerly Zoologisches Museum Berlin [ZMB]), Berlin.

Access to types and topotypic material

From a taxonomic perspective, the only means to properly address the question of the species limits, in the Linnaean sense, of the various named forms in the *C. pumilus* complex occurring in the eastern portion of Africa and the western Indian Ocean islands was to measure newly collected animals and compare them to type specimens. This material included the lectotype (SMF 4311) and associated type series (SMF 11917, 11918, 12372, 12377) of nominate *pumilus* from Massawa [other spellings include Massaua and Mitsiwa], Eritrea, collected by E. Rüppell and published by Cretzschmar (1830–1831). The lectotype is a mounted specimen that Mertens (1925) noted as having the skull unextracted; subsequently, the skull was removed and cleaned. The occipital portion of the cranium is missing, but otherwise the specimen is in relatively good condition. Further, we examined the syntypes of *limbatus* (ZMB 537, 538) as designated by Turni and Kock (2008). Finally, a key aspect to resolve which of the multiple clades within this *pumilus* species complex is referable to the nominate form, was to extract DNA from an individual (USNM 38032) collected at the type locality (see section "DNA isolation, PCR and sequencing of the nearly 120 year old sample from Massawa,

Eritrea"). USNM 38032 was also directly compared to the types of nominate *pumilus* and *limbatus* in the SMF and ZMB, respectively.

Morphological study

Three different types of measurements were recorded from adult specimens of the *C. pumilus* complex (*C. pumilus* sensu lato—eastern Madagascar (n=231), Aldabra (n=9), Comoros (n=63), Kenya (n=61), South Africa (n=18), and *C. leucogaster* – Madagascar (n=129)): external, cranial, and dental. Only adults were used in the analyses presented herein and these were defined as having fused basisphenoid-basioccipital sutures, as well as fully erupted adult dentitions. Given that members of this genus regularly show sexual dimorphism in cranio-dental measurements, but not necessarily external measurements (Bouchard 1998; Peterson *et al*. 2005; Ratrimomanarivo *et al*. 2009a), conclusions supported by many of the variables measured herein, we have separated adult males and females in our descriptive and statistical comparisons. In morphometric comparisons of Malagasy *C. leucogaster*, which show some clinal variation, we have used specimens from the central western portion of Madagascar, which is the terre typica of this animal (OTU 3 of Ratrimomanarivo *et al*. 2009a).

Five different external measurements were taken by FHR and SMG in a similar fashion for the recent Malagasy and Comoros collections using a millimeter ruler to a precision of 0.5 mm: total length, tail length, hindfoot length (excluding claw), ear length, and forearm length. Body mass in grams was also recorded with a Pesola spring balance at an accuracy of 0.5 g. Associated with potential differences in measurement technique, a certain level of caution is needed in comparisons of external measurements made by these two field workers and those data gleaned from museum specimens.

Ten cranial and eight dental measurements, largely following Freeman (1981), were made by FHR and SMG using digital calipers accurate to 0.1 mm (acronym for each measurement presented in parentheses using Freeman's system). Several comparisons were made to verify that these two researchers measured specimens in the same fashion and that differences in measurements of the same individuals were less than 1% different. Measurements included in our analyses are: CRANIAL MEASUREMENTS: greatest skull length (GSL), from posterior-most part of occipital to anterior-most point of premaxillary bone; condyloincisive length (CON INCI), from occipital condyle to anterior-most point of incisors; greatest zygomatic breadth (ZYGO BR), width taken across zygomatic arches at the widest point; postorbital width (POST ORB), dorsal width at most constricted part of skull; breadth at mastoids (MASTOID), greatest breadth across skull at mastoid processes; palatal length (PALATE), from posterior border of hard palate to anterior edge of premaxillary bone; lacrimal width (LACR WID), width across rostrum dorsally at lacrimal protuberances; condylocanine length (CON CANI), from midpoint of mandibular condyle to anterior border of alveolus of lower canine; temporal moment arm (MOM1 COR), length from middle of mandibular condyle to tip of coronoid process; masseter moment arm (MOM2 ANG), length from middle of mandibular condyle to tip of angular process; and DENTAL MEASUREMENTS: anterior palatal width (C^1-C^1), taken across the outer alveolar borders of the upper canines; posterior palatal width (M^3-M^3), taken across the outer alveolar borders of the third upper molars; upper molariform row (UP MOLR), length from PM^4 to M^3 (alveolar); maxillary toothrow (MTR), length from anterior alveolar border of canine to posterior alveolar border of M^3; height upper canine (UP CANIN), greatest length from point immediately dorsal to cingulum to end of tooth; width of M^3 (M3 WIDTH), greatest lateral-medial width of tooth; and lower toothrow (LOWER TR), length from posterior alveolar border of m_3 to alveolar border of c_1. Finally, details were recorded for each specimen on the degree of anterior palate emargination using the classification of Freeman (1981, variable C65). Terminology for cranial and dental characters follows Bates and Harrison (1997), Csorba *et al*. (2003), and Debaeremaeker and Fenton (2003).

Statistical analyses

To evaluate patterns of morphological variation in members of the *C. pumilus* complex the statistical package STATISTICA AX version 7.0 was used for all univariate and multivariate analyses. Cranial and dental measurements were analyzed separately following the conclusions of Ranivo and Goodman (2007) that

these two types of variables do not necessarily co-vary. T-tests were used to examine patterns of sexual dimorphism in regional populations, which in general showed differences for cranial and dental variables. Principal Component Analysis (PCA), using log-transformed data, was utilized to distinguish the different populations in three-dimensional space.

Genetic study

Our objectives with the genetic analyses were 1) to identify which clade represents nominate *pumilus*; 2) to determine if *C. leucogaster* can be differentiated from *C.* '*pumilus*' on Madagascar and other populations in the western Indian Ocean region; and 3) to evaluate the relationships of the different populations of the *C.* '*pumilus*' species on other western Indian Ocean islands (Comoros Archipelago and Aldabra).

Genetic variation in *Chaerephon* spp. was investigated using mitochondrial cytochrome *b* (n=89) and D-loop (n=92) sequencing (Appendix 1). Samples of *Chaerephon* were obtained from numerous localities in eastern and western Madagascar and other western Indian Ocean islands, including, Pemba, Zanzibar, Aldabra, Anjouan, Grande Comore, Mayotte, and Mohéli. *Chaerephon* specimens from Massawa (Eritrea), Yemen, and mainland Tanzania were used for comparative purposes. Samples used as outgroups included *Mops leucostigma* G. M. Allen, 1918, *M. midas* (Sundevall, 1843), and *Otomops martinesseni* (Matschie, 1897). A short sequence of *C. chapini* (GenBank acc. numb. AY591329.1) was used as an outgroup for a second analysis including the type specimen of *C. pumilus* and based on 206 nucleotides of the mitochondrial cytochrome *b* gene.

DNA isolation, PCR, and sequencing

The following protocols were used on all samples with the exception of the century-old individual (USNM 38032), from Massawa. Total genomic DNA was extracted from tissues, preserved in 80% ethanol or EDTA, using DNeasy® DNA isolation kit (Qiagen, Cape Town). The cytochrome *b* gene was amplified as two overlapping fragments with primers pairs L 14723 - H 15553 and L15146 - H15915 (Irwin *et al.* 1991).

Polymerase Chain Reaction (PCR)-amplifications were performed in 25 µl reaction volumes each consisting of 9 µl genomic DNA solution (containing 30 ng DNA), 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl $MgCl_2$ (25 mM) (Super-Therm), 0.5 µl dNTP mix (10 mM) (Roche Diagnostics), 0.2 µl *Taq* polymerase (5 U/µl) (Super-Therm) and 4 µl each of forward and reverse primer (6 µM). Thermal cycling parameters consisted of an initial denaturation step at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min 30s, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min.

The D-loop region was PCR-amplified using the primers pair P - F from Wilkinson and Chapman (1991). Where samples failed to amplify using this primer set, the primers pair P - E was used, F being nested within E (Wilkinson and Chapman 1991). PCR-amplifications were performed in 25 µl reaction volumes, as described above. Thermal cycling parameters consisted of initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min 30 s, extension 72°C for 2 min, and a final extension step at 72°C for 7 min.

For both cytochrome *b* and D-loop amplifications, target fragments were purified from excised gel bands using a QIAquick® Gel Extraction Kit (Qiagen Inc.). Purified DNA fragments were sequenced in the forward and reverse directions on an ABI 3730 capillary sequencer at Inqaba Biotechnical Industries Pty. Ltd., Hatfield, Pretoria, South Africa.

DNA isolation, PCR and sequencing of the nearly 120 year old sample from Massawa, Eritrea

Small muscle scrapings obtained from the skull of a *C. pumilus* specimen (USNM 38032) collected on 2 May 1890 at the type locality of Massawa, were sent to Lakehead University (Thunder Bay, Ontario, Canada) Paleo-DNA Laboratory Genetic Testing Service for cytochrome *b* sequencing. A standard proteinase K extraction, containing 1X TNE Buffer, 20% SDS, 0.39M DTT and Proteinase K was carried out on these tissue fragments. This was followed by silica bead purification. Additional size exclusion column purification was carried out to help remove inhibitors, if present. The above extraction procedure was repeated once. A

standard Platinum Taq DNA Polymerase PCR reaction was carried out using a primer pair designed from *Chaerephon* cytochrome *b* sequence alignments and amplifying a fragment of 206 bp (Bat 1F 5' CCTAATAATCCAAATTTTGACAGG 3' - Bat 1R 5' CTCCTATTTTTCATGTTTCTG 3'; annealing T°: 50°C). The DNA sample was amplified multiple times with both sets of primers. All PCR reaction products were separated by electrophoresis in 6% polyacrylamide gels, which were stained with ethidium bromide for visualization of PCR product. The single PCR product was sequenced as detailed above.

Sequence analyses

Electropherograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia). Sequence alignments were generated under BioEdit version 7 (Hall 1999) and its accessory CLUSTAL W alignment application (Thompson *et al*. 1994), and corrected manually by visual inspection for alignment errors.

Two cytochrome *b* alignments were created. One of these, trimmed to a common length of 206 nucleotides, determined by the length of sequence which was obtained from the Massawa specimen, was used to show the relationships of major clades within the *Chaerephon pumilus* complex in order to determine which clade represents nominate *pumilus*. A second cytochrome *b* alignment, which included all samples excluding the Massawa sample, was trimmed to a common length of 1031 nucleotides. For this sample series, a D-loop alignment of 337 nucleotides was also built.

Haplotype analysis of all datasets was carried out using the program DnaSP 4.10 (Rozas *et al*. 2003). Modeltest (Posada & Crandall 1998) was used to select the model of nucleotide substitution which best fits each sequence dataset. The HKY+I model was selected using the AIC information criterion for both cytochrome *b* datasets (206 and 1031 nucleotides) and for the D-loop dataset.

Bayesian analysis was implemented in Mr Bayes 3.0 (Hulsenbeck & Ronquist 2001) using flat priors. For all analyses, four Markov chains were run for 15 million generations each, and the first 500,000 trees discarded as burn-in.

PAUP* 4.0b10 (Swofford 2002) was used to estimate nucleotide divergence and generate neighbor-joining and maximum parsimony trees. For parsimony analysis, we used the random additions sequence option (n=100) for discrete, unordered characters. The shortest tree was obtained using the heuristic search option under tree bisection-reconnection (TBR) branch swapping option. The degree of support for each node of the resulting tree was estimated using bootstrap re-sampling analysis (1000 pseudoreplicates; Felsenstein 1985).

Results and Discussion

Which clade is the real *Chaerephon pumilus* sensu stricto?

Bayesian, maximum parsimony (MP), and neighbor-joining (NJ) analyses based on 206 nucleotides of the mitochondrial cytochrome *b* gene (Figure 2) provide strong support (posterior probability (pp.) 1.00, bootstrap 100% (MP and NJ) for the monophyly of the major *Chaerephon* clades that form the subject of this study. *Chaerephon chapini* is sister to the other *Chaerephon* taxa, which form a moderately well-supported group in MP (91%) and NJ (94%) analyses, but not in Bayesian analyses (pp. 0.85). Although this first analysis was limited to 206 nucleotides of the cytochrome *b* gene, we were able to establish the distinctness of *C. pumilus* from Massawa, and its sister-relationship with *C. pumilus* from Yemen (node well supported in MP (83%) and NJ (90%) analyses). The comments below are based on a more complete cytochrome *b* sequence alignment (1031 nucleotide) and a 332-nucleotides control region alignment (for a detailed discussion, see the section below entitled 'Molecular genetics').

Chaerephon pumilus from Yemen (Clade A; established above as sister to *C. pumilus* sensu stricto from Massawa), is sister to three major clades (B, C2a, and C2b) of *Chaerephon* from the western Indian Ocean region. Clades B (from eastern Madagascar) and C2a (from the Comoros and Aldabra) are strongly supported, whilst clade C2b is moderately supported. Clade A is separated from Clades B (2.98%), C2a

(2.86%), and C2b (2.67%) by cytochrome *b* genetic distances (Table 1) falling toward the lower end of the range reported for sister-species of bats (2.3% to 14.7%; Baker & Bradley 2006).

TABLE 1. HKY+I genetic distances (%) (below diagonal) based on 1031 base pairs of cytochrome *b* gene between haplotypes of different taxa of *Chaerephon* and different outgroups of the genera *Mops* and *Otomops*. Mean HKY+I genetic distances between clades (above diagonal). The clade assignments are based on those noted in Figure 4, namely: Clade C2a = *C. pusillus* (Comoros & Aldabra), Clade C1 = *C. pumilus* sensu lato (Tanzania), Clade C2b = *C. leucogaster*, Clade B = *C. atsinanana* **sp. nov**. (referred to in the first portion of the text as *C. 'pumilus'*), Clade A = *C. pumilus* (Yemen). Abbreviation: H = haplotype.

		Clade C2a				Clade C1		Clade C2b								Clade B						Cl. A			
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24
Clade C2a	H1					2.25		1.31								3.07					2.86				
	H2	0.10																							
	H3	0.20	0.29																						
	H4	0.29	0.39	0.10																					
Clade C1	H5	2.20	2.30	2.40	2.50			1.68								2.46					2.55				
	H6	1.99	2.09	2.20	2.30	0.20																			
Clade C2b	H7	0.99	1.09	1.19	1.29	1.79	1.59									2.29					2.67				
	H8	0.89	0.99	1.09	1.19	1.69	1.49	0.10																	
	H9	1.19	1.29	1.39	1.49	1.79	1.59	0.39	0.29																
	H10	1.09	1.19	1.29	1.39	1.69	1.49	0.29	0.20	0.10															
	H11	1.19	1.29	1.39	1.49	1.79	1.59	0.39	0.29	0.20	0.10														
	H12	1.19	1.29	1.39	1.49	1.79	1.59	0.39	0.29	0.20	0.20	0.20													
	H13	1.29	1.39	1.49	1.59	1.89	1.69	0.49	0.39	0.29	0.20	0.10	0.10												
	H14	1.19	1.29	1.39	1.49	1.79	1.59	0.39	0.29	0.20	0.10	0.20	0.20	0.29											
Clade B	H15	3.23	3.33	3.44	3.54	2.71	2.71	2.61	2.51	2.61	2.51	2.61	2.61	2.71	2.61						2.98				
	H16	3.12	3.23	3.33	3.44	2.71	2.71	2.51	2.40	2.51	2.40	2.51	2.51	2.61	2.51	0.89									
	H17	2.92	3.02	3.12	3.23	2.40	2.40	2.30	2.20	2.30	2.20	2.30	2.30	2.40	2.30	0.39	0.69								
	H18	2.61	2.71	2.82	2.92	2.20	2.20	2.00	1.89	2.00	1.89	2.00	2.00	2.10	2.00	0.59	0.49	0.39							
	H19	2.71	2.81	2.92	3.02	2.30	2.30	2.10	1.99	2.10	1.99	2.10	2.10	2.20	2.10	0.69	0.59	0.49	0.10						
Clade A	H20	3.32	3.32	3.64	3.80	2.55	2.55	2.71	2.55	2.70	2.55	2.70	2.70	2.86	2.55	3.48	2.85	3.17	2.70	2.70					
M. midas	H21	13.13	13.24	13.13	13.00	12.86	12.86	12.38	12.51	12.63	12.51	12.63	12.38	12.51	12.63	13.24	12.61	12.99	12.50	12.61	12.47				
M. leucostigma	H22	12.61	12.72	12.86	12.74	12.72	12.47	12.50	12.62	12.75	12.62	12.75	12.50	12.62	12.75	12.50	11.85	12.35	11.99	12.10	11.96	13.91			
M. condylurus	H23	11.76	11.87	12.01	12.13	12.24	11.99	11.77	11.90	12.02	11.90	12.02	12.02	12.15	12.02	12.15	11.50	12.00	11.64	11.75	11.77	13.68	2.81		
O. martiensseni	H24	16.95	17.07	16.69	16.82	16.83	17.09	16.70	16.83	16.96	16.83	16.96	16.70	16.83	16.96	17.10	16.94	16.96	16.82	16.94	15.28	17.93	18.40	17.59	

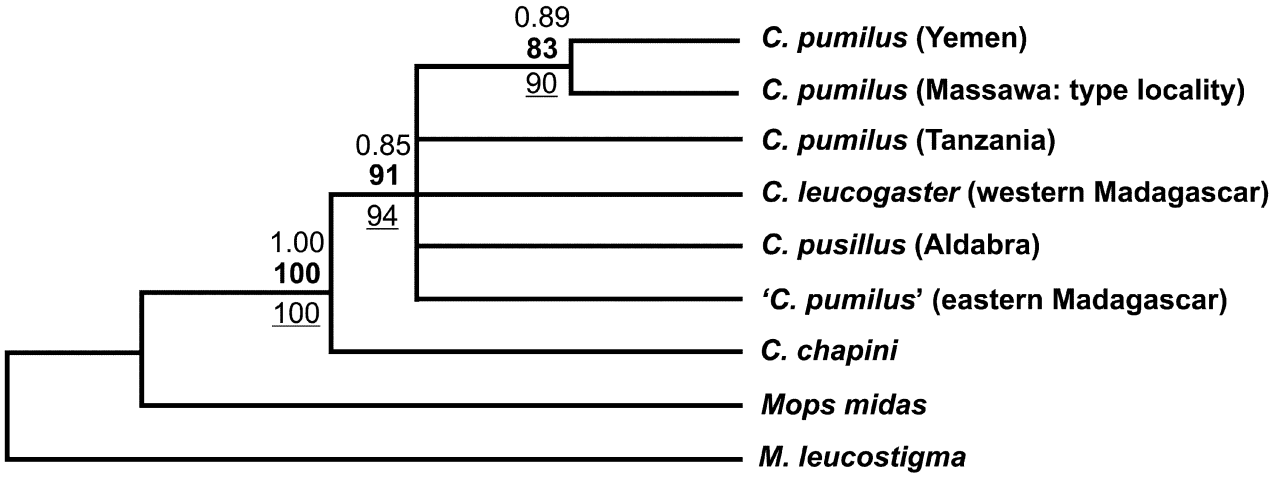

FIGURE 2. Dendrogram based on analyses of 206 nucleotides of the mitochondrial cytochrome *b* gene illustrating relationships between *Chaerephon pumilus* from the type locality of Massawa, Eritrea, and other *Chaerephon* clades: *C. pumilus* sensu lato (Tanzania), *C. pumilus* sensu stricto (Yemen), *C. leucogaster* (western Madagascar), *C. pusillus* (Aldabra), and *C.* '*pumilus*' (eastern Madagascar, described herein as *C. atsinanana* **sp. nov.**). All sequenced individuals belonging to the above-mentioned taxa clustered into monophlyletic groups. *Chaerephon chapini* from mainland Africa, *Mops leucostigma*, and *M. midas* are used as outgroups. Nodal support is indicated according to the following analyses: Bayesian posterior probability in standard font (top), bootstrap value (1000 pseudo-replicates) for maximum parsimony analysis in bold font (middle), and neighbor-joining analysis underlined (bottom). Bootstrap values of less than 50% and posterior probabilities of less than 0.7 are not indicated.

We thus consider that there is support for the species-level distinctness of topotypic material of *C. pumilus* (Massawa) from the above-mentioned *Chaerephon* taxa from the western Indian Ocean. Relationships involving the Yemen sample will be discussed in a paper on *Chaerephon* species in mainland Africa. Aspects of patterns of morphological variation in *C. pumilus* from Massawa are discussed in the "Comparisons" section below under the new species description.

Are Malagasy and Comorian populations of *Chaerephon leucogaster* and *C.* '*pumilus*' distinct from one another?

Recent research using morphological characters has found that populations of a broadly distributed Malagasy molossid, *Mops leucostigma*, are morphologically distinct in the mesic eastern portion of the island from animals in the drier western parts (Ratrimomanarivo *et al*. 2008). However, genetically the same animals used in the morphological study from the eastern and western sides of Madagascar cannot be differentiated (Ratrimomanarivo *et al*. 2008) and the morphological differences are presumed to be adaptive. Based on literature (e.g. Peterson *et al*. 1995), *C. leucogaster* occurs on the western side of Madagascar and *C.* '*pumilus*' on the eastern side, with *C. leucogaster* in many ways being a slightly smaller form of eastern *C.* '*pumilus*'. Hence, this begs the question if these two *Chaerephon* spp. are not simply a parallel case to *M. leucostigma*. Further, the general differences in wing coloration, with *leucogaster* being dominated by white or translucent wing membranes and '*pumilus*' from eastern Madagascar by dark wing membranes, may possibly parallel the situation relating to white-winged *limbatus* and dark-winged *pumilus* from eastern and southern Africa (Kingdon 1974). In order to properly interpret genetic and morphological patterns in the *C. pumilus* complex, it is imperative to demonstrate that Malagasy *C. leucogaster* and *C.* '*pumilus*' are distinct lineages. The critical question is whether the morphological differences between these animals are correlated with genetic distinctiveness? Below we present several lines of evidence that show Malagasy *C. leucogaster* and *C.* '*pumilus*' are distinct lineages and do not represent the same taxon; in certain cases these differences are discussed in greater detail in the species description presented towards the end of this paper. The question of morphological and genetic variation within Malagasy, Comorian, and certain offshore African island populations of *C. leucogaster* has been discussed elsewhere (Ratrimomanarivo *et al*. 2009a).

Geographical distributions and vocalizations

Recent fieldwork on Madagascar has found one case for the sympatric occurrence of animals referable to *C. leucogaster* and *C.* '*pumilus*' based on morphological and genetic sequence data (see below). At Manakara along the southeastern coast (Figure 1), an individual of *C. leucogaster* (FMNH 185228) was captured in a day roost within a school that held numerous *C.* '*pumilus*' (FMNH 185306-185318). A similar case has been found at a day roost site on Mayotte, where both *C. leucogaster* (FMNH 194019, 194028) and *C.* '*pumilus*' (FMNH 194036) occurred. Hence, animals referable to these two lineages do occur in sympatry, thus fulfilling an important requirement of the biological species concept (Mayr 1963).

The previous idea that *C.* '*pumilus*' from eastern Madagascar has a more upland distribution (Eger and Mitchell 2003) needs to be corrected, as it is now known in this zone across an elevational range from near sea-level to 1100 m (Peterson *et al*. 1995; data presented herein). The previous reports of *C. leucogaster* from Ile Sainte-Marie, an eastern near-shore island, are incorrect and these records are from the site of Sainte Marie de Marovoay, to the southeast of Mahajanga (Rakotonandrasana and Goodman 2007).

Russ *et al*. (2001) provided details on the echolocation calls of Malagasy animals referable to *C. leucogaster* and *C.* '*pumilus*' based on external measurements and coloration, but without associated voucher specimens or DNA samples. Hence, current data on the echolocation information of these two taxa are too sparse to be properly incorporated herein.

External morphology

A number of authors have mentioned that Malagasy bats referable to *C. leucogaster* are smaller than *C.* '*pumilus*' (Eger & Mitchell 2003; Peterson *et al*. 1995). Our measurement data (Table 2) show that for two variables, total length and forearm length, which do not show sexual dimorphism, these two species show little overlap and differences are statistically significant (total length, $t=29.2$, $df=453$, $P<0.0001$; forearm length, $t=31.8$, $df=456$, $P<0.0001$). On Madagascar, animals of this species complex with a forearm length greater than 38 mm are referable to *C.* '*pumilus*' from eastern Madagascar.

In all of the 280 *C. leucogaster* specimens examined, the leading edge of the wing and at least the middle portion of the membrane were white or translucent. In contrast, of the 239 specimens of *C.* '*pumilus*' from eastern Madagascar studied, the majority (80.3%) had dark leading wing edges and largely dark wings, while 26 individuals (10.9%) had white leading wing edges and middle portions of the membrane, and 21 individuals (8.8%) had white leading wing edges and the complete membrane (excluding the distal tip) white. There was a tendency for *C.* '*pumilus*' from eastern coastal areas to have whiter wings compared to those from more upland areas. For example, of the 44 bats from Vangaindrano (near sea-level) examined, 19 (36%) had white leading wing edges and most of the membrane, while for an upland site such as Moramanga (820 m) of the 26 specimens studied, not one had a white leading edge to the wing and the membrane was blackish or with a small translucent window. In many respects the wing coloration of Malagasy *C. leucogaster* and a certain proportion of coastal eastern *C.* '*pumilus*' is similar to east African populations of *C. pumilus* classically assigned to *limbatus*. This is presumably why *leucogaster* was considered a synonym of *limbatus* by some authorities (e.g., Dobson 1878). There are notable differences in pelage coloration and antitragus shape between these two taxa, which are discussed in detail in the species description below.

Cranial morphology

Malagasy individuals of *C. leucogaster* and *C.* '*pumilus*' are separable based on cranial morphology and measurements. In the latter taxon, all of the 10 cranial variables show significant levels of sexual dimorphism (Table 3). With the exception of POST ORD, MOM1 COR, and MOM2 ANG, the balance of the measured variables shows a pronounced difference between *C. leucogaster* and *C.* '*pumilus*' and in several cases no overlap in measurements. For example, amongst male specimens, there is a slight overlap between the two species in GSL, with those measuring more than 16.3 mm being referable to *C.* '*pumilus*' and for females, there is no overlap between the two species and individuals measuring 16.0 mm or greater are identifiable as *C.* '*pumilus*' from eastern Madagascar.

TABLE 2. External measurements (in millimeters), except for body mass (in grams), of adult *Chaerephon atsinanana* **sp. nov**. (referred to in the first portion of the text as *C.* '*pumilus*') and *C. leucogaster* from Madagascar, and other members of the *C. pumilus* complex, from Aldabra and Anjouan, Grande Comore, and Mohéli (=*C. pusillus*), and from eastern Africa (Kenya) and the Kwa-Zulu Natal Province of South Africa (=*C. pumilus* sensu lato). Measurements are presented as mean ± standard deviation, minimum-maximum measurements, and number of specimens. T-test comparisons are presented for the Madagascar samples of *C. atsinanana* to test for sexual dimorphism (n.s. – not significant). Measurements in bold were made by FHR or SMG using consistent techniques. We have not located a specimen from Massawa, Eritrea, with external measurements made by the field collector.

	Total length	Tail length	Hindfoot length	Ear length	Forearm length	Body mass
C. atsinanana						
Holotype						
FMNH 185259	90	32	6	16	39	10
Eastern Madagascar						
Adult male	95.1 ± 2.79 90–101, n=87	33.6 ± 1.74 29–39, n=89	5.9 ± 0.35 5–7, n=89	16.7 ± 0.61 15–18, n=89	39.2 ± 0.91 37–42, n=89	11.0 ± 1.00 8.8–14.5, n=89
Adult female	94.6 ± 2.36 90–101, n=141	33.5 ± 1.71 27–37, n=141	6.0 ± 0.25 5–7, n=141	16.4 ± 0.62 15–18, n=141	39.1 ± 0.86 37–41, n=142	11.6 ± 1.72 9.0–16.5, n=142
Sexual dimorphism	n.s.	n.s.	t=2.63, df=228 P=0.009	t=3.73, df=228 P=0.0002	n.s.	t=3.25, df=229 P=0.001
C. leucogaster						
Madagascar – central west						
Adult male	86.4 ± 2.73 80–93, n =50	30.7 ± 1.95 26–37, n =50	5.3 ± 0.53 5–7, n =50	15.7 ± 0.75 14–17, n =16	35.2 ± 1.05 33–37, n =50	7.48 ± 0.73 5.6–10.0, n =50
Adult female	86.4 ± 2.22 82–92, n =79	30.7 ± 1.97 27–36, n =79	5.2 ± 0.50 5–7, n =79	15.7 ± 0.68 13–17, n =79	35.2 ± 1.03 33–37, n =79	7.6 ± 0.55 6.5–9.0, n=33
C. pusillus						
Aldabra						
Adults Sexes combined	79, n=1	30.1 ± 1.17 29–32, n=6	5.8 ± 0.39 5–6, n=6	14.8, n=1	38.0 ± 0.53 37–39, n=9	6.4 ± 1.00 5.0–7.5, n=5
Comoros						
Adult male	88.4 ± 1.80 85–92, n=29	30.7 ± 1.17 28–33, n=29	5.0 ± 0.09 5–6, n=29	17.4 ± 0.56 16–18, n=29	36.9 ± 0.75 36–38, n=29	7.2 ± 0.66 6.1–8.3, n=29
Adult female	88.3 ± 2.10 84–93, n=34	30.7 ± 1.63 27–36, n=34	5.1 ± 0.38 4–6, n=34	17.1 ± 0.34 16–18, n=34	37.2 ± 0.73 36–38, n=34	8.7 ± 1.51 5.9–11.5, n=34

TABLE 2 (continued)

	Total length	Tail length	Hindfoot length	Ear length	Forearm length	Body mass
C. pumilus						
Kenya						
Adult male	95.8 ± 4.32 87–104, n=24	33.2 ± 3.18 29–41, n=24	9.1 ± 0.61 8–10, n=24	16.8 ± 1.55 13–19, n=24	39.6 ± 1.65 37–43, n=23	10.2 ± 1.34 4–8, n=18
Adult female	95.9 ± 3.38 90–103, n=37	31.9 ± 3.06 20–37, n=37	9.3 ± 0.74 8–11, n=37	17.1 ± 0.95 13–18, n=37	39.5 ±1.63 36–42, n=33	10.2 ± 1.31 8.5–14, n=31
South Africa						
Adult male	87.6 ± 5.34 80–94, n=7	31.3 ± 4.39 25–37, n=7	7.2 ± 0.91 6–9, n=7	14.6 ± 1.79 11–17, n=7	36.9 ± 1.57 34–39, n=8	9, n=1
Adult female	88.8 ± 3.56 86–94, n=5	29.6 ± 1.14 28–31, n=5	6.9 ± 0.22 6.5–7, n=5	15.4 ± 0.55 15–16, n=5	38.3 ± 0.79 37–39, n=10	6, n=1

TABLE 3. Cranial and mandible measurements (in millimeters) of *Chaerephon atsinanana* **sp. nov**. (referred to in the first portion of the text as *C.* '*pumilus*') and *C. leucogaster* from Madagascar, and other members of the *C. pumilus* complex from Aldabra and Anjouan, Grande Comore, and Mohéli in the Comoros (=*C. pusillus*), nominate *pumilus* from Eritrea, and from eastern Africa (Kenya) and from the Kwa-Zulu Natal Province of South Africa (=*C. pumilus* sensu lato). Measurements are presented as mean ± standard deviation, minimum-maximum measurements, and number of specimens. T-test comparisons associated with sexual dimorphism are presented for the Madagascar samples of *C. atsinanana*.

	GSL	CON INCI	ZYGO BR	POST ORB	MASTOID	PALATE	LACR WID	CON CANI	MOM1 COR	MOM2 ANG
C. atsinanana										
Holotype										
FMNH 185259	17.0	15.5	10.6	3.9	9.9	6.6	5.9	11.0	3.1	3.2
Eastern Madagascar										
Adult males	17.0 ± 0.27 16.0–17.5, n=87	16.0 ± 0.26 15.2–16.5, n=87	10.7 ± 0.21 10.0–11.2, n=87	3.9 ± 0.10 3.5–4.1, n=87	10.0 ± 0.17 9.5–10.6, n=87	6.9 ± 0.20 6.5–7.7, n=87	6.1 ± 0.24 5.3–7.3, n=87	11.2 ± 0.22 10.5–11.7, n=87	3.3 ± 0.12 2.9–3.8, n=87	3.4 ± 0.17 2.6–3.7, n=87
Adult females	16.8 ± 0.25 16.0–17.4, n=140	15.7 ± 0.25 14.9–16.4, n=140	10.4 ± 0.19 9.8–10.8, n=140	3.8 ± 0.11 3.5–4.1, n=140	9.9 ± 0.17 9.5–10.3, n=140	6.7 ± 0.16 6.3–7.2, n=140	5.9 ± 0.18 5.4–6.3, n=140	10.9 ± 0.21 10.3–11.5, n=140	3.2 ± 0.11 2.9–3.5, n=140	3.2 ± 0.17 2.1–3.5, n=140
Sexual dimorphism	t=6.61 P<0.0001	t=9.69 P<0.0001	t=9.26 P<0.0001	t=2.96 P=0.003	t=5.48 P<0.0001	t=8.06 P<0.0001	t=7.15 P<0.0001	t=8.47 P<0.0001	t=4.41 P<0.0001	t=6.85 P<0.0001
C. leucogaster										
Madagascar – central west										
Adult males	15.4 ± 0.38 14.3–16.2, n=50	14.5 ± 0.35 13.5–15.2, n=50	9.6 ± 0.23 9.0–10.1, n=50	3.6 ± 0.13 3.3–3.9, n=50	9.1 ± 0.19 8.6–9.4, n=50	6.2 ± 0.21 5.8–6.7, n=50	5.6 ± 0.22 5.8–6.7, n=50	10.0 ± 0.31 9.1–10.7, n=50	2.9 ± 0.13 2.5–3.2, n=50	2.9 ± 0.15 2.6–3.2, n=50
Adult females	15.2 ± 0.35 14.1–15.8, n=77	14.2 ± 0.32 13.4–14.9, n=77	9.5 ± 0.20 8.9–9.9, n=76	3.5 ± 0.14 3.3–3.8, n=77	9.0 ± 0.19 8.5–9.4, n=77	6.0 ± 0.19 5.6–6.5, n=77	5.4 ± 0.24 4.8–6.0, n=77	9.8 ± 0.27 9.2–10.3, n=75	2.9 ± 0.11 2.6–3.1, n=75	2.8 ± 0.13 2.5–3.2, n=75
C. pusillus										
Aldabra										
Adults										
Sexes combined	15.2 ± 0.39 14.6–15.7, n=6	–	9.0 ± 0.17 8.7–9.2, n=7	3.1 ± 0.04 3.0–3.1, n=7	8.8 ± 0.17 8.5–9.0, n=6	6.3 ± 0.22 6.0–6.6, n=6	5.0 ± 0.18 4.7–5.7, n=7	–	–	–
Comores										
Adult males	15.7 ± 0.22 15.2–16.0, n=29	14.7 ± 0.19 14.3–15.0, n=29	9.7 ± 0.16 9.3–9.9, n=29	3.4 ± 0.12 3.3–3.7, n=29	9.2 ± 0.18 8.8–9.4, n=29	6.5 ± 0.13 6.2–6.7, n=29	5.6 ± 0.16 5.2–5.8, n=29	10.3 ± 0.19 9.9–10.7, n=29	3.0 ± 0.11 2.8–3.2, n=29	3.1 ± 0.12 2.8–3.3, n=29
Adult females	15.4 ± 0.21 14.9–15.7, n=33	14.4 ± 0.20 14.1–14.8, n=33	9.4 ± 0.18 9.0–9.8, n=29	3.4 ± 0.09 3.2–3.6, n=29	9.1 ± 0.16 8.8–9.4, n=29	6.3 ± 0.16 5.9–6.6, n=33	5.3 ± 0.12 5.1–5.5, n=29	10.0 ± 0.18 9.7–10.3, n=33	2.9 ± 0.09 2.8–3.1, n=33	2.9 ± 0.11 2.7–3.1, n=33

TABLE 3 (continued)

	GSL	CON INCI	ZYGO BR	POST ORB	MASTOID	PALATE	LACR WID	CON CANI	MOM1 COR	MOM2 ANG
C. p. pumilus Massawa, Eritrea Lectotype (SMF 4311)	-	-	9.6	3.3	-	6.3	-	10.3	-	-
Adults Sexes combined	15.7 ± 0.15 15.6–15.9, n=3	14.6 ± 0.15 14.4–14.7, n=3	9.9 ± 0.17 9.6–10.0, n=4	3.5 ± 0.13 3.3–3.6, n=4	9.0 ± 0.15 8.9–9.2, n=3	6.4 ± 0.08 6.2–6.7, n=6	5.4 ± 0.17 5.2–5.6, n=4	10.3 ± 0.15 10.1–10.4, n=3	2.9 ± 0.15 2.7–3.0, n=4	3.0 ± 0.06 3.0–3.1, n=3
C. pumilus Kenya Adult males	17.2 ± 0.43 16.3–17.9, n=25	16.1 ± 0.40 15.4–16.8, n=25	10.7 ± 0.34 10.0–11.2, n=25	3.9 ± 0.15 3.6–4.1, n=25	10.0 ± 0.20 9.5–10.4, n=25	7.1 ± 0.26 6.7–7.7, n=25	6.0 ± 0.25 5.6–6.6, n=25	11.4 ± 0.34 10.7–12.0, n=25	3.4 ± 0.13 3.2–3.6, n=25	3.3 ± 0.26 2.7–3.9, n=25
Adult females	16.9 ± 0.40 16.0–17.8, n=38	15.8 ± 0.42 15.1–17.0, n=38	10.4 ± 0.28 9.9–10.9, n=38	3.9 ± 0.11 3.5–4.1, n=38	9.9 ± 0.19 9.5–10.3, n=38	7.0 ± 0.25 6.6–7.7, n=39	5.9 ± 0.24 5.5–6.4, n=38	11.1 ± 0.37 10.1–11.9, n=39	3.3 ± 0.14 3.0–3.6, n=39	3.2 ± 0.23 2.6–4.0, n=39
South Africa Adult males	16.7 ± 0.53 15.9–17.5, n=8	15.9 ± 0.59 14.9–16.9, n=8	10.4 ± 0.33 9.9–10.9, n=8	3.8 ± 0.13 3.6–4.0, n=8	9.8 ± 0.24 9.4–10.2, n=8	7.0 ± 0.33 6.6–7.6, n=8	5.9 ± 0.33 5.2–6.3, n=8	11.2 ± 0.49 10.3–11.9, n=8	3.1 ± 0.25 2.7–3.4, n=8	3.1 ± 0.15 2.9–3.3, n=8
Adult females	16.9 ± 0.45 16.3–17.5, n=10	16.0 ± 0.38 15.4–16.6, n=10	10.4 ± 0.23 10.0–10.8, n=10	3.8 ± 0.16 3.5–4.0, n=10	9.8 ± 0.19 9.5–10.1, n=10	7.0 ± 0.19 6.7–7.3, n=10	6.0 ± 0.22 5.5–6.2, n=11	11.2 ± 0.28 10.8–11.6, n=10	3.2 ± 0.18 3.0–3.4, n=10	3.1 ± 0.17 2.9–3.4, n=10

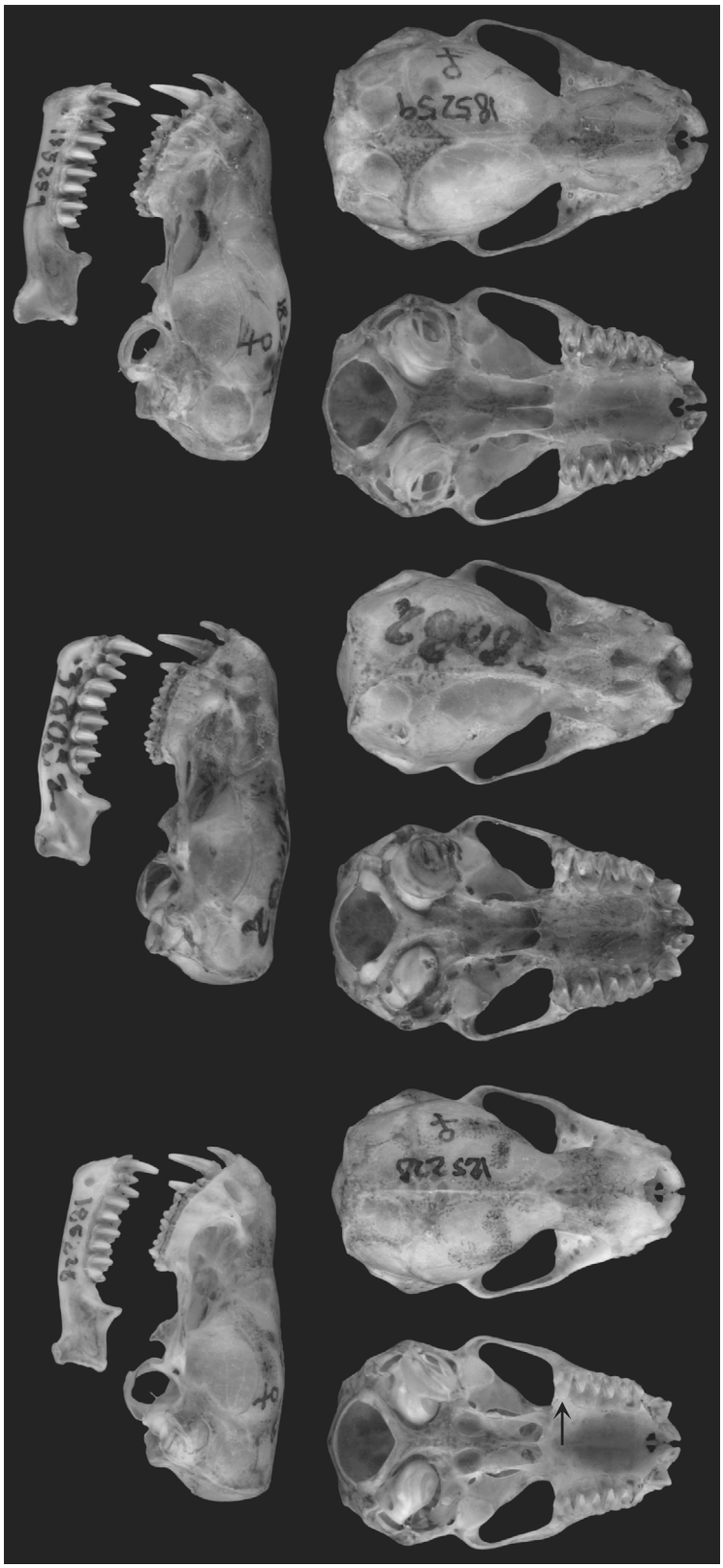

FIGURE 3. Different views of skull and mandible of an adult female *C.* '*pumilus*' (eastern Madagascar, described herein as *Chaerephon atsinanana* **sp. nov.**) (left set - FMNH 185259, holotype, GSL = 17.0 mm) obtained at Farafangana; adult female *C. pumilus* sensu stricto (middle set - USNM 38032, GSL = 15.9 mm) collected at Massawa, Eritrea, which is the type locality of this species; and adult female *C. leucogaster* (right set - FMNH 185228, GSL = 15.4 mm) obtained from Manakara: **upper row,** dorsal and ventral views of crania and **lower row pair**, lateral view of crania and mandibles. Arrows indicate differences between the three taxa in the M^3 hypoconal flange. (Photograph taken by John Weinstein, FMNH image.)

TABLE 4. Dental measurements (in millimeters) of *Chaerephon atsinanana* **sp. nov**. (referred to in the first portion of the text as *C.* '*pumilus*') and *C. leucogaster* from Madagascar, and other members of the *C. pumilus* complex from Aldabra and Anjouan, Grande Comore, and Mohéli in the Comoros (=*C. pusillus*), nominate *pumilus* from Eritrea, and from eastern Africa (Kenya) and from the Kwa-Zulu Natal Province of South Africa (=*C. pumilus* sensu lato). Measurements are presented as mean ± standard deviation, minimum-maximum measurements, and number of specimens. T-test comparisons are presented for the Madagascar samples of *C. atsinanana* (n.s. – not significant).

	C^1-C^1	M^3-M^3	UP MOL R	MTR	UP CANIN	M^3WIDTH	LOWER TR
C. atsinanana							
Holotype							
FMNH 185259	4.5	7.5	4.7	6.4	2.7	1.9	6.8
Eastern Madagascar							
Adult males	4.9 ± 0.18 4.4–5.2, n=86	7.8 ± 0.22 6.9–8.3, n=86	4.8 ± 0.11 4.6–5.1, n=86	6.5 ± 0.13 6.1–6.7, n=86	2.8 ± 0.02 2.2–3.2, n=86	1.9 ± 0.07 1.7–2.0, n=86	6.8 ± 0.16 6.3–7.2, n=86
Adult females	4.6 ± 0.15 4.2–5.1, n=140	7.7 ± 0.18 7.3–8.1, n=140	4.7 ± 0.11 4.4–5.1, n=139	6.7 ± 0.16 6.3–7.2, n=140	2.5 ± 0.13 2.0–3.0, n=137	1.9 ± 0.07 1.7–2.0, n=136	6.7 ± 0.14 6.2–7.0, n=139
Sexual dimorphism	t=11.17 P<0.0001	t=3.36 P<0.0009	t=2.98 P=0.003	t=7.04 P<0.0001	t=14.29 P<0.0001	n.s.	t=8.54 P<0.0001
C. leucogaster							
Madagascar – central west							
Adult males	4.2 ± 0.18 3.8–4.7, n=49	6.8 ± 0.19 6.4–7.2, n=49	4.2 ± 0.14 4.0–4.5, n=49	5.7 ± 0.17 5.4–6.1, n=49	2.5 ± 0.14 2.0–2.8, n=49	1.7 ± 0.09 1.4–1.8, n=49	6.1 ± 0.21 5.6–6.6, n=49
Adult females	4.0 ± 0.16 3.6–4.4, n=71	6.8 ± 0.19 6.4–7.5, n=71	4.2 ± 0.11 5.3–5.9, n=71	5.7 ± 0.13 5.3–5.9, n=71	2.2 ± 0.12 1.9–2.5, n=71	1.7 ± 0.09 1.5–1.9, n=71	6.0 ± 0.18 5.6–6.5, n=71
C. pusillus							
Aldabra							
Adults							
sexes combined	3.8 ± 0.22 3.4–4.1, n=7	6.5 ± 0.21 6.1–6.7, n=6	4.4 ± 0.26 4.0–4.7, n=5	5.5 ± 0.15 5.2–5.7, n=7	-	-	-

TABLE 4 (continued)

	C^1-C^1	M^3-M^3	UP MOL R	MTR	UP CANIN	M^3 WIDTH	LOWER TR
Comores							
Adult males	4.4 ± 0.12 4.1–4.6, n=28	7.0 ± 0.15 6.7–7.3, n=28	4.4 ± 0.10 4.2–4.5, n=28	5.9 ± 0.10 5.7–6.1, n=28	2.5 ± 0.08 2.3–2.6, n=28	1.7 ± 0.06 1.6–1.8, n=28	6.3 ± 0.12 6.0–6.5, n=28
Adult females	4.1 ± 0.13 3.9–4.4, n=33	6.9 ± 0.17 6.6–7.3, n=33	4.3 ± 0.08 4.2–4.5, n=33	5.8 ± 0.10 5.6–5.9, n=33	2.2 ± 0.12 1.9–2.5, n=33	1.6 ± 0.05 1.5–1.7, n=33	6.1 ± 0.11 5.9–6.3, n=33
C. p. pumilus Massawa, Eritrea Lectotype (SMF 4311)	4.0	7.0	4.3	5.8	2.6	1.7	6.2
Sexes combined	4.1 ± 0.14 3.9–4.3, n=6	7.2 ± 0.08 7.0–7.2, n=6	4.5 ± 0.12 4.3–4.6, n=6	5.9 ± 0.12 5.8–6.1, n=6	2.6 ± 0.05 2.5–2.6, n=6	1.8 ± 0.05 1.7–1.8, n=6	6.4 ± 0.12 6.2–6.5, n=6
C. pumilus subsp? Kenya							
Adult males	4.9 ± 0.22 4.5–5.3, n=23	7.6 ± 0.32 7.1–8.3, n=24	4.8 ± 0.25 4.4–5.3, n=24	6.4 ± 0.23 6.0–6.9, n=24	2.8 ± 0.18 2.5–3.2, n=22	1.8 ± 0.12 1.7–2.1, n=24	6.9 ± 0.21 6.5–7.5, n=24
Adult females	4.6 ± 0.21 4.1–5.2, n=38	7.5 ± 0.26 7.0–8.0, n=38	4.7 ± 0.19 4.3–5.1, n=38	6.3 ± 0.20 5.9–6.7, n=38	2.5 ± 0.17 2.2–2.9, n=38	1.8 ± 0.11 1.6–2.1, n=38	6.7 ± 0.30 5.4–7.4, n=38
South Africa Adult males	4.8 ± 0.17 4.6–5.0, n=7	7.7 ± 0.23 7.2–7.9, n=7	4.7 ± 0.10 4.5–4.8, n=7	6.4 ± 0.16 6.2–6.6, n=7	2.9 ± 0.16 2.7–3.1, n=7	1.7 ± 0.05 1.7–1.8, n=7	6.9 ± 0.20 6.6–7.2, n=7
Adult females	4.6 ± 0.17 4.3–4.9, n=9	7.6 ± 0.17 7.3–7.8, n=9	4.7 ± 0.13 4.5–4.8, n=9	6.4 ± 0.10 6.2–6.5, n=9	2.7 ± 0.15 2.5–2.9, n=8	1.8 ± 0.09 1.6–1.9, n=9	6.8 ± 0.11 6.7–7.0, n=9

Consistent cranial characters have also been identified that allow the separation of these two taxa, particularly when comparisons are made between individuals of the same sex (Figure 3). In dorsal view, the rostral portion of the nasal in *C.* '*pumilus*' from Madagascar is distinctly more inflated than in *C. leucogaster* and with a distinct anterio-lateral expansion. The infraorbital foramen in most individuals of *C. leucogaster* shows a basin-like structure, which is rarely present in *C.* '*pumilus*'. Further, in *C.* '*pumilus*' the interorbital region shows a more pronounced constriction relative to the width of the skull across the zygomatic arches. Further, in *C.* '*pumilus*' the nasal orifice has an oblong shape, as compared to *C. leucogaster*, which has the interorbital region less constricted and the nasal orifice distinctly more rounded. Another clear difference is the form of the palatal foramen, which in *C.* '*pumilus*' is open (state 2 and 3 in character C65 of Freemen 1981) and in *C. leucogaster* closed (state 1 in character C65 of Freemen 1981). The basisphenoid pits in *C.* '*pumilus*' are larger, more rounded, and approaching the basioccipital septum, as compared to the notably smaller, slightly oblong, and separated structures in *C. leucogaster.*

Dental morphology

Based on tooth measurements and structural characters, it is possible to separate individuals of *C. leucogaster* and *C.* '*pumilus*'. In the latter taxon, six of seven dental variables show notable differences between males and females (Table 4). When segregated by sex, the mean measurements in *C.* '*pumilus*' are consistently greater than in *C. leucogaster* and for certain variables (e.g., UP MOL R in males and MTR in females), there is no overlap between these two taxa. In lateral view, the distal portion of the PM^3 cusp in *C.* '*pumilus*' is slightly longer than the cingulum of the C, while in *C. leucogaster*, the PM^3 cusp does not reach or is the same length as the C cingulum. One consistent difference is the hypoconal flange of the M^3 in *C.* '*pumilus*', which is larger and extends more laterally than in *C. leucogaster* (Figure 3).

Molecular genetics

Cytochrome *b*

In a set of analyses based on 1031 nucleotides of the mitochondrial cytochrome *b* gene (Figure 4), *Chaerephon* forms a strongly supported clade. *C.* '*pumilus*' from eastern Madagascar also forms a strongly-supported monophyletic group (B), which is sister to the *C. pumilus* clade from Yemen (A) and a mixed *C. pumilus* sensu lato/*C. leucogaster* clade (C) (support 0.72, 81%, 92%). Clade B comprises five haplotypes (15, 16, 17, 18, 19) (Appendix 2), separated by a mean genetic distance of 0.52% (0.10% – 0.89%). The genetic distances separating Clade B from the other clades are as follows: Clade A (*C. pumilus*, Yemen) 2.98%, Clade C1 (*C. pumilus* sensu lato Tanzania) 2.46%, Clade C2a (*C.* '*pumilus*' Comoros and Aldabra) 3.07% and Clade C2b (*C. leucogaster*) 2.29% (Table 1). For a discussion of genetic distances and species designations, see the 'Conclusions' section below.

Clade C comprises two subclades; subclade C1 is strongly supported and contains *C. pumilus* sensu lato from Tanzania, while subclade C2 (support 0.85, 65%, 96%) is further subdivided into clades C2a and C2b. Tanzanian clade C1 was included for reference purposes in this paper, which focuses on *Chaerephon* species from western Indian Ocean islands, and comprises two haplotypes (5, 6), separated by a mean genetic distance of 0.52%. The genetic distances separating Clade C1 from the other clades are as follows: Clade A (*C. pumilus*, Yemen) 2.55%, Clade B (*C.* '*pumilus*' eastern Madagascar) 2.46%, Clade C2a (*C.* '*pumilus*' Comoros and Aldabra) 2.25%, and Clade C2b (*C. leucogaster*) 1.68% (Table 1).

Clade C2a is strongly supported and comprises *C. pusillus* from Aldabra and samples originally assigned to *C. pumilus* originating from all four islands of the Comoros Archipelago. This clade comprises four haplotypes (1, 2, 3, 4) separated by a mean genetic distance of 0.23% (range 0.10% – 0.39%). Interestingly, each of these four haplotypes is found on two or three of the islands of the Comoros (Figure 4); this low level of genetic structure is consistent with regular gene flow even though these islands are separated by 40 – 80 km of ocean. Further, a single haplotype (3) is representative of *C. pusillus* as well as *C.* '*pumilus*' samples from the islands of Mayotte and Grande Comore, indicating a lack of distinctness between these taxa, which are referable to a single species. The genetic distances separating Clade C2a from the other clades are as follows:

Clade A (*C. pumilus*, Yemen) 2.86%, Clade B (*C.* '*pumilus*' eastern Madagascar) 3.07%, Clade C1 (*C. pumilus* sensu lato Tanzania) 2.25%, and Clade C2b (*C. leucogaster*) 1.31% (Table 1).

Clade C2b, less supported, comprises samples morphologically-identified as *C. leucogaster* and originating from western Madagascar, Pemba and Zanzibar (Tanzania), and Mayotte. The eight haplotypes that constitute this clade (7, 8, 9, 10, 11, 12, 13, 14) were separated by a mean genetic distance of 0.25% (range 0.10% – 0.49%). The genetic distances separating Clade C2b from the other clades are as follows; Clade A (*C. pumilus*, Yemen) 2.67%, Clade B (*C.* '*pumilus*' eastern Madagascar) 2.29%, Clade C1 (*C. pumilus* sensu lato Tanzania) 1.68%, Clade C2a (*C.* '*pumilus*' Comoros and Aldabra) 1.31% (Table 1). *Chaerephon leucogaster* samples with haplotype 9 were found in western Madagascar as well as on Mayotte and Pemba. A sample representative of the most common *C. leucogaster* haplotype from western Madagascar (haplotype 10) was found in sympatry with specimens of *C.* '*pumilus*' **(**haplotype 15) in a roost site in Manakara, eastern Madagascar.

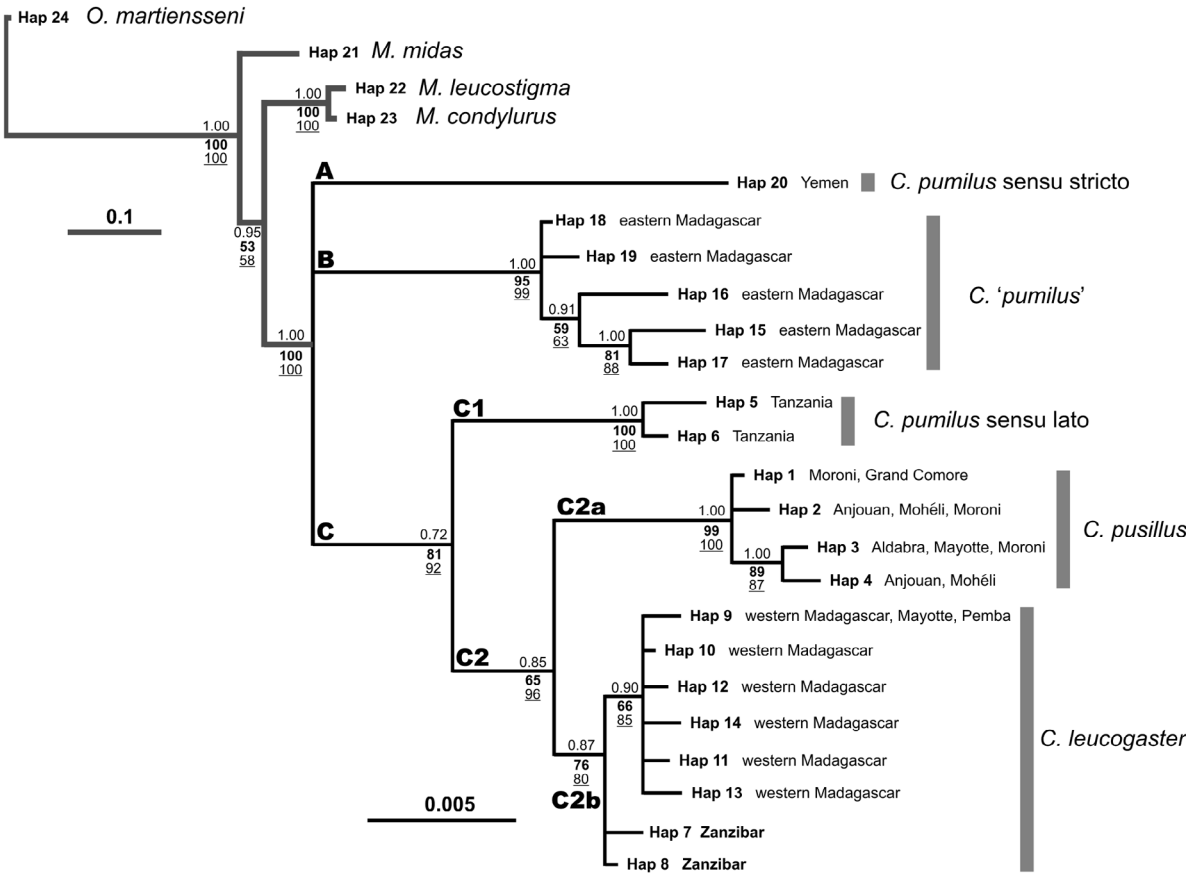

FIGURE 4. Tree based on analyses of 1031 nucleotides of the mitochondrial cytochrome *b* gene illustrating relationships between 20 haplotypes of *Chaerephon* spp. with respect to the outgroups *Mops leucostigma* from Madagascar (haplotypes 22 and 23), *M. midas* from Madagascar (haplotype 21), and *Otomops martiensseni* from Kenya (haplotype 24). Nodal support is indicated according to the following analyses used: Bayesian posterior probability in standard font (top), bootstrap value (1000 pseudo-replicates) for maximum parsimony analysis in bold font (middle), and neighbor-joining analysis underlined (bottom). Bootstrap values of less than 50% and posterior probabilities of less than 0.7 are not indicated. *Chaerephon* '*pumilus*' from eastern Madagascar is described herein as *C. atsinanana* **sp. nov.**

D-loop

The structure of trees obtained from Bayesian, maximum parsimony, and neighbor-joining analyses of 332 bp of the mitochondrial D-loop (Figure 5) are congruent with those obtained from the cytochrome *b* analysis (Figure 4). There is very strong support for the monophyly of the major *C. pumilus* sensu lato clades. *Chaerephon* '*pumilus*' from eastern Madagascar forms a strongly-supported clade (B) which is sister to three

other clades: Clade A is the *C. pumilus* clade from Yemen; Clade C2a includes *C. pusillus* from Aldabra and samples originally assigned to *C.* '*pumilus*' originating from Grande Comore; Clade C2b (less supported) comprises samples morphologically identified as *C. leucogaster* and originating from western Madagascar and Pemba (Tanzania). Information on the distribution of the different haplotypes is presented in Appendix 3.

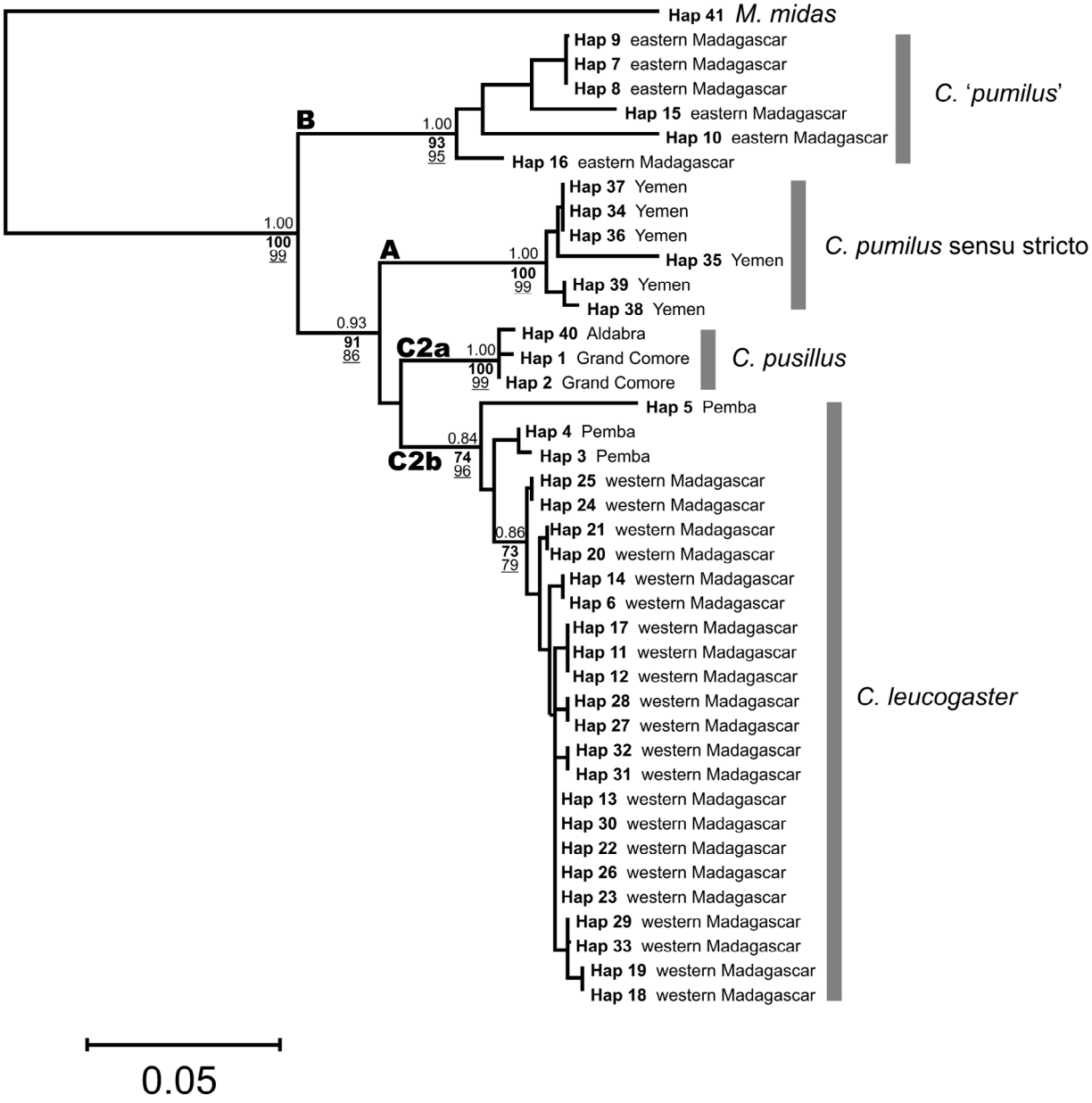

FIGURE 5. Tree based on analyses of 332 nucleotides of the mitochondrial D-loop illustrating relationships between 40 haplotypes of *Chaerephon* spp. with respect to the outgroup *M. midas* from Madagascar (haplotype 41). Nodal support is indicated according to the following analyses used: Bayesian posterior probability in standard font (top), bootstrap value (1000 pseudo-replicates) for maximum parsimony analysis in bold font (middle), and neighbor-joining analysis underlined (bottom). Bootstrap values of less than 50% and posterior probabilities of less than 0.7 are not indicated. Clade designations A, B, C2a, and C2b match those in Fig. 6. *Chaerephon* '*pumilus*' from eastern Madagascar is described herein as *C. atsinanana* **sp. nov.**

Conclusions

On the basis of different morphological and genetic characters, we conclude that *C. leucogaster* and *C.* '*pumilus*' from eastern Madagascar are diagnosably distinct from one another and should be considered

separate species. Genetically, *C. 'pumilus'* forms a strongly supported monophyletic clade, which is separated from the monophyletic *C. leucogaster* clade by a genetic distance of 2.29% (cytochrome *b*; Table 1). This distance is at the upper limit (2.3%) of the range of intraspecific cytochrome *b* genetic distances reported for bats based on 25 species from 10 genera (Baker & Bradley 2006). However, 2.3% is also the lower limit of the interspecific distances they report. None of the bats reported by Baker and Bradley (2006) are molossids; Nabholz *et al*. (2008) report strong variations in the mitochondrial mutation rate between mammalian families, which raises the possibility that the mutation rate in the Molossidae may be lower than that in other families of bats. Although they are largely allopatric, these two genetic forms (*leucogaster* and '*pumilus*') occur in sympatry at Manakara, and, most important to their designation as separate species, appears as valid morphological/biological species.

Animals from the Comoros Archipelago previously assigned to *C. pumilus* fall within the *C. pusillus* clade and should be referred to this taxon. *Chaerephon pusillus* is essentially a 100%-supported monophyletic clade, and therefore a valid phylogenetic species. Although it is separated from *C. leucogaster* by a low genetic distance of 1.31%, it is a geographically circumscribed clade, restricted to the Comoros and Aldabra, which occurs in sympatry with the morphologically distinct form, *leucogaster*, on Mayotte.
Below we name and describe the Madagascar populations formerly assigned to *C. pumilus*.

Systematics

Family Molossidae Gill, 1872

Genus *Chaerephon* Dobson, 1874

Chaerephon atsinanana sp. nov. (Figures 3-7)

Madagascar free-tailed bat

Holotype. FMNH 185259, adult female (not in reproductive condition), body preserved in 12% formalin and subsequently transferred to 70% ethanol, skull removed and cleaned. Original field number Fanja H. Ratrimomanarivo (RHF) 1060. Muscle tissue preserved in EDTA and housed in the FMNH under the same catalog number. This specimen was used in both the morphological and molecular analyses.

Type locality. Madagascar: Province de Fianarantsoa, Farafangana, Collèges d'Enseignement Général (CEG) Fenoarivo, 22°49.275'S, 47°49.860'E, 10 m. Captured in a village on 26 April 2005 with a mist net placed between buildings.

Paratypes. Includes eight additional specimens from the type locality (FMNH 185260-185268). All of these specimens were used in the morphological analyses and FMNH 185260 in the molecular analyses.

Referred specimens (specimens **not** used in morphological analysis in **bold** and those used in the molecular analysis underlined). *Province d'Antsiranana*: Andapa, 500 m (FMNH 154064). *Province de Fianarantsoa*: Vangaindrano (ville), bureau circonscription scolaire, 23°21.300'S, 47°35.763'E, 10 m, (FMNH 185229, 185230, 185231-185238); Vangaindrano (ville), Dispensaire public, Ampasy, 23°21.426'S, 47°35.813'E, 10 m (FMNH **185239-185240**, 185241, **185242-185243**, 185244-185256, **185257**, 185258); Commune Mahabo, chalet de marché, 23°11.316'S, 47°40.745'E, 30 m (FMNH 185269-185271, **185272**, 185273-185278); Commune Ampahatelo, maison du domaine d'Akamasoa, 23°20.678'S, 47°35.778'E, 20 m (FMNH 185279-185282); Vohipeno, Commune Vohitrindry, Quartier Fenoarivo, grenier N. 1, 22°21.997'S, 47°50.206'E, 35 m (FMNH 185283-185285, 185286, 185287-185292); Vohipeno, quartier Ambohimanarivo, bureau de Fivondronana, 22°21.242'S, 47°50.421'E, 30 m (FMNH 185293-185296, **185297**, 185298-185305); Manakara, Manakara be, EPP Tanambe, salle A, 22°09.418'S, 48°01.009'E, 15 m (FMNH 185306-185311, **185312**, 185313, 185314, 185315, 185316-185318); Ifanadiana (ville), Hôpital CSB II Mazavatakona, 21°18.394'S, 47°38.144'E, 459 m (FMNH 185319-185321, 185322, 185323-185328); Ifanadiana (ville), école Lycée, salle I, 21°17.904'S, 47°38.264'E, 460 m (FMNH **185329**, 185330-185335, **185336**, 185337-185338); Ranomafana (Ifanadiana), maison d'habitation, 610 m (FMNH 188081-188082, **188083**, 188084-188087, 188088, 188089, 188090-188091); Ifanadiana (MNHN **1985.437-1985.440**).*Province de Toamasina*: Périnet-Analamazaotra, 140 km E Tana (BMNH **76.1896**, **76.1897**); Moramanga, 950 m (MCZ **45098-45099**, **45107**, **45582-45583**); Ambodiriana, 20 km SW Périnet, 975 m (ROM **42071-42072**); Périnet (Andasibe),

CIBA, 18°53.747'S, 48°24.907'E, 950 m (FMNH 184491, **184492**, 184493-184495); Périnet (Andasibe), cité CIBA, 18°55.410'S, 48°25.246'E, 980 m (FMNH 184496-184499, **184500**, 184501-184508); Périnet (MNHN **1985.475**); Beforona, bureau de poste, 18°53.349'S, 48°34.683'E, 560 m (FMNH 184509-184518); Beforona, ex hotel villa Martin'son, 18°53.320'S, 48°34.711'E, 560 m (FMNH 184519-184522); Beforona, école CEG, 18°53.334'S, 48°34.792'E, 560 m (FMNH 184523-184524); Ambatondrazaka, Andrarabarikely, 17°49.784'S, 48°25.112'E, 1000 m (FMNH **184651-184652**, 184653, **184654-184656**, 184657, **184658-184659**, 184660); Sabotsy Anjiro, maison d'habitation, 18°53.672'S, 47°58.401'E, 850 m (FMNH 184661-184666, **184667**); Sabotsy Anjiro, maison d'habitation No. 2, 18°53.711'S, 47°58.391'E, 850 m (FMNH 184668-184672, **184673**, 184674-184676); Sabotsy Anjiro, Andranoalina, maison d'habitation No. 3, 18°52.945'S, 47°58.245'E, 850 m (FMNH 184677, 184678, 184679-184685, **184686**); Toamasina (ville), menuiserie Mangarano, 18°08.441'S, 49°22.670'E, 10 m (FMNH 187797—187799, 187800-187803); Toamasina (ville), EPP Ambohijafy, 18°07.569'S, 49°24.128'E, 10 m (FMNH 187804—187805, 187806, 187807-187813); Toamasina (ville), menuiserie Mangarano, 18°08.441'S, 49°22.670'E, 10 m (FMNH **187814-187815**); Fanandrana, EPP, 18°15.122'S, 49°16.067'E, 40 m (FMNH 187816-187822); Brickaville, bureau commune, 18°49.317'S, 49°04.343'E, 10 m (FMNH 187823); Ranomafana, hôpital CSB II, 18°57.661'S, 48°50.655'E, 50 m (FMNH 187824-187830, **187831**, 187832-187833); Ranomafana, église catholique, 18°57.636'S, 48°50.845'E, 90 m (FMNH 187834, 187835, 187836, 187837); Moramanga (ville), Lycée technique, 820 m (FMNH 188112-188118, **188119**, 188120-188121); Moramanga (ville), grande salle de l'EPP près du Lycée, 820 m (FMNH 188122-188126, **188127**, 188128, **188129**, 188130); Moramanga (ville), école primaire privée, 820 m (FMNH **188131**); Anjiro, Marozevo, maison d'habitation II, 815 m (FMNH **188142**, 188143, **188144**); Moramanga (ville), Sahanofata, 835 m (FMNH **188132**, 188133, **188134**, 188135-188137, **188138**, 188139-188140, **188141**); Station Forestière d'Ivoloina, près du gîte Mahatsinjo, 18°03.406'S, 49°21.635'E, 15 m (UADBA **RB-20**, **RB-21**); Andreba, Railroad Station (USNM **328771-328772**); 3 km E Périnet (USNM **341734-341743**).

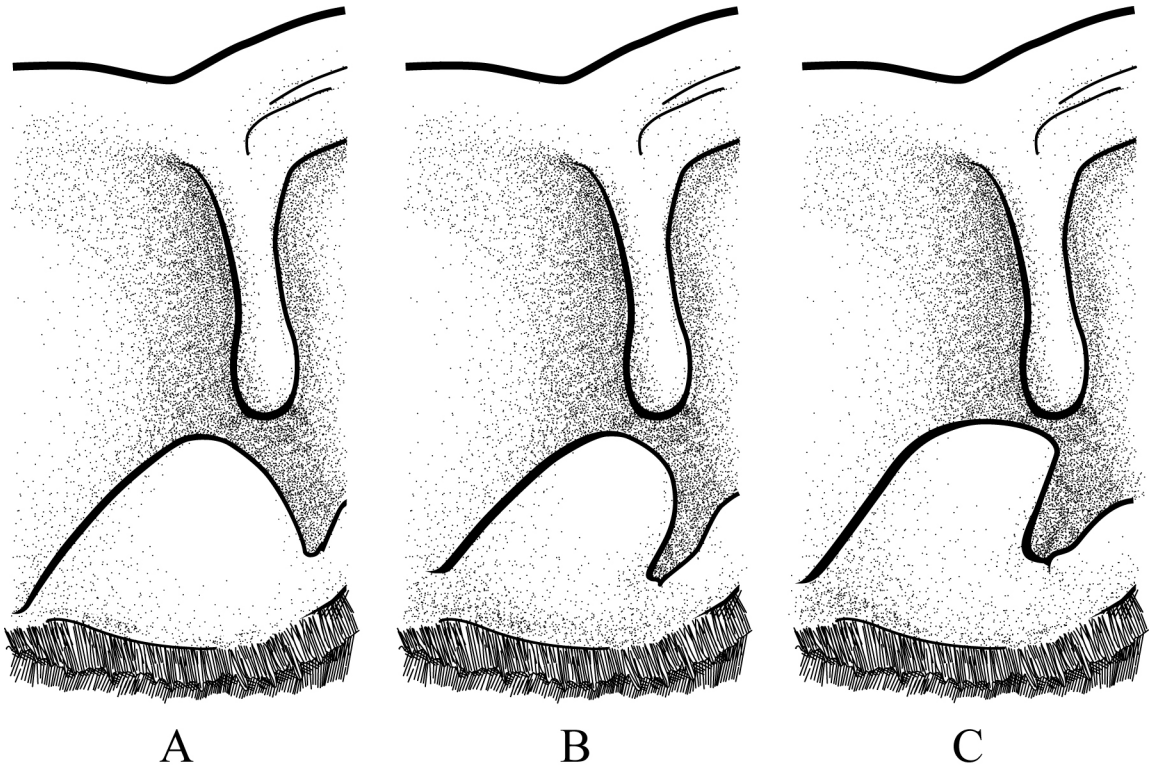

FIGURE 6. Lateral view of central portion of external right ear and antitragus of different *Chaerephon* spp.: **A** – *C. atsinanana* **sp. nov.** (FMNH 188144) from Madagascar: Province de Toamasina, Commune Anjiro, Marozevo; **B** – *C. leucogaster* (FMNH 188644) from Madagascar: Province d'Antsiranana, Nosy Komba, Ampangorinana; **C** – *C. pumilus* sensu stricto (BMNH 19.7.7.334) from Massawa, Eritrea, the type locality of this taxon.

Etymology. The name *atsinanana* is derived from the Malagasy word meaning "from the east."

Diagnosis. A relatively small molossid bat with a forearm of 37-42 mm. The ears are notably shorter than the head and they are united by a short band of skin. The antitragus is large and broad, the anterior margin slightly angled and posterior margin rounded, and blunt tip (Figure 6a). The tragus has a broad attachment. Skull and mandible relatively large for a small member of *Chaerephon*. The nasal is inflated and in dorsal view, the rostrum is expanded anterior-laterally (Figure 3). The cranial portion of the frontal, parietal, and supraoccipital, as well as the squamosal, are inflated. The basisphenoid pits are large, deep, slightly oval, and approaching the basioccipital septum. Basioccipital pits are either not present or indistinct. Palatal foramen is open, but not forming a deep cleft. The individual teeth, particularly molariform, relatively robust. The P^3 is prominent and aligned towards the outer margin of the toothrow. Lingual portion of upper molariform teeth elongated, particularly the hypoconal flange of M^1, the paracone and hypoconal flange of M^2, and the hypoconal flange in M^3.

Given present taxon and character sampling, the species is further diagnosed by the following strict synapomorphies in the cytochrome *b* gene (the first nucleotide given is the ancestral state, followed by the nucleotide position in the cytochrome *b* gene, followed by the derived state; substitutions that result in an amino acid change are indicated in bold text):
Hap 15: C108T, A126T, A147G, A238T, T282C, T309C, T351C, C352T, A516G, C573T, T579C, G709A, G711A, A753G, A1020G, G1038A, C1101T, G1104A.
Hap 16 and 19: C108T, A238T, T282C, T309C, T351C, C573T, T579C, G711A, A1020G, G1038A, C1101T, G1104A.
Hap 17: C108T, A147G, A238T, T282C, T309C, T351C, C352T, C573T, T579C, G711A, A753G, A1020C, G1038A, C1101T, G1104A.
Hap 18: C108T, A238T, T282C, T309C, T351C, C573T, T579C, G711A, A1020G, G1038A, C1101T, G1104A.

Sympatrically occurring Molossidae. At the holotype site in Farafangana near the Collèges d'Enseignement Général (CEG) Fenoarivo, only *C. atsinanana* was captured, but *Mops leucostigma* was also found in this village. Across the range of *C. atsinanana*, it is known to occur in sympatry, in virtually all cases in synanthropic settings, with *C. leucogaster* (at Manakara [Ratrimomanarivo *et al*. 2009a]), *Mormopterus jugularis* (Ratrimomanarivo *et al*. 2009b), and *Mops leucostigma* (Ratrimomanarivo *et al*. 2008).

Description. The lectotype of nominate *pumilus* (SMF 4311, an adult female based on the examination of the specimen's external genitalia) from Massawa, Eritrea, has a partially damaged skull, while other specimens from the type series collected in Massawa, particularly SMF 11918, are in excellent condition. In the Senckenberg Museum, these specimens were compared to USNM 38032 (an adult female), also from Massawa, and all are morphologically similar with the exception of some subtle differences that are best considered falling within the range of intraspecific variation. In subsequent comparisons, USNM 38032 was used to represent the cranial and dental character states in nominate *pumilus*.

External characters. The forearm length of the lectotype of nominate *pumilus* (SMF 4311) taken from the outstretched wing of the mounted specimen measured 38.6 mm. This falls within the range of *C. atsinanana*. Given the style of preparation and the state of the specimens, it was difficult to compare USNM 143167 or individuals of *C. atsinanana* to the type series of nominate *pumilus* in the SMF. Herein, comparisons are based on the specimens from Eritrea, Saati - USNM 143167 (an adult male) and from Massawa - USNM 143168, USNM 38032, and BMNH 19.7.7.3347 (all females) from Massawa.

On the basis of the material available, in fluid preserved specimens of nominate *pumilus* and *C. atsinanana*, the fleshy portion of the ears, including the band connecting them, have similar morphology. However, the antitragus of *C. atsinanana* is notably broad, with an angular anterior edge, and terminating with a rectangular-blunt tip, while in nominate *pumilus* the structure is less thickset, with a less angular anterior margin, more angular posterior margin, and slightly rounded tip (Figure 6). Further, the short tragus in *C. atsinanana* is distinctly broader and with an extensive portion attaching to the ear, compared to nominate *pumilus* that has a narrower articular surface (not illustrated). In *C. leucogaster*, the antitragus is small, proportionately not wide, the two lateral margins being largely similar in shape, and terminates with a rounded

edge and in *C. atsinanana* it is notably larger and broader, anterior margin slightly angled and posterior margin rounded, and blunt tipped (Figure 6). The tragus of *C. leucogaster* is slightly longer and narrower at the base than the shorter and broader structure in *C. atsinanana*.

The dorsum, throat, and chest in *C. leucogaster* is dark brown, as compared to *C. atsinanana* with a blackish-brown dorsum, brown throat, and dark brown chest; in the latter taxon there is considerable variation (Figure 7). The abdomen in *C. leucogaster* tends to have a large whitish area, although in a few individuals this is not extensive and restricted to the mid-ventral area, while in *C. atsinanana* the venter is a dark brown and in a few rare cases, there is a small white mid-ventral patch. In general, *C. atsinanana* have a distinct whitish or beige strip of hairs at the base of the wings (plagiopatagium), also found in most African animals assigned to *C. pumilus*, although considerable intraspecific variation can be found on Madagascar within adults of the same population (Figure 7). This pelage trait is largely unknown from Malagasy *C. leucogaster.*

Skull and dentition. The skull of *C. atsinanana* is notably more massive than in nominate *pumilus* (Figure 3). In all cases, the average skull measurements of *C. atsinanana* are larger than those of nominate *pumilus* and for certain variables (GSL, CON INCI, MASTOID), there is no overlap in measurements between these two species (Table 3).

In dorsal view, there is a notable inflation of the nasal in *C. atsinanana* as compared to nominate *pumilus* (Figure 3), resulting in a distinct antero-lateral expansion of this portion of the rostrum. When examined from a lateral view, the lacrimal ridge has a more angular shape in nominate *pumilus*, particularly along the facies orbitalis of the frontal. Further, in *C. atsinanana* the cranial portion of the frontal, parietal, and supraoccipital, as well as the squamosal, are notably more inflated than in nominate *pumilus*. In ventral view, the basisphenoid pits in *C. atsinanana* are larger, deeper, slightly oval, and approaching the basioccipital septum; this is in contrast to the notably smaller, shallower, rounded, and separated structures in nominate *pumilus* (Figure 3). Further, in nominate *pumilus* shallow basioccipital pits are present and these structures are very indistinct or absent in *C. atsinanana*. The form of the palatal foramen in the two taxa is similar, being open (state 2 or 3 in character C65 of Freemen 1981), but not with a deep cleft. Some caution is needed when assessing this character, as certain skulls, which have not been thoroughly cleaned, particularly with Dermestidae beetles, retain remnant tissue and cartilage. A case in point is the holotype of *C. atsinanana* (Figure 3), in which the remaining attachment between the premaxillae is cartilaginous. This character is also highly variable in African populations of *C. pumilus* (Taylor 1999b).

The condylocanine length (CON CANI) in *C. atsinanana* is on average longer than in nominate *pumilus*, but there is broad overlap in measures of the temporal and masseter moment arms of the mandible (MOM1 COR, MOM2 ANG) and lower toothrow. The lower molariform teeth in these two taxa have similar morphology and cusp structure, but the width of individual teeth is wider in *C. atsinanana* than in nominate *pumilus.*

With one exception, the average measurements for the dental variables are larger in *C. atsinanana* than in nominate *pumilus*; the exception is UP CANIN in nominate *pumilus* (sexes combined), which on average is slightly longer (2.6 mm) than in female *C. atsinanana* (2.5 mm). Hence, individual molariform teeth in *C. atsinanana* are distinctly more robust than in nominate *pumilus*, particularly when comparing adult individuals of the same sex. The P^3 in nominate *pumilus* is notably reduced in size, being peg-like, and is aligned in the middle of the toothrow, as compared to in *C. atsinanana* in which this tooth is notably larger and aligned towards the outer margin of the toothrow. In general, the cusp morphology of the upper molariform teeth of these two species are similar, although in *C. atsinanana* there is a distinct elongation of the portion of the tooth lingual to the commissures, particularly the hypoconal flange of M^1 the, the paracone and hypoconal flange of M^2, and the hypoconal flange and more open commissure of M^3.

Patterns of morphological variation in members of the C. pumilus *complex in the western Indian Ocean.* In order to provide further insight into patterns of morphological variation in a multivariable sense of *C. atsinanana* with other western Indian Ocean islands and east African populations of *C. pumilus* sensu lato, a PCA analysis was conducted separately for males and females for different cranial and dental variables (Table 5). In order to maximize the number of specimens that could be used in these comparisons, a few variables with numerous cases of missing data were excluded from the analysis. In general, two different groups occur

in the projections of PC 1 plotted against PC 3 (Figure 8): a cluster of points composed of *C. atsinanana* from Madagascar and *C. pumilus* sensu lato from Kenya and a second cluster composed of *C. pusillus* from the Comoros and Aldabra, which in most cases included *C. pumilus* sensu stricto from Eritrea. For the various comparisons, with the sexes and types of variables separated, the total percentage of the explained cumulative variance for the first three axes surpasses 94%, with the first axis explaining a minimum of 82% (Table 5). The second axis adds an additional 6.4—7.8 %, indicating that size is the major factor separating the two groups.

FIGURE 7. View of the ventrum of three individuals of *Chaerephon atsinanana* **sp. nov**. captured the same day and from the same colony at Andasibe, showing the range of variation in ventrum coloration (left to right): Male (FMNH 184504, RHF 068), female (FMNH 184506, RHF 070), and female (FMNH 184508, RHF 072).

TABLE 5. Factor loadings of principal component analyses of cranial and dental characters of male and female *Chaerephon pusillus* from Aldabra and the Comoros (excluding Mayotte), *C. atsinanana* **sp. nov**. from Madagascar, *C. pumilus* sensu stricto from Eritrea, and *C. pumilus* sensu lato from Kenya. Several variables were removed from the analyses in cases of numerous missing values to maximize the number of specimens used. See Figure 9 for plots of these analyses.

Cranial variables

	Male			Female		
	PC 1	PC 2	PC 3	PC 1	PC 2	PC 3
GSL	-0.961	0.120	0.016	-0.961	0.102	0.015
PALATE	-0.813	0.556	-0.109	-0.821	0.561	-0.049
LACR WID	-0.860	-0.317	-0.382	-0.910	-0.123	0.309
POST ORB	-0.900	-0.153	0.311	-0.895	-0.200	-0.354
ZYGO BR	-0.962	-0.038	0.021	-0.958	-0.101	0.060
MASTOID	-0.949	-0.128	0.107	-0.939	-0.183	0.005
Eigen value	4.961	0.465	0.266	5.026	0.423	0.227
% total variation explained	82.7	90.5	94.9	83.8	90.9	94.7

Dental variables

	Male			Female		
	PC 1	PC 2	PC 3	PC 1	PC 2	PC3
C^1-C^1	-0.891	0.442	0.090	-0.926	-0.326	-0.120
M^3-M^3	-0.944	-0.039	-0.324	-0.948	-0.122	0.277
UP MOL	-0.936	-0.264	0.169	-0.921	0.353	0.038
MTR	-0.962	-0.115	0.070	-0.955	0.097	-0.196
Eigen value	3.488	0.280	0.146	3.517	0.255	0.131
% total variation explained	87.2	94.2	97.9	87.9	94.3	97.6

On the basis of morphological characters and different cranio-dental measurements, as well as molecular genetics, it has been shown that animals referable to nominate *pumilus* obtained in the vicinity of the type locality in Eritrea are distinct from Malagasy specimens. The PCA analysis of cranial and dental measurements is concordant with this conclusion (Figure 8; Table 5), with a broad separation for both males and females for individuals collected in Eritrea and Madagascar. The single exception is a male from Eritrea, which shows, overlap for the dental variables with individuals from Madagascar and Kenya. Further, the PCA analysis closely groups individuals from the Comoros and Aldabra, which is in agreement with the molecular analysis, and gives morphological support to these populations being united under the name *pusillus*. The overlap between individuals from Kenya and Madagascar, which are in different clades of the *C. pumilus* complex, is presumed to be associated convergence in aspects of craniodental size, although, as outlined above, several morphological characters separate African and Malagasy members of this species complex.

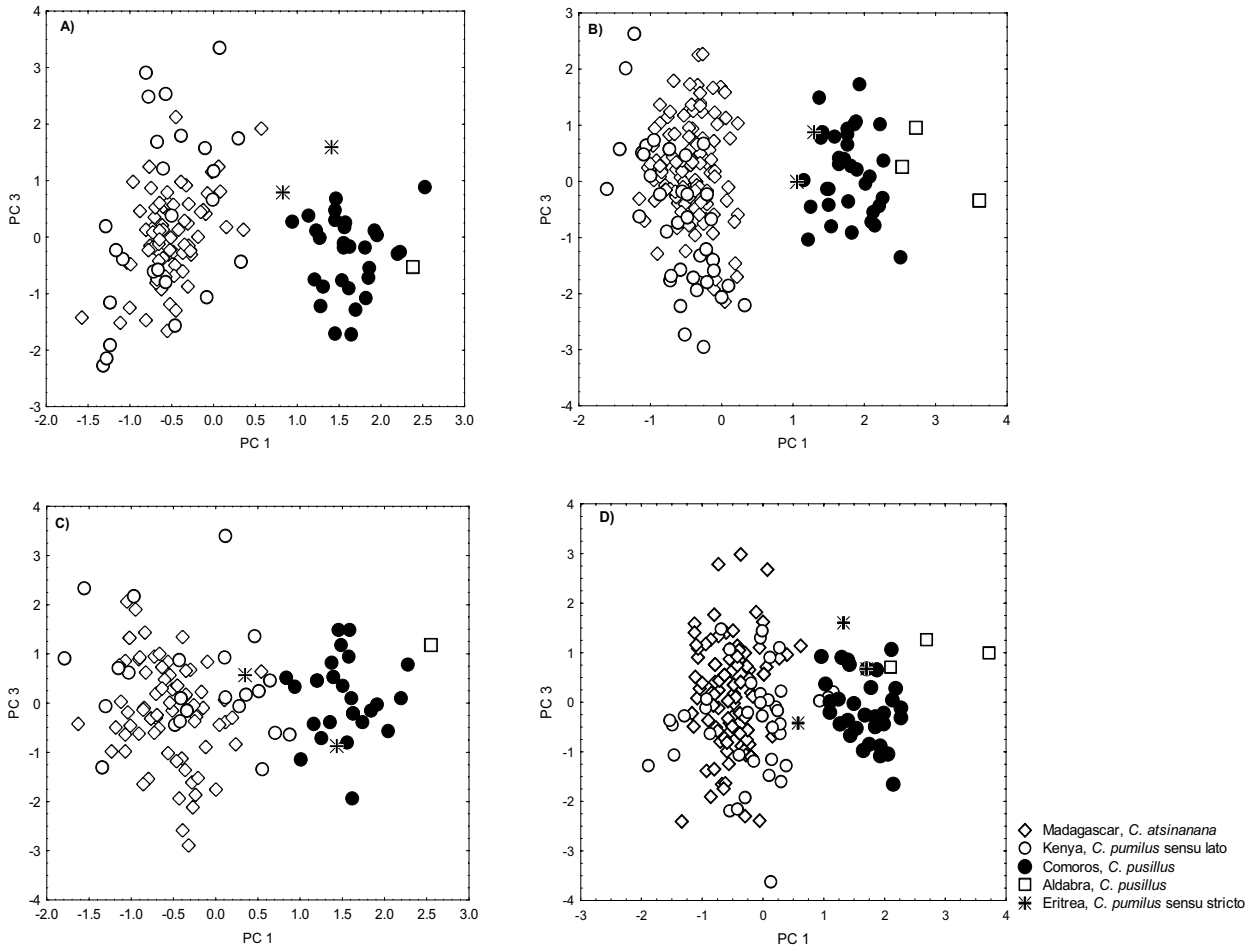

FIGURE 8. Projection of PC factors 1 and 3 for *Chaerephon pusillus* from Aldabra and the Comoros (excluding Mayotte), *C. atsinanana* **sp. nov.** from Madagascar, *C. pumilus* sensu stricto from Eritrea, and *C. pumilus* sensu lato from Kenya. A: cranial variables - males, B: cranial variables - females, C: dental variables - males, and D: dental variables - females. See Table 5 for the PCA factor loadings.

Distribution, biology and conservation status. *Chaerephon atsinanana* is known from numerous localities across the eastern half of Madagascar from near sea level to over 1100 m (Figure 1). Of the 44 day roost sites located over the past few years, all are in synanthropic settings (schools, churches, occupied houses). Most of these buildings have distinct architectural styles, with the roofs 4—6 m off the ground, with or without air ventilation holes in the gables, and metal roofs. All of these sites are in urban or at least rural areas and outside of natural forest. During our exploration of Madagascar, we have never located a natural day-roost site of this species. Hence, we assume that since the construction of fixed permanent structures on

the island, within the past hundred years or so, local populations of this species have increased and its distribution may have concordantly expanded. While there is some evidence of hunting of this species for bush-meat (Goodman *et al*. 2008), it is remarkably adaptable to human transformation of the natural landscapes of the island (Harper *et al*. 2007) and occupying buildings for day roosts. Hence, there is no immediate conservation concern associated with the short or medium-term future of *C. atsinanana*. The same holds true for *C. pusillus* from the Comoros, which particularly on Mohéli, Grande Comoro, and Anjouan are abundant in certain architectural styles of abandoned or occupied buildings. In contrast, *C. pusillus* from the western Seychelles, specifically Aldabra Atoll, is not known to occupy the few buildings on the island, indicating that day roost sites might be more limited.

Taxonomic conclusions

Several authors have underlined that the current taxonomical classification of the *Chaerephon pumilus* sensu lato complex, as a single species, does not reflect the evolutionary history of this group (e.g., Simmons 2005; Taylor *et al*. 2009). Here, based on the isolation of DNA from a tissue sample recovered from an older specimen collected at the type locality (Massawa, Eritrea), we were able to establish which clade represents *C. pumilus* sensu stricto. Thereafter, with a combination of molecular genetics and morphological characters several different aspects could be established: 1) *C. leucogaster*, which is genetically nested within *C. pumilus* sensu lato, was diagnostically distinct to animals classically referred to *C. pumilus* from eastern Madagascar and at one site on the island they are known to occur in sympatry; 2) subsequently, to refer all of the animals within the *C. pumilus* sensu lato clade to a single species was not concordant to phylogenetic patterns and it was necessary to consider the Malagasy population of *C.* '*pumilus*' as a separate evolutionary unit; and 3) a range of morphological characters allow the separation of Malagasy animals from nominate *pumilus* and the Malagasy animals are described herein as a new species to science, *C. atsinanana*. Another important aspect is that size alone, specifically associated with cranio-dental measurements, should not be used as a taxonomic character, as an overlay of the phylogenetic results and morphological results indicate, for example, that similarities in size between *C. atsinanana* and *C. pumilus* from Kenya is convergent. The research presented herein is the first step in resolving a portion of the evolutionary history of the *C. pumilus* species complex.

Movements of volant animals between continental areas and western Indian Ocean islands

Even though numerous islands in the western Indian Ocean are several hundred kilometers from the nearest continental landmass, there is evidence for different animal groups of regular migration and irregular movements. This is at least in part associated with seasonal wind patterns of the Inter-Tropical Convergence Zone (ITCZ) (Anderson 2009; Dijkstra 2007; Pedgley *et al*. 1995). Evidence for movements of bats between Africa and Madagascar can be found in the molossid *Mops midas*, which was formerly thought to have distinctive African and Malagasy populations. Recent research shows no morphological or genetic differentiation between these populations (Ratrimomanarivo *et al*. 2007) and either movements of these bats across the Mozambique Channel or recent colonization of Madagascar can best explain this observation. Although the Comoros Archipelago is separated from the nearest portion of Madagascar by about 300 km of open water, several molossids are shared between these two areas (*M. leucostigma* and *Chaerephon leucogaster*) and show no genetic differences. Hence, these taxa either only recently colonized this archipelago or there is at least occasional dispersal movements between Madagascar and the Comoros that maintain the genetic similarity. Another example is two small species of *Miniopterus* (Family Miniopteridae) bats that are shared in common between northern Madagascar and two Comorian islands (Grande Comore and Anjouan) that are morphologically and genetically similar (Goodman *et al*. 2009; Weyeneth *et al*. 2008). None of the bat taxa mentioned in this paragraph is known from the outer western Seychelles island of Aldabra, which forms a point of a nearly equilateral triangle between northern Madagascar and the Comoros (Figure 1).

The case presented here for animals of the *C. pumilus* species complex shows a different pattern in the western Indian Ocean from those mentioned above for other bat species. The Malagasy animals, named herein as a new species endemic to the island, are morphologically and genetically distinct from regional

islands and mainland Africa. The population of *C. pusillus* from the western Seychelles atoll of Aldabra cluster with that of the Comoros, rather than Madagascar. For numerous other species of volant vertebrates (bats and birds), the origin of the Aldabra fauna is mixed between the Comoros and Madagascar (e.g., Goodman & Ranivo 2008; O'Brien *et al*. 2009; Pasquet *et al*. 2007; Warren *et al*. 2003). Hence, in the case of these 9-17 g *Chaerephon* bats, these water barriers have been associated with the isolation and subsequent differentiation of populations after successful dispersal and colonization events. In contrast, within the Comoros, there is no apparent genetic structure between with the individual islands, which are separated by 40 and 80 km, indicating that this distance is regularly traversed by these bats and giving rise to intra-archipelago panmixia of populations.

The next step

Our definitions of morphological and molecular characters associated with *C. pumilus* sensu stricto and *C. leucogaster* sensu stricto (Ratrimomanarivo *et al*. 2009a; herein), as well as the delimitation of taxa in the *C. pumilus-leucogaster* complex from the western Indian Ocean, sets the stage for further resolution of the species-complex from the African continent. This is notably complicated by the various named forms within this complex that have been placed in synonymy and the need to either obtain sequence data from type series or topotypic material to resolve in a taxonomic sense the names that should be applied to the different clades. For example, the status of various small-sized taxa assigned to *C. leucogaster* from west Africa (see Rosevear 1965) needs to be critically evaluated. Additionally, the taxonomic status of the different *C. pumilus* sensu lato clades identified in Taylor *et al*. (2009) needs to be assessed in the light of wider molecular sampling and comparison of other types of data, such as echolocation and karyotypes.

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APPENDIX 1. Details of samples used in the genetic analyses. The samples are ordered as they appear in the haplotype lists (Appendices 2 and 3). Museum numbers include the Field Museum of Natural History (FMNH), National Museum of Natural History [formerly The United States National Museum (USNM)], and the National Museum of Prague (NMP). Uncatalogued specimens are denoted with collector numbers which include PB = Petr Benda. Samples from Nosy Be and Nosy Komba, near-shore islands of Madagascar, are denoted, as are from Pemba and Zanzibar, offshore islands of Tanzania.

Taxon	Geographic coordinates		Museum or collector number	Country/island	Genbank acc. numb. D-loop	Genbank acc. numb. Cyt *b*	Sex
	Latitude	**Longitude**					
Chaerephon pusillus	12°49.923'S	45°08.215'E	FMNH 194019	Mayotte	-	GQ 489152	F
	12°49.923'S	45°08.215'E	FMNH 194020	Mayotte	-	GQ 489153	M
	11°41'26.3"S	11°41'26.3"E	FMNH 194183	Grande Comore	-	GQ 489140	F
	11°41'26.3"S	11°41'26.3"E	FMNH 194186	Grande Comore	-	GQ 489141	F
	11°41'26.3"S	11°41'26.3"E	FMNH 194189	Grande Comore	-	GQ 489142	F
	11°41'26.3"S	11°41'26.3"E	FMNH 194192	Grande Comore	-	GQ 489143	F
	11°82.750'S	43°45.444'E	FMNH 194214	Grande Comore	GQ 489113	-	F
	11°82.750'S	43°45.444'E	FMNH 194217	Grande Comore	GQ 489114	-	M
	11°88.970'S	43°42.000'E	FMNH 194220	Grande Comore	GQ 489115	-	F
	11°88.970'S	43°42.000'E	FMNH 194226	Grande Comore	GQ 489116	-	F
	11°41.263'S	43°15.250'E	FMNH 194250	Grande Comore	-	GQ 489138	F
	11°41.263'S	43°15.250'E	FMNH 194251	Grande Comore	-	GQ 489139	F
	11°41.263'S	43°15.250'E	FMNH 194256	Grande Comore	-	GQ 489144	F
	11°41.263'S	43°15.250'E	FMNH 194258	Grande Comore	-	GQ 489145	F
	12°49.923'S	45°08.215'E	FMNH 194024	Mayotte	-	GQ 489149	F
	12°53.609'S	45°08.550'E	FMNH 194035	Mayotte	-	GQ 489150	M
	12°53.609'S	45°08.550'E	FMNH 194036	Mayotte	-	GQ 489151	M
	12°09.552'S	44°25.952'E	FMNH 194323	Anjouan	-	GQ 489146	F
	12°12.010'S	44°28.014'E	FMNH 194329	Anjouan	-	GQ 489147	F
	12°12.010'S	44°28.014'E	FMNH 194334	Anjouan	-	GQ 489148	F
Chaerephon pumilus	13°20'N	43°43'E	NMP PB 3667	Yemen	-	GQ 489112	F
	15°44'N	43°37'E	NMP PB 3752	Yemen	-	GQ 489111	F
	13°02'N	44°34'E	NMP PB 3626	Yemen	GQ 489128	-	M
	14°09'N	43°31'E	NMP PB 3685	Yemen	GQ 489129	-	F
	15°26'N	43°29'E	NMP PB 3154	Yemen	GQ 489130	-	F
	13°08'N	44°51'E	NMP PB 3606	Yemen	GQ 489131	-	F
	13°08'N	44°51'E	NMP PB 3619	Yemen	GQ 489132	-	M
Chaerephon pumilus	-	-	USNM 38032	Eritrea	GQ 867179	-	
Chaerephon pusillus	09°23.339'S	46°12.142'E	FMNH 205318	Aldabra	GQ 489133	GQ 489154	F
	09°23.339'S	46°12.142'E	FMNH 205319	Aldabra	GQ 489134	GQ 489155	F

Chaerephon pumilus sensu lato	01.01751°S	031.54976°E	FMNH 193055	Tanzania	-	GQ 489156			
	01.01751°S	031.54976°E	FMNH 192938	Tanzania	-	GQ 489157			
Chaerephon leucogaster	05°35.400'S	39°13.800'E	FMNH 198093	Zanzibar	-	GQ 489160	F		
	05°35.400'S	39°13.800'E	FMNH 198149	Zanzibar	-	GQ 489158	F		
	05°35.400'S	39°13.800'E	FMNH 198151	Zanzibar	-	GQ 489159	M		
	05°35.400'S	39°13.800'E	FMNH 198152	Zanzibar	-	GQ 489161	F		
	05°35.400'S	39°13.800'E	FMNH 198091	Zanzibar	-	GQ 489162	F		
	05°35.400'S	39°13.800'E	FMNH 198092	Zanzibar	-	GQ 489163	M		
	05°35.400'S	39°13.800'E	FMNH 198150	Zanzibar	-	GQ 489165	M		
	05°35.400'S	39°13.800'E	FMNH 198153	Zanzibar	-	GQ 489164	F		
	12°49.923'S	45°08.215'E	FMNH 194028	Mayotte	-	EU716040	M		
	12°49.923'S	45°08.215'E	FMNH 194023	Mayotte	-	GQ 489166	M		
	12°49.923'S	45°08.215'E	FMNH 194019	Mayotte	-	EU 716041	F		
	04°34.200'S	39°25.200'E	FMNH 192886	Pemba	EU727534	EU716003	M		
	04°34.200'S	39°25.200'E	FMNH 192889	Pemba	-	EU716004	M		
	04°96.487'S	39°71.456'E	FMNH 192891	Pemba	GQ 489117	-	M		
	04°96.487'S	39°71.456'E	FMNH 192819	Pemba	GQ 489118	-	M		
	15°42.778'S	46°18.752'E	FMNH 184604	Madagascar	EU727485	-	F		
	15°42.778'S	46°18.752'E	FMNH 184605	Madagascar	EU727486	-	F		
	15°42.778'S	46°18.752'E	FMNH 184606	Madagascar	EU727487	-	F		
	15°42.778'S	46°18.752'E	FMNH 184607	Madagascar	EU727488	-	F		
	15°42.778'S	46°18.752'E	FMNH 184608	Madagascar	EU727517	-	M		
	16°42.062'S	46°04.304'E	FMNH 184922	Madagascar	EU727502	EU716039	F		
	16°42.062'S	46°04.304'E	FMNH 184923	Madagascar	EU727503	EU716006	M		
	22°54.546'S	44°31.574'E	FMNH 184259	Madagascar	-	EU716005	M		
	22°54.546'S	44°31.574'E	FMNH 184263	Madagascar	EU727461	-	F		
	22°54.546'S	44°31.574'E	FMNH 184264	Madagascar	EU727470	-	M		
	16°42.062'S	46°04.304'E	FMNH 184924	Madagascar	EU727504	EU716007	M		
	16°26.173'S	47°09.329'E	FMNH 184954	Madagascar	EU727511	EU716014	F		
	16°20.229'S	46°50.794'E	FMNH 184955	Madagascar	EU727512	EU716015	M		
	16°20.229'S	46°50.794'E	FMNH 184956	Madagascar	EU727513	EU716016	M		
	16°20.229'S	46°50.794'E	FMNH 184957	Madagascar	EU727514	EU716017	F		
	16°20.229'S	46°50.794'E	FMNH 184958	Madagascar	EU727515	EU716018	F		
	16°20.229'S	46°50.794'E	FMNH 184959	Madagascar	EU727516	EU716019	F		
	15°54.245'S	46°35.873'E	FMNH 185020	Madagascar	EU727525	GQ 489167	F		
	15°54.245'S	46°35.873'E	FMNH 185021	Madagascar	EU727526	-	M		
	15°54.245'S	46°35.873'E	FMNH 185022	Madagascar	EU727527	-	F		

15°54.245'S	46°35.873'E	FMNH 185027	Madagascar	EU727528	-	F		
15°54.245'S	46°35.873'E	FMNH 185028	Madagascar	EU727529	-	M		
15°54.245'S	46°35.873'E	FMNH 185029	Madagascar	EU727530	-	M		
15°54.245'S	46°35.873'E	FMNH 185030	Madagascar	EU727530	-	F		
23°23.704'S	43°43.219'E	FMNH 184239	Madagascar	EU727483	EU716036	M		
23°23.704'S	43°43.219'E	FMNH 184240	Madagascar	EU727474	EU716037	F		
23°23.704'S	43°43.219'E	FMNH 184238	Madagascar	EU727471	EU716038	M		
23°23.704'S	43°43.219'E	FMNH 184237	Madagascar	EU727462	-	F		
13°21.095'S	48°11.307'E	FMNH 188496	Nosy Be	GQ 489137	GQ 489178	F		
16°42.062'S	46°04.304'E	FMNH 184925	Madagascar	EU727505	EU716008	M		
16°42.062'S	46°04.304'E	FMNH 184926	Madagascar	EU727506	EU716009	F		
16°57.452'S	46°49.433'E	FMNH 184915	Madagascar	EU727496	-	F		
16°57.452'S	46°49.433'E	FMNH 184916	Madagascar	EU727497	-	M		
16°57.452'S	46°49.433'E	FMNH 184917	Madagascar	EU727498	-	F		
16°57.452'S	46°49.433'E	FMNH 184919	Madagascar	EU727500	-	M		
16°57.452'S	46°49.433'E	FMNH 184920	Madagascar	EU727519	-	M		
16°24.807'S	46°45.876'E	FMNH 184973	Madagascar	EU727518	-	M		
16°24.807'S	46°45.876'E	FMNH 184974	Madagascar	EU727524	-	F		
16°26.173'S	47°09.329'E	FMNH 184950	Madagascar	EU727507	-	F		
16°26.173'S	47°09.329'E	FMNH 184951	Madagascar	EU727508	EU716012	F		
16°26.173'S	47°09.329'E	FMNH 184952	Madagascar	EU727509	-	F		
16°26.173'S	47°09.329'E	FMNH 184953	Madagascar	EU727510	EU716013	F		
16°06.961'S	46°45.400'E	FMNH 184975	Madagascar	EU727521	EU716020	M		
16°06.961'S	46°45.400'E	FMNH 184976	Madagascar	-	EU716021	M		
16°06.961'S	46°45.400'E	FMNH 184977	Madagascar	EU727522	EU716022	M		
16°06.961'S	46°45.400'E	FMNH 184978	Madagascar	-	EU716023	M		
16°06.961'S	46°45.400'E	FMNH 184979	Madagascar	EU727484	EU716024	F		
17°11.000'S	46°50.947'E	FMNH 184896	Madagascar	EU727489	-	F		
17°11.000'S	46°50.947'E	FMNH 184897	Madagascar	EU727490	-	F		
17°11.036'S	46°51.000'E	FMNH 184898	Madagascar	EU727491	-	F		
17°11.036'S	46°51.000'E	FMNH 184899	Madagascar	EU727492	-	M		
17°11.036'S	46°51.000'E	FMNH 184900	Madagascar	EU727493	-	F		
17°11.036'S	46°51.000'E	FMNH 184901	Madagascar	EU727494	-	F		
17°11.036'S	46°51.000'E	FMNH 184902	Madagascar	EU727495	-	M		
22°09.418'S	48°01.009'E	FMNH 185228	Madagascar	-	EU716031	F		
13°24.308'S	48°18.201'E	FMNH 187750	Nosy Be	EU727464	EU716030	M		
13°22.012'S	48°18.927'E	FMNH 187751	Nosy Be	EU727478	-	M		
13°22.012'S	48°18.927'E	FMNH 187753	Nosy Be	EU727467	-	F		

	13°22.012'S	48°18.927'E	FMNH 187756	Nosy Be	EU727501	-	M
	13°22.012'S	48°18.927'E	FMNH 187754	Nosy Be	EU727468	EU716026	M
	13°22.012'S	48°18.927'E	FMNH 187755	Nosy Be	EU727469	EU716027	M
	13°24.254'S	48°16.425'E	FMNH 188495	Nosy Be	GQ 489136	-	F
	13°21.099'S	48°11.307'E	FMNH 188497	Nosy Be	EU727475	-	F
	13°21.099'S	48°11.307'E	FMNH 188498	Nosy Be	EU727476	EU716028	F
	13°21.099'S	48°11.307'E	FMNH 188499	Nosy Be	EU727477	-	M
	13°21.099'S	48°11.307'E	FMNH 188500	Nosy Be	EU727463	-	F
	13°24.308'E	48°18.201'S	FMNH 187752	Nosy Be	EU727479	EU716030	
	13°26.562'S	48°20.874'E	FMNH 188640	Nosy Komba	EU727479	EU716032	F
	13°26.562'S	48°20.874'E	FMNH 188641	Nosy Komba	EU727480		F
	13°26.562'S	48°20.874'E	FMNH 188642	Nosy Komba	EU727481	EU716033	F
	13°26.562'S	48°20.874'E	FMNH 188643	Nosy Komba	EU727482	EU716034	F
	13°26.562'S	48°20.874'E	FMNH 188644	Nosy Komba	EU727472	EU716035	M
C. atsinanana **sp. nov.** (eastern Madagascar)	23°21.300'S	47°35.763'E	FMNH 185230	Madagascar	-	GQ 489168	F
	22°82.130'S	47°83.100'E	FMNH 185260	Madagascar	GQ 489119	-	M
	22°36.660'S	47°83.677'E	FMNH 185286	Madagascar	GQ 489120	GQ 489170	F
	22°15.700'S	48°01.682'E	FMNH 185315	Madagascar	GQ 489121	-	M
	18°14.070'S	49°37.783'E	FMNH 187797	Madagascar	GQ 489122	-	M
	18°15.020'S	49°41.105'E	FMNH 187799	Madagascar	GQ 489123	-	M
	18°96.060'S	48°84.742'E	FMNH 187834	Madagascar	GQ 489124	-	F
	18°96.060'S	48°84.742'E	FMNH 187836	Madagascar	GQ 489125	-	F
	21°25.760'S	47°45.592'E	FMNH 188088	Madagascar	GQ 489126	-	F
	21°25.760'S	47°45.592'E	FMNH 188089	Madagascar	GQ 489127	-	F
	22°49.275'S	47°49.860'E	FMNH 185259	Madagascar	-	GQ 489169	F
	22°21.997'S	47°50.206'E	FMNH 185286	Madagascar	-	-	F
	22°09.418'S	48°01.009'E	FMNH 185314	Madagascar	-	GQ 489171	F
	18°08.441'S	49°22.670'E	FMNH 187798	Madagascar	-	GQ 489172	F
	18°49.317'S	49°04.343'E	FMNH 187823	Madagascar	-	GQ 489173	M
	18°57.636'S	48°50.845'E	FMNH 187834	Madagascar	-	GQ 489174	F
	21°18.394'S	47°38.144'E	FMNH 185322	Madagascar	-	GQ 489175	F
	21°15.456'S	47°27.355'E	FMNH 188089	Madagascar	-	GQ 489176	F
	18°52.945'S	47°58.245'E	FMNH 184678	Madagascar	-	GQ 489177	F

APPENDIX 2. Haplotypes defined by analysis of 1031 nucleotides of the cytochrome *b* gene of *Chaerephon* species. Museum numbers include the Field Museum of Natural History (FMNH) and National Museum Prague (NMP). Uncatalogued specimens are denoted with collector numbers which include PB = Petr Benda.

Taxon	Hap	N	Sample codes
C. pusillus (Comoros and Aldabra)	1	6	FMNH 194250, 194251, 194183, 194186, 194189, 194192
	2	2	FMNH 194019, 194020
	3	8	FMNH 194256, 194258, 194024, 194035, 194036, 194020, FMNN 205318, FMNH 205319
	4	3	FMNH 194323, 194329, 194334
C. pumilus sensu lato (Tanzania)	5	1	FMNH 193055
	6	1	FMNH 192938
C. leucogaster	7	4	FMNH 198149, 198151, 198093, 198152
	8	4	FMNH 198091, 198092, 198153, 198150
	9	8	FMNH 194028, 194023, 194019, 192889, 192886, 192823, 184922, 184923
	10	14	FMNH 184259, 184924, 184954, 184955, 184956, 184957, 184958, 184959, 188496, 185020, 184239, 184240, 184238, 185228
	11	4	FMNH 184925, 184926, 184951, 184953
	12	4	FMNH 184975, 184976, 184977, 184978
	13	1	FMNH 184979
	14	9	FMNH 187754, 187755, 188498, 187750, 188640, 188641, 188642, 188643, 188644
C. atsinanana **sp. nov.** (eastern Madagascar)	15	4	FMNH 185230, 185259, 185286, 185314
	16	1	FMNH 187798
	17	2	FMNH 187823, 187834
	18	2	FMNH 185322, 188089
	19	1	FMNH 184678
C. pumilus (Yemen)	20	2	NMP PB-3752, NMP PB-3667

APPENDIX 3. Haplotypes defined by analysis of 332 nucleotides of the D-loop of *Chaerephon* species. Museum numbers include the Field Museum of Natural History (FMNH) and National Museum Prague (NMP). Uncatalogued specimens are denoted with collector numbers, which include PB = Petr Benda.

Taxon	Hap	N	Sample codes
C. pusillus (Comoros)	1	1	FMNH 194214
	2	3	FMNH 194217, 194220, 194226
C. leucogaster	3	1	FMNH 192886
	4	1	FMNH 192891
	5	1	FMNH 192819
	6	15	FMNH 187750, 187751, 187752, 187753, 187754, 187755, 187756, 188495, 188497, 188498, 188499, 188500, 188640, 188643, 188644
C. atsinanana **sp. nov**. (eastern Madagascar)	7	1	FMNH 185260
	8	1	FMNH 185286
	9	1	FMNH 185315
	10	2	FMNH 187797, 187799
C. leucogaster	11	3	FMNH 184237, 184238, 184240
	12	1	FMNH 184239
	13	5	FMNH 188496, 184922, 184923, 184955, 185028
	14	2	FMNH 188641, 188642
C. atsinanana **sp. nov.** (eastern Madagascar)	15	2	FMNH 187834, 187836
	16	2	FMNH 188088, 188089
C. leucogaster	17	2	FMNH 184263, 184264
	18	4	FMNH 184604, 184606, 184607, 184608
	19	1	FMNH 184605
	20	3	FMNH 184896, 184897, 184898
	21	4	FMNH 184899, 184900, 184901, 184915
	22	1	FMNH 184902
	23	1	FMNH 184916
	24	1	FMNH 184917
	25	2	FMNH 184919, 184920
	26	2	FMNH 184924, 184954
	27	5	FMNH 184925, 184926, 184950, 184951, 184953
	28	1	FMNH 184952
	29	1	FMNH 184956
	30	3	FMNH 184957, 184958, 184959
	31	1	FMNH 184973
	32	1	FMNH 184974
	33	9	FMNH 184975, 184977, 184979, 185020, 185021, 185022, 185027, 185029, 185030
C. pumilus (Yemen)	34	1	NMP PB-3626
	35	1	NMP PB-3685
	36	1	NMP PB-3154
	37	1	NMP PB-3606
	38	1	MNP PB-3619
	39	1	NMP PB-3667
C. pusillus (Aldabra)	40	2	FMNH 205318, FMNH 205319

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Cross-genus amplification and characterisation of microsatellite loci in the little free tailed bat, *Chaerephon pumilus* s. l. (Molossidae) from South Eastern Africa

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Microsatellite loci for *Chaerephon pumilus* sensu lato from south eastern Africa were cross-amplified using primers developed for the Mexican free-tailed bat, *Tadarida brasiliensis*. Two dinucleotide and four tetranucleotide loci were recovered and genotyped for 74 bats, yielding 9 to 15 alleles per locus. The observed and expected heterozygosities were 0.06 to 0.84 and 0.54 to 0.81 respectively, and the PIC values ranged from 0.51 to 0.80, indicative of considerable variability within the sample. There was no evidence of linkage disequilibrium among pairs of loci, or of deviation from Hardy-Weinberg equilibrium. These six loci were informative in studies of population genetic structure of *C. pumilus* sensu lato.

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

INTRODUCTION

Microsatellite markers have become a powerful tool in investigations of population genetic structure, but can be time-consuming and expensive to develop *ab initio*. It is often more viable to develop markers by cross-amplification using primers published for a related species or genus (Wilson et al., 2004; Zhou et al., 2009), although the number of loci which amplify and are polymorphic tend to decrease with increasing divergence between the taxa in question (Moore et al., 1991; Peakall et al., 1998).

The little free-tailed bat, *Chaerephon pumilus* Cretzchmar, 1830-31 (Chiroptera: Molossidae) has a broad distribution across sub-Saharan Africa, extending to the Arabian Peninsula and islands in the Western Indian Ocean (Peterson et al., 1995; Bouchard, 1998; Simmons, 2005). Goodman et al. (2010) showed that the nominate form from Massawa (Eritrea) was genetically distinct from forms bearing this name found elsewhere on the African continent, referred to here as *C. pumilus* sensu lato (s. l.).

Little has been published about the roosting habits and social structure of these nocturnal insectivorous bats. Taylor et al. (2009) reported four mitochondrial clades of *C. pumilus* s. l. in south eastern Africa separated by intra-specific level cytochrome *b* genetic distances of 0.6 to 0.9% (Baker and Bradley, 2006). It has been hypothesised *inter alia* that these clades are the result of social isolation mechanisms such as philopatry, that they arose through introgression created by past hybridization events, and that they represent speciation in progress. In order to further investigate these issues we decided to assess the population genetic structure of this species

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Table 1. Characteristics of six *C. pumilus* s. l. microsatellite loci cross-amplified using primers developed for *T. brasiliensis* (Russell et al., 2005).

Locus	Repeat motif	Genbank accession number	Ta (°C)	Number of alleles	Allele size (nt)	Number of repeats	PIC	Ho	He	Primer sequence (5'-3')
Tabr A10	TAGA TGGA	KC896691	60	9	178-254	8 - 23 3 - 8	0.69	0.51±0.05	0.69±0.03	F:AAGTGGTTGGGCGTTGTC R:GCGATGCACTGCCTTGAGA
Tabr D10	GATA	KC896693	60	13	331-379	2-14	0.80	0.81±0.07	0.81±0.03	F:CCCCACTCATTTATCCATCCACA R:ATCTCGCAGCTATTGAAGTA
Tabr D15	GATA	KC896692	60	10	148-284	4 - 38	0.51	0.06±0.05	0.54±0.14	F:AGTCCTGGCTCCTATTCTCATTG R:CTATCCGTCTACCTGTCCGTCTAT
Tabr E 9	GA	KC896694	60	15	329-365	6 - 24	0.79	0.84±0.07	0.80±0.03	F: GTTTGTCTTCCCCACTGA R: CTTAGGACAGGAGAAGTCA
Tabr H 6	TAGA	KC896695	60	14	139-318	4 - 49	0.61	0.46±0.04	0.64±0.04	F:ATCTCTCCAGTCCTTACCA R:TTTACCCTCCACAGTCTCA
Tabr A30	GA	KC896690	65	9	240-296	5 - 33	0.61	0.78±0.60	0.64±0.04	F:AGTCGCGGGTTTGATTCCAGTTA R:ACCCCTTCCCTTTGTTCCTTCAG

Locus, name of locus; Ta, PCR annealing temperature; nt, nucleotides; PIC, polymorphism information content; Ho, observed heterozygosity; He, expected heterozygosity; F, forward; R , reverse; Tabr, *Tadarida brasiliensis*.

Based on nuclear microsatellite markers. Our approach was to cross-amplify hypervariable microsatellites reported for another molossid genus, the South American free-tailed bat, *Tadarida brasiliensis* (Russell et al., 2005), in order to identify markers which were appropriately variable in our sample of the little free-tailed bat, *C. pumilus* s. l.

This strategy, if successful, was aimed at producing markers useful in the analyses of population genetic structure, kinship and colony structure of populations of this bat in south eastern Africa and possibly also other regions of Africa.

MATERIALS AND METHODS

Analyses were carried out on 74 samples of *C. pumilus* s. l. from South Eastern Africa (Table 2). TheDNeasy® blood and tissue Kit (QIAGEN Inc., QiagenStraße 1,40724 Hilden, Germany) was used to isolate genomic DNA. Polymerase chain reaction (PCR) amplifications were performed in 25 µl volumes containing: 9 µl DNA (3 ngμl^{-1}), 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 *Taq* polymerase (5 u/µl) (Super-Therm) and 4 µl of each primer (6 µM) (forward and reverse) per reaction.

The thermal cycling parameters were: 95°C for 1 min, followed by 39 cycles of 95°C for 30 s, annealing temperature for 30 s, 72°C for 2 min, followed by 72°C for 10 min. The optimal annealing temperature for each primer pair was standardised using gradient PCR (Table 1). The reaction mix comprised 1 µl of PCR product labelled with the dyes 5' 6-FAM or 5' HEX, and 0.5 µl of a ROX500 size standard, brought to 15 µl with Hi-Di Formamide (Applied Biosystems, agents: LifeTechnologies, 200 Smit Street, Fairland, Johannesburg). STRs were genotyped on an ABI 3500 genetic analyzer (Applied Biosystems) at the South African Sugar Research Institute, Mount Edgecombe, South Africa. Raw allelic peak data were analysed using STR and v. 2.2.30 (Locke et al., 2000).

Genalex (Peakall and Smouse, 2006) was used to calculate the observed and expected heterozygosities (Ho and He). The polymorphism information content (PIC) was calculated using a web-based PIC calculator (Kemp, 2002).

Table 2. Details of specimens used in this study.

Field number	Locality in South Eastern Africa	Latitude	Longitud E
C. pumilus			
UWWW1CP1	Umbilo Waste Water	29.846 S	30.890 E
UWWW1CP3	Umbilo Waste Water	29.846 S	30.890 E
UWWW1CP4	Umbilo Waste Water	29.846 S	30.890 E
UWWW1CP5	Umbilo Waste Water	29.846 S	30.890 E
UWWW1CP6	Umbilo Waste Water	29.846 S	30.890 E
URPV1CP1	Paradise Valley	29.831 S	30.892 E
URPV1CP2	Paradise Valley	29.831 S	30.892 E
URPV1CP3	Paradise Valley	29.831 S	30.892 E
URPV1CP4	Paradise Valley	29.831 S	30.892 E
URPV1CP5	Paradise Valley	29.831 S	30.892 E
URPV2CP6	Paradise Valley	29.831 S	30.892 E
URPV2CP7	Paradise Valley	29.831 S	30.892 E
URPV2CP8	Paradise Valley	29.831 S	30.892 E
PNT1	Pinetown	29.828 S	30.866 E
PNT2	Pinetown	29.828 S	30.866 E
PH1	Phinda: Swilles	27.695 S	32.356 E
PH2	Phinda: Swilles	27.695 S	32.356 E
PH3	Phinda: Swilles	27.695 S	32.356 E
PH4	Phinda: Swilles	27.695 S	32.356 E
PH5	Phinda: Swilles	27.695 S	32.356 E
PH6	Phinda: Swilles	27.695 S	32.356 E
PH7	Phinda: Swilles	27.695 S	32.356 E
PH8	Phinda: Swilles	27.695 S	32.356 E
PH9	Phinda: Swilles	27.695 S	32.356 E
PH11	Phinda: Swilles	27.695 S	32.356 E
EH1	Effingham Heights	29.769 S	31.010 E
EH2	Effingham Heights	29.769 S	31.010 E
EH3	Effingham Heights	29.769 S	31.010 E
EH4	Effingham Heights	29.769 S	31.010 E
EH5	Effingham Heights	29.769 S	31.010 E
EH6	Effingham Heights	29.769 S	31.010 E
EH7	Effingham Heights	29.769 S	31.010 E
EH8	Effingham Heights	29.769 S	31.010 E
EH9	Effingham Heights	29.769 S	31.010 E
EH10	Effingham Heights	29.769 S	31.010 E
EH11	Effingham Heights	29.769 S	31.010 E
EH12	Effingham Heights	29.769 S	31.010 E
EH13	Effingham Heights	29.769 S	31.010 E
EH14	Effingham Heights	29.769 S	31.010 E
EH15	Effingham Heights	29.769 S	31.010 E
EH16	Effingham Heights	29.769 S	31.010 E
EH17	Effingham Heights	29.769 S	31.010 E
CH1	Chatsworth	29.930 S	30.925 E
D1	Durban Int. Airport	29.967 S	30.942 E
D2	Hell’s Gate	28.067 S	32.421 E
D4	Hell’s Gate	28.067 S	32.421 E
D5	Hell’s Gate	28.067 S	32.421 E
D6	Hell’s Gate	28.067 S	32.421 E
D7	Hell’s Gate	28.067 S	32.421 E
D8	uMkhuze Game Reserve	27.583 S	32.217 E

Table 2. Contd.

D9	uMkhuze Game Reserve	27.583 S	32.217 E
D10	Amanzimtoti	30.05 S	30.883 E
D11	Amanzimtoti	30.05 S	30.883 E
D12	Morningside	29.833 S	31.00 E
D13	CROW	Unknown	
D14	Hell's Gate	28.067 S	32.421 E
D15	Hell's Gate	28.067 S	32.421 E
D16	CROW rehab	Unknown	
D17	Hell's Gate	28.067 S	32.421 E
D18	Bluff	29.933 S	31.017 E
D19	Ballito	29.533 S	31.217 E
D20	Bluff	29.933 S	31.017 E
D22	Amanzimtoti	30.05 S	30.883 E
D26	Umbilo	29.833 S	31.00 E
D27	Athlone Park	30.016 S	30.917 E
D29	Pinetown	29.817 S	30.85 E
D30	Illovo	30.1 S	30.833 E
D34	Park Rynie	30.317 S	30.733 E
D35	SZ: Mlawula	26.192 S	32.005 E
D36	SZ: Wylesdale	25.819 S	31.292 E
D37	Durban City Hall	29.858 S	31.025 E
D39	Durban	29.867 S	31.00 E
D40	Yellowwood Park	29.917 S	30.933 E
D43	Durban	Unknown	

RESULTS AND DISCUSSION

Three of the nine loci initially tested were discarded, as it was either not possible to amplify them across all samples, or because the banding pattern was too ambiguous to score. The data were checked for errors in scoring due to stuttering, large allele dropout or null alleles using Micro-checker (van Oosterhout et al., 2004). Individuals with missing data at more than two loci were discarded.

All individuals were genotyped for the loci TabrA10, TabrD10, TabrD15, TabrE9, TabrH6 and TabrA30 (Russell et al., 2005). There was no evidence of linkage disequilibrium among pairs of microsatellite loci after standard Bonferroni correction, and none of the 6 loci showed significant deviation from Hardy Weinberg equilibrium ($p>0.05$). The genotyped loci were all polymorphic, yielding 9 to 15 (mean 11.67) alleles per locus. This is considerably lower than the 15 to 55 (mean 36.7) alleles per locus reported by Russell et al. (2005) for the confamilial South American bat, *T. brasiliensis*. A finding of lower levels of polymorphism in microsatellites cross-amplified from another genus is likely to be related to the degree of divergence between the genera in question (Moore et al., 1991; Peakall et al., 1998). The smaller sampling range used in this study may also be reflected in the lower number of alleles recovered; we sampled bats over a north/ south distance of less than 1000 km, whereas Russell et al. (2005) compared bat populations from Texas and Argentina, which are separated by a much greater distance and are therefore more likely to be divergent. Nonetheless, the expected (He) and observed (Ho) heterozygosities over all samples ranged from 0.54 to 0.81 and 0.06 to 0.84, respectively, and the PIC values ranged from 0.51 to 0.80 (Table 1), indicating considerable variability within our sample (Mukesh et al., 2011).

Although it is commonly assumed that microsatellite loci differ among individuals only in the number of units of a single repeat (Guyer and Collins, 1993), many studies have shown that their sequence variation may be more complex (Bull et al., 1999). Five of the cross-amplified *C. pumilus* s. l. loci contained the same repeat motif as *T. brasiliensis*. The repeat motif of locus TabrA10, however, was a tetranucleotide (TAGA) in *C. pumilus* s. l. compared with a diucleotide (GA) in *T. brasiliensis*. We also recovered a short stretch of a second tetranucleotide repeat (TGGA) adjacent to the TAGA repeat at locus TabrA10. Thus, it appears that this locus in *C. pumilus* s. l. may be a compound microsatellite (Weber, 1990) which arose by mutation and replication slippage (Tautz and Schlötterer, 1994) in the period since *C. pumilus* s. l. and *T. brasiliensis* last shared a common ancestor. Analyses of complex microsatellites can underestimate variability, as sequencing has revealed differences between such alleles which are identical in length (Bull et al., 1999).

In conclusion, the six polymorphic microsatellite loci reported here are sufficiently variable to prove useful in analyses of mating and paternity studies, as well as in studies of population genetic structure of *C. pumilus* s. l. from south eastern Africa, and possibly other members of the *C. pumilus* species complex from Africa and the western Indian Ocean region.

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Genetically and geographically isolated lineages of a tropical bat (Chiroptera: Molossidae) show demographic stability over the late Pleistocene

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The newly described molossid bat, *Chaerephon atsinanana* Goodman *et al*., 2010, endemic to eastern Madagascar, shows notably high levels of phylogeographic and genetic structure compared with allopatric *Chaerephon leucogaster* Grandidier, 1869 from western Madagascar. Such highly significant structuring of haplotypes among altitudinally and latitudinally stratified population groups is contrary to the expected panmixia in strong flying bats. The null model of concordance in historical demographic patterns across these two *Chaerephon* species was not supported. Mismatch and Bayesian skyline analyses indicated ancient stable *C. atsinanana* populations of constant size during the last two major Pleistocene glacial periods, making retreat into and expansion from glacial refugia an unlikely explanation for such high levels of structure, in accordance with expectations for tropical bats. Analyses were consistent with post-refugial population expansion in the less diverse and structured *C. leucogaster* during the end of the last Pleistocene glacial period. We hypothesise that the pronounced genetic structuring in *C. atsinanana* may result from female philopatry. Furthermore, differing demographic histories of the two species may have been shaped by differing climate or habitat preferences, consistent with evidence from MaxEnt ecological niche modelling, which shows differences in variables influencing the current predicted distributions. Fossil Quaternary pollen deposits further indicate greater stability in past climatic patterns in eastern versus western Madagascar. *Biological Journal of the Linnean Society*, 2012, **106**, 18–40.

ADDITIONAL KEYWORDS: *Chaerephon* – environmental niche modelling – genetic structuring – historical demography – Madagascar – phylogeography.

INTRODUCTION

Here we examine the population genetic structure, historical demography, and predicted distribution of *Chaerephon atsinanana* Goodman *et al*., 2010, a newly described species of molossid bat that is endemic to eastern Madagascar (Goodman *et al*., 2010), and contrast this with patterns shown by another member of the genus occurring on Madagascar, *Chaerephon leucogaster* Grandidier, 1869. The family Molossidae (Order Chiroptera), commonly known as free-tailed or mastiff bats, comprises 17 genera and about 100 species (Simmons, 2005), and is globally widespread, occurring on every continent except Antarctica and on numerous isolated islands. These generally robust bats tend to be strong flyers,

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with long narrow wings, and catch their insect prey in flight. A study based on nuclear (*Rag2*) and mitochondrial (cytochrome *b*) data (Lamb *et al*., 2011) shows *C. atsinanana* to be sister to a clade comprising *C. leucogaster*, *Chaerephon pusillus* Miller, 1902 (Comoros and Aldabra) and *Chaerephon pumilus* Cretzschmar, 1830–1831 (southern Africa).

Comparative studies of closely related taxa distributed within a region can shed light on the role of population processes and historical events in shaping their current distribution and genetic structure. Such taxa might be expected to show similarities in genetic structure based on their shared histories (Zink, 1996). Conversely, marked differences in genetic structure may be attributed to contrasting life history or ecological traits (Chen *et al*., 2010).

In bats, social structure, which can be reflected in genetic structure, is largely determined by roosting ecology (Chen *et al*., 2010). Dispersal ability is also a key force in shaping the demography of natural populations (Proctor *et al*., 2004). Both *C. atsinanana* and *C. leucogaster* show similar roosting patterns and flight capabilities and, therefore, dispersal capabilities, as indicated by aerodynamic aspects of wing shape (Taylor *et al.*, in press). Based on these aspects we would expect them to show similar patterns of genetic structure.

The influence of glacial changes in tropical and subtropical regions is often minimal (Lessa, Cook & Patton, 2003). The null model for the effect of glacial changes is concordance in historical demographic and genetic patterns across the two Malagasy *Chaerephon* species, *C. leucogaster* and *C. atsinanana* (Weir & Schluter, 2004). As there is evidence for expansion of populations of *C. leucogaster* (Ratrimomanarivo *et al*., 2009), and of the broadly distributed Malagasy bat, *Myotis goudoti* (Smith, 1834) (Weyeneth, Goodman & Ruedi, 2011), from Pleistocene refugia, we would expect to find a similar expansion of *C. atsinanana*. Anticipated genetic signatures of range expansions might include star-shaped haplotype networks, an excess of singletons, and unimodal mismatch distributions (Excoffier, Foll & Petit, 2009).

In accord with relatively low levels of phylogeographic structure in *C. leucogaster* and a general expectation of panmixia over large geographic expanses in strong flying bats such as Molossidae (Russell, Medellin & McCracken, 2005), we would anticipate relatively low levels of structuring in *C. atsinanana*. However, the degree of spatial partitioning of genetic diversity in bats is surprisingly variable, and some species show high levels of spatial structure on small (e.g. Kerth, Mayer & König, 2000) or large (e.g. Miller-Butterworth, Jacobs & Harley, 2003) geographic scales.

Mammalian species that show high levels of population substructure are frequently separated by non-traversable vicariant barriers (Avise *et al*., 1987). Malagasy forms of *Chaerephon*, however, have relatively broad elevational distributions, and are likely to be capable of traversing potential geographic barriers such as passes within mountain ranges and river valleys occurring within their preferred habitat. Thus, when high levels of population substructure occur in *Chaerephon* or other molossid species in Madagascar, the cause is unlikely to be vicariance.

If Quaternary climatic vicissitudes were similar in the eastern and western portions of Madagascar, the absence of concordance in historical demographic and genetic patterns between *C. leucogaster* and *C. atsinanana* would suggest the role of factors other than Pleistocene-era climatic and habitat oscillations in shaping the demography of *C. atsinanana*. For example, simulations carried out by Knowles & Alvarado-Serrano (2010) indicate that significant levels of genetic differentiation can occur as a consequence of the demography of the expansion process across a heterogeneous environment, and do not necessarily depend on long-term isolation of refugial populations. Significant levels of genetic differentiation may also occur as a result of behavioural and/or social characteristics of a species, such as the tendency of females and/or males to return consistently to, or remain in, a roost or area (philopatry) (e.g. Worthington-Wilmer *et al*., 1994).

Adaptation to different ecological factors or habitats is an alternative explanation for lack of concordance in patterns between the two Malagasy *Chaerephon* species. For example, the species-specific demographic histories of five passerine bird species in Tibet were shown by Qu *et al*. (2010) to depend on habitat requirements. *Chaerephon leucogaster* and *C. atsinanana* are essentially allopatrically distributed. *Chaerephon atsinanana* is common in the mesic eastern portion of Madagascar (Fig. 1), across an elevational range from near sea level to 1100 m a.s.l. (Goodman *et al*., 2010). *Chaerephon leucogaster* is found across an elevational range of 0–920 m a.s.l., and is confined almost exclusively to the more extensive and drier western portion of the island (Fig. 1), although there is one record of an individual occurring in sympatry with *C. atsinanana* at Manakara, on the south-east coast (Ratrimomanarivo *et al*., 2009).

Both *C. atsinanana* and *C. leucogaster* are adapted to the high levels of human-induced environmental change on Madagascar, and have established synanthropic day roosts in buildings. During surveys at numerous sites in Madagascar, *C. atsinanana* was never found roosting in a natural setting (Goodman *et al*., 2010). These authors report that only 1.4% of

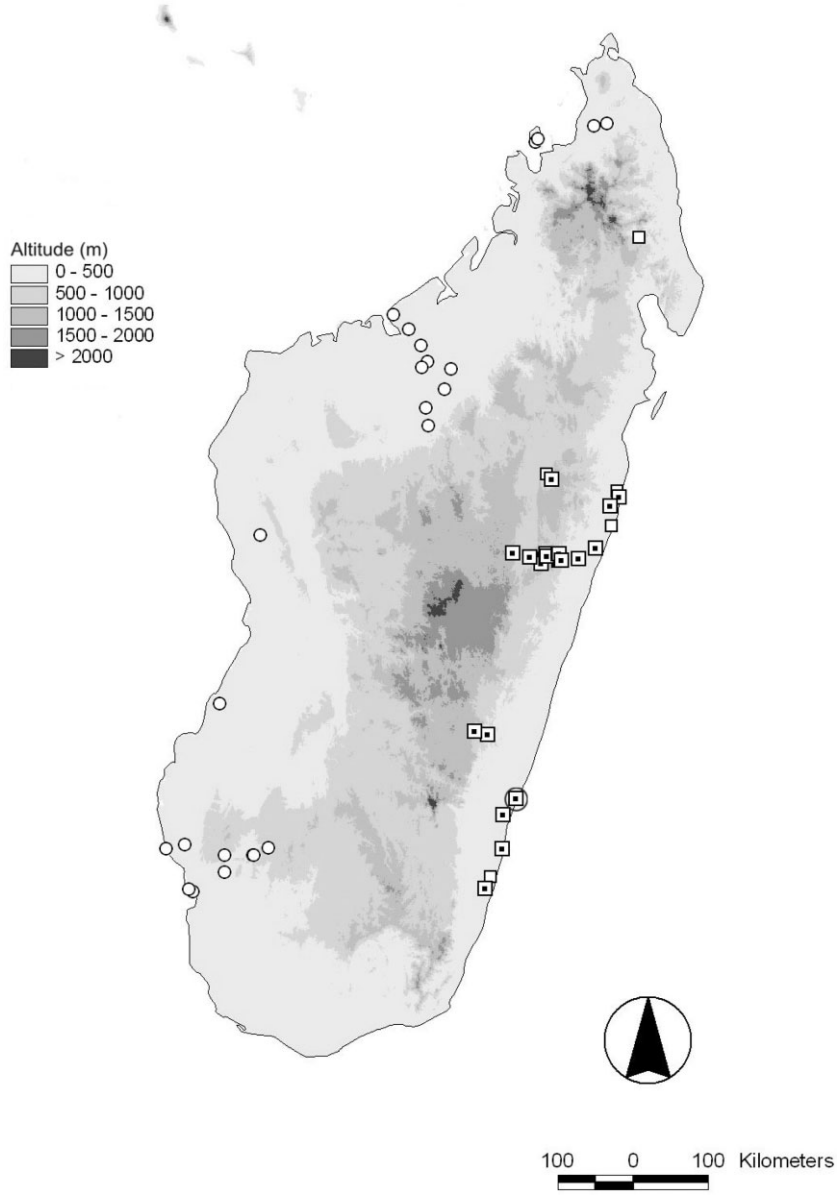


Figure 1. Map of the Madagascar region illustrating the range and collection localities of specimens of *Chaerephon atsinanana* and *Chaerephon leucogaster* reported on in this study; ⊡, *C. atsinanana*; ○, *C. leucogaster*.

known *C. leucogaster* day roost sites are in natural settings. Neither of these species is recorded to roost in caves (Goodman, 2011).

The goal of this study was to use mitochondrial cytochrome *b* and control region sequence data to examine the population genetic structure, historical demography, and predicted distribution of *C. atsinanana*, and to contrast this with similar information for its Malagasy congener, *C. leucogaster*. We aimed to test the null model that, based on similar roosting behaviour and dispersal capability, there is an expectation of concordance in historical demographic and genetic patterns for *C. leucogaster* and *C. atsinanana* (Weir & Schluter, 2004). Lack of concordance in historical demographic and genetic patterns might be explained by behavioural differences, or differences in physiological tolerance or habitat associations between the two *Chaerephon* species. We used ecological niche modelling (maximum entropy or MaxEnt method; Phillips, Andersen & Schapire, 2006) to examine the predicted distributions of *C. atsinanana* and *C. leucogaster*, and the factors most strongly influencing them, including bioclimatic factors, vegetational habitat associations, and, given the

known synanthropic tendencies of molossids in Madagascar, human influence on the landscape.

MATERIAL AND METHODS

Study animals

This study presents new sequence analyses for *C. atsinanana* (Appendix), and compares these with information previously published for *C. leucogaster* (Ratrimomanarivo *et al.*, 2009). Some new analyses of the *C. leucogaster* data set are presented here for comparison.

Sampling

The mitochondrial cytochrome *b* and/or control region were sequenced for between one and 13 samples of *C. atsinanana* from each of 17 localities spanning the distribution range of this species (Appendix). The median number of samples from each locality was four for cytochrome *b* (total 62) and six (total 107) for the control region. Cytochrome *b* trees were constructed using the following molossid taxa as out-groups: *C. leucogaster* (Madagascar), *C. pusillus* (Comoros and Aldabra), *Mops condylurus* Smith, 1833 (mainland Africa), *Mops leucostigma* Allen, 1918 (Madagascar) and *Mops midas* Sundevall, 1843 (Madagascar) (Appendix). Animals were handled in accordance with the guidelines of the American Society of Mammalogists (Sikes, Gannon & The Animal Care and Use Committee of the American Society of Mammalogists, 2011). Tissue samples used in this study included wing punches and liver, heart, kidney, or muscle tissue stored in 80% ethanol or lysis buffer.

DNA isolation, polymerase chain reaction (PCR) amplification, and sequencing

DNA was isolated from tissue samples using a DNeasy® DNA isolation kit (QIAGEN Inc.). PCR amplifications were performed in 25-μL volumes. Each reaction contained 9 μL of DNA (3 ng μL^{-1}), 0.8 μL of sterile water, 2.5 μL of 10 X reaction buffer (Super-Therm), 4 μL of 25 mM $MgCl_2$ (Super-Therm), 0.5 μL of 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Roche), 0.2 μL of *Taq* polymerase (5 U μL^{-1}) (Super-Therm), and 4 μL of each primer (6 μM) (forward and reverse) per reaction.

The cytochrome *b* gene was PCR amplified as two overlapping double-stranded fragments using primer pairs: L14723 (5′-ACCAATGCAATGAAAAATCATC GTT-3′) and H15553 (5′-TAGGCAAATAGGAAATAT CATTCTGGT-3′); L15146 (5′-CATGAGGACAAATAT CATTCTGAG-3′) and H15915 (5′-TCTCCATTTCT GGTTTACAAGAC-3′) (Irwin, Kocher & Wilson, 1991). The thermal cycling parameters used were: 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 finally followed by 72 °C for 10 min. The control region was PCR amplified as a single fragment using primers P (5′-TCCTACCATCAGCACCCAAAGC-3′) and E (5′-CCTGAAGTAGGAACCAGATG-3′) (Wilkinson & Chapman, 1991). The thermal cycling parameters used were: 94 °C for 4 min, followed by 40 cycles of 94 °C for 60 s, 55 °C for 90 s and 72 °C for 120 s, and finally followed by 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 (Hatfield, Pretoria, South Africa). All fragments were sequenced in both directions to allow for the reconciliation of ambiguous positions. Sequences were deposited in GenBank (Appendix). Sequences were aligned using the CLUSTAL W option (Thompson, Higgins & Gibson, 1994) of BioEdit 5.0.9 for Windows 95/98/NT, and by visual inspection.

Phylogenetic analysis of cytochrome *b* sequence data

Cytochrome *b* sequences were analysed using the neighbour-joining and maximum parsimony methods in PAUP 4.0b10 (Swofford, 2002) and Bayesian inference as implemented in MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). We used jModelTest 0.1.1 (Posada, 2008), applying Akaike's information criterion to determine the most appropriate evolutionary model (GTR + G) to use in neighbour-joining, genetic distance and Bayesian analyses. For parsimony analyses, starting trees were obtained by stepwise addition. The addition sequence was random, with one tree held at each step and with ten replicates. A total of 1000 bootstrap replicates were carried out for both maximum parsimony and neighbour-joining analyses. 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 20 000 trees as burn-in, in each case having checked in a preliminary run that this was more than sufficient to achieve stationarity. The results were presented as a single haplotype tree (Fig. 2) with support from all analysis methods indicated at the nodes.

Population genetic analyses

Because of its higher variability, the control region data set was primarily used for population genetic analyses. Relationships among *C. atsinanana* mitochondrial DNA control region sequences were analysed using a variety of approaches.

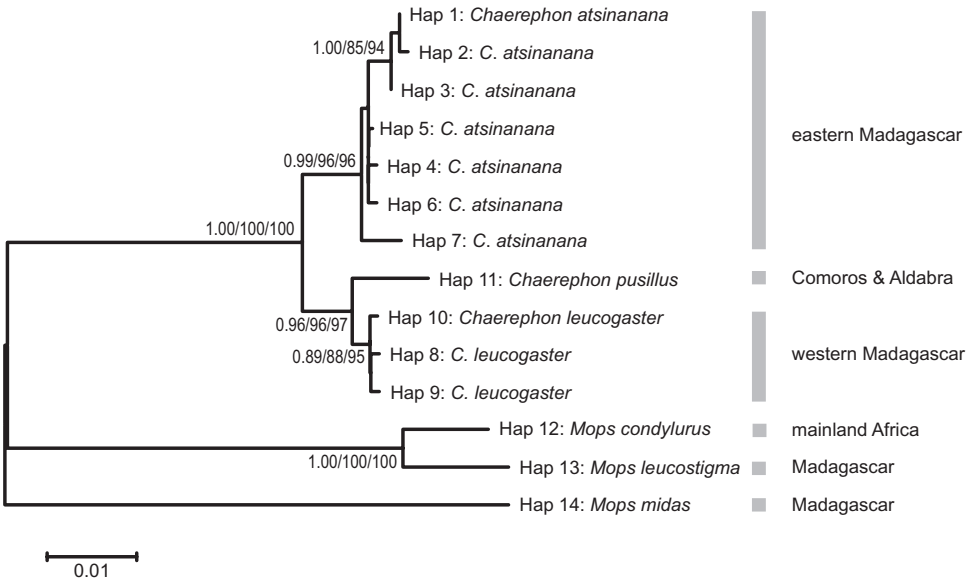


Figure 2. Bayesian inference tree based on an analysis of 863 nucleotides of the mitochondrial cytochrome *b* gene illustrating evolutionary relationships between *Chaerephon atsinanana* haplotypes and out-groups. Nodal support values, including those from congruent maximum parsimony and neighbour-joining analyses, are indicated as: Bayesian posterior probability/maximum parsimony bootstrap percentage/neighbour-joining bootstrap percentage.

Table 1. Genetic distances between haplotypes of *Chaerephon atsinanana*

Haplotype	1	2	3	4	5	6	7
1	–	1.01	0.70	0.82	0.82	0.94	0.82
2	3.82	–	0.58	0.70	0.23	0.12	0.70
3	6.35	5.44	–	0.12	0.35	0.47	0.12
4	5.47	3.00	6.31	–	0.47	0.58	0.23
5	5.48	3.00	6.32	0.01	–	0.12	0.47
6	3.84	6.35	7.23	6.33	6.34	–	0.59
7	3.04	5.49	8.14	5.47	5.48	0.74	–
8	2.65	5.06	7.68	5.90	5.90	4.25	3.44

Below diagonal: mean HKY + G genetic distances (%) between haplotype groups based on an analysis of 301 nucleotides of the mitochondrial control region. Above diagonal: mean GTR + G genetic distances (%) between haplotype groups based on an analysis of 863 nucleotides of the mitochondrial cytochrome *b* gene.

Haplotype analyses

We used DnaSP 5.10 (Librado & Rozas, 2009) to generate haplotype data files from the cytochrome *b*, control region, and concatenated cytochrome *b*–control region data sets. We used the control region data set for analyses of molecular variance (AMOVA). Genetic distances between the control region and between cytochrome *b* haplotypes were analysed in PAUP 4.0b10 (Swofford, 2002) using the most appropriate evolutionary models, HKY + G and GTR + G, respectively, determined in jModelTest 0.1.1 (Posada, 2008) (Table 1). Statistical parsimony haplotype networks were created in TCS from the control region and concatenated control region–cytochrome *b* data sets.

Analysis of molecular variance

We tested for significant variance in the distribution of control region sequences between individuals, populations, and groups of populations using AMOVA, carried out in ARLEQUIN 3.0 (Excoffier, Laval & Schneider, 2005). Fixation indices were calculated in a conventional fashion and their significance tested using a non-parametric permutation approach, as described in Excoffier, Smouse & Quattro (1992). Three designs were used for *C. atsinanana*: (1) no grouping of populations; (2) populations divided into groups north and south of 20°S; and (3) populations divided into three altitudinal groups (0–100, 101–600, and 601–1000 m a.s.l.) (Fig. 3C; Tables 2 & 3). The designs

Table 2. Analysis of molecular variance within and among population groups and populations of *Chaerephon atsinanana* and *Chaerephon leucogaster*

	Grouping criterion	Source of variation	Fixation indices	% of variation	*P* value
C. atsinanana	No groups		FST: 0.994		
C. atsinanana	Latitude*	Among groups	FCT: 0.277	27.76	0.033
		Among populations within groups	FSC: 0.932	67.36	0.000
		Within populations	FST: 0.951	4.88	0.000
		Among groups	FCT: 0.449	44.85	0.035
C. atsinanana	Altitude†	Among populations within groups	FSC: 0.997	54.92	0.000
		Within populations	FST: 0.951	0.22	0.000
C. leucogaster	No groups		FST: 0.792	–	–
C. leucogaster	Latitude‡	Among groups	FCT: 0.038	3.82	0.141
		Among populations within groups	FSC: 0.468	45.02	0.000
		Within populations	FST: 0.488	51.17	0.000

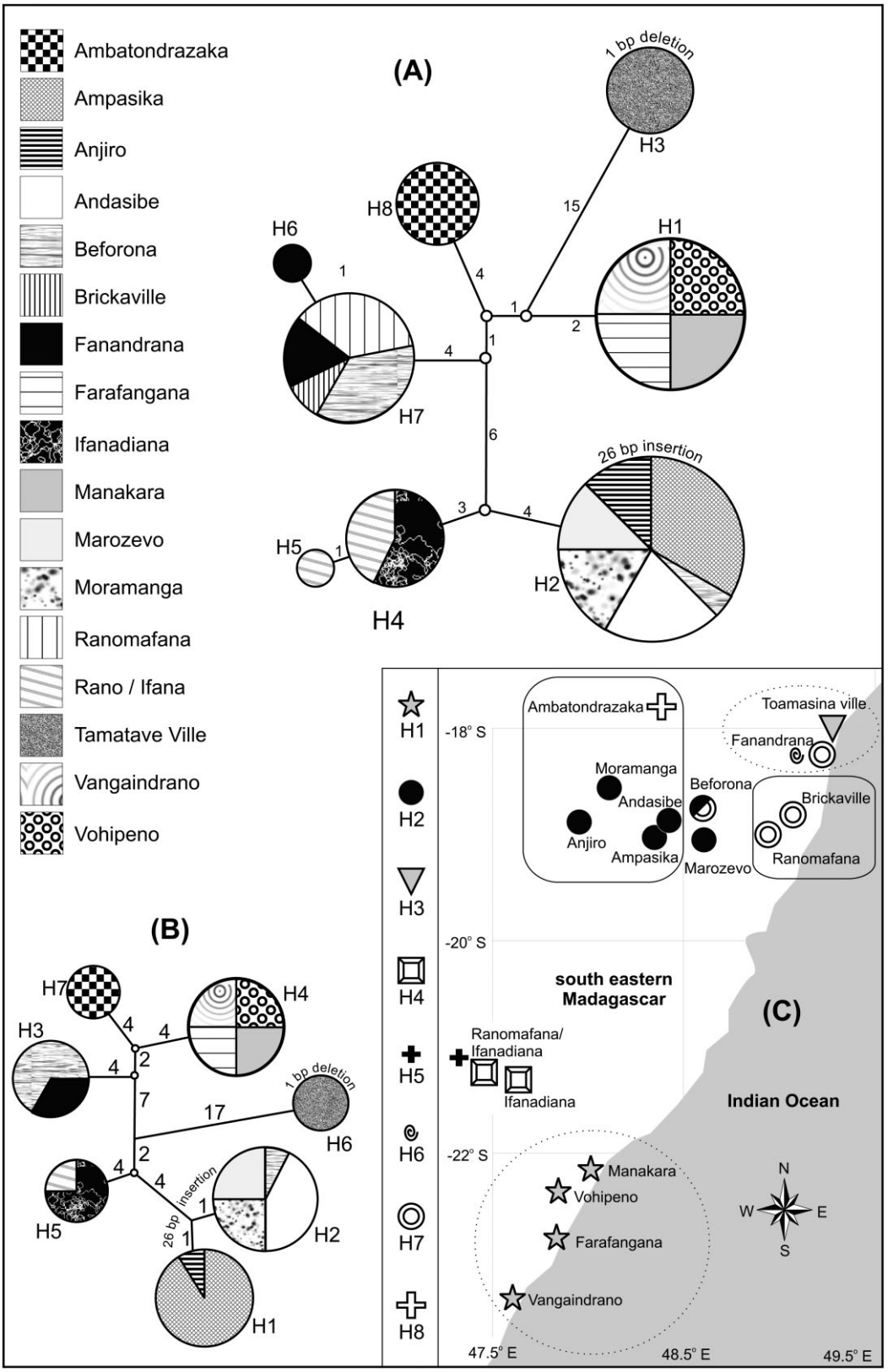
*North and south of 20°S.
†0–100 m a.s.l.; 101–600 m a.s.l.; 601–1000 m a.s.l.
‡13°S; 15–17°S; 22–23°S.

tested for *C. leucogaster* were: (1) no grouping of populations; and (2) populations divided into northern, central, and southern groups (see Ratrimomanarivo *et al*., 2009).

Population structure and historical demography

The historical demography of *C. atsinanana* was inferred through a variety of methods and compared with similar data obtained previously for *C. leucogaster* (Ratrimomanarivo *et al*., 2009). Following Rogers & Harpending (1992) and Russell *et al*. (2005), we calculated nucleotide diversity (π) and halotype diversity (h) (Nei, 1987). To test for deviations from neutrality (as would be expected under population expansion), we estimated F^* and D^* (Fu & Li, 1993), and F_s (Fu, 1997). In Fu and Li's D^* and F^* tests, departures from neutrality are estimated as a deviation between estimates of nucleotide diversity derived from external branches of a phylogeny and from the total number of mutations (D^*) or from the average pairwise diversity (F^*). Fu's Fs is the probability of observing a random sample with a number of alleles equal to or smaller than the observed value, given the observed level of diversity. A negative Fs is evidence for an excess number of alleles, derived for example from a recent population expansion, whereas a positive Fs might indicate an allele deficiency derived from a recent population bottleneck. We used mismatch distribution analysis to estimate whether each population group was stationary or had undergone a historical population expansion. High h with low π, a unimodal mismatch distribution, significant F_s but non-significant D^* and F^*, and a high ratio of the number of segregating sites (S) to the average number of pairwise differences (d) ($S : d$) are indicators of a historical population expansion event (Russell *et al*., 2005, and references therein). The above analyses were carried out with DNASP 4.0 (Rozas *et al*., 2003). Based on the distribution of pairwise nucleotide differences (mismatch distribution), the time since expansion, tau (τ), could be calculated in mutational units. Using an estimated D-loop mutation rate (μ) of 1.73×10^{-7} mutations per site per generation (Rogers & Harpending, 1992), we approximated the absolute time since expansion using the formula $\tau = 2ut$, where u was calculated as the product of the mutation rate and sequence length (301 bp), and t was the time (in generations, estimated as 1 year) since expansion.

Past population dynamics of *C. atsinanana* and *C. leucogaster* were also estimated with a Bayesian skyline plot implemented in BEAST 1.2 (Drummond *et al*., 2005). The parameter m (the number of grouped intervals) was set to five. The Markov chain Monte Carlo (MCMC) analysis was run for 10 000 000 generations (sampled every 1000 iterations), of which the first 10% was discarded as burn-in. The substitution model used was HKY. In order to test the robustness of the plots, we used three different mutation rates, covering a ninefold variation: 0.56×10^{-7}, 1.73×10^{-7} and 5.19×10^{-7} mutations per site per generation. The Bayesian skyline plot was created using TRACER 1.2.1 (Rambaut & Drummond, 2009). A Mantel test for isolation by distance was carried out on the control region data set in R (http://www.R-project.org).


Ambatondrazaka
Ampasika
Anjiro
Andasibe
Beforona
Brickaville
Fanandrana
Farafangana
Ifanadiana
Manakara
Marozevo
Moramanga
Ranomafana
Rano / Ifana
Tamatave Ville
Vangaindrano
Vohipeno
(A)
1 bp deletion
H3
H8
H6
H1
15
4
1
2
1
4
H7
6
26 bp insertion
3
4
H5
1
H2
H4
(B)
H7
H4
H3
4
4
4
2
7
17
1 bp deletion
H6
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Andasibe
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Figure 3. A, statistical parsimony network based on the analysis of 301 nucleotides of the mitochondrial control region of 107 *Chaerephon atsinanana* specimens from eastern Madagascar. B, statistical parsimony network based on the analysis of 1164 nucleotides of the concatenated mitochondrial cytochrome *b* (863 nt) and control region (301 nt) of 54 *C. atsinanana* specimens from eastern Madagascar. C, distribution of eight control region haplotypes among *C. atsinanana* sampling sites in eastern Madagascar. Also illustrated is the arrangement of samples into altitude groups used in the analysis of molecular variance. Localities encircled by dotted lines, 0–100 m a.s.l.; localities encircled by solid lines, 601–1000 m a.s.l.; localities that are not outlined, 101–600 m a.s.l. Latitudinal groups: north and south of 20°S; H, haplotype.

Ecological niche modelling

The MaxEnt method (Phillips *et al*., 2006) was used to model the predicted distributions of *C. atsinanana* and *C. leucogaster*. The occurrence records from existing museum specimens of *C. atsinanana* yielded 52 georeferenced localities. The removal of duplicates (from the same 2.5×2.5 arc minute grid) led to a final number of 22 occurrence records. For direct comparison of the two species included in this study, we also re-analysed the data set of 49 occurrence records (25 records after removing duplicates) of *C. leucogaster* analysed by Ratrimomanarivo *et al*. (2009) using the same environmental variables used in this study as described below (Appendix).

Altitude and eight bioclimatic variables (WORLDCLIM 1.4; http://www.worldclim.org/; Hijmans *et al*., 2005) were originally chosen, reflecting means, extremes, and seasonal variation of temperature and precipitation: mean annual temperature (Bio1); temperature seasonality (Bio4); maximum temperature of warmest month (Bio5); minimum temperature of coldest month (Bio6); annual precipitation (Bio12); precipitation of wettest month (Bio13); precipitation of driest month (Bio14); and precipitation seasonality (Bio15). To correct for potential statistical overfitting caused by high correlation coefficients between certain bioclimatic variables, we summarized the pattern of correlations between the original set of eight bioclimatic variables by means of an unweighted pair-group method with averages (UPGMA) correlation phenogram. Individual variables were selected from three distinct (independent) clusters (data not shown): Bio1, Bio4, and Bio12. Given the known synanthropic tendencies of molossid bats, which typically roost in the roofs and eaves of human structures in Madagascar, we also included a fourth variable, human footprint, which is a global map of human influence on the landscape, available at a resolution of 1 km^2 (Sanderson *et al*., 2002). Finally, to capture possible vegetational habitat associations, we included as a fifth variable: terrestrial ecoregions of the world (Olson *et al*., 2001).

For both bat species, the environmental data were set to a spatial grid resolution of 2.5 arc minutes and clipped to an area encompassing Madagascar. The MaxEnt model was run with 100% presence records used for training, with the regularization multiplier set to 2.0, maximum number of iterations set to 1500, convergence threshold set to 1×10^{-5} and output format set to logistic. Duplicate records (in the same 2.5×2.5 arc minute grid) were excluded. Model performance was assessed with the 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 of sensitivity versus 1 – specificity). As MaxEnt produces a continuous probability (ranging from 0 to 1.0), the continuous model output was transformed to a map representing probabilities of occurrence. 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 their absolute, contribution to the model.

RESULTS

CYTOCHROME *B*

Of the 863 nucleotides of the *C. atsinanana* cytochrome *b* gene, 12 characters were variable and parsimony informative, whereas 151 were invariant. There were no singletons. Sixty-two *C. atsinanana* samples yielded seven cytochrome *b* haplotypes (Appendix; Fig. 2). Analysis of cytochrome *b* haplotypes yielded a single maximum parsimony tree, which was congruent with the trees produced using the neighbour-joining and Bayesian inference methods (Fig. 2). All *Chaerephon* haplotypes formed a very strongly supported monophyletic clade (posterior probability of 1.00; maximum parsimony bootstrap 100%; neighbour-joining bootstrap 100%) with respect to the *Mops* out-group taxa. Within this was a strongly supported *C. atsinanana* clade (0.99, 96%, 96%): relationships among the seven haplotypes comprising this clade were largely unresolved, although haplotypes 1, 2, and 3 formed a well-supported group (1.00, 85%, 94%). GTR + G genetic distances between haplotypes ranged from 0.12 to 1.01% (mean 0.51%) (Table 1). The *C. atsinanana* clade was sister to a strongly supported out-group (*C. leucogaster* and *C. pusillus*) clade (0.96, 96%, 97%), in which *C. pusillus* appeared sister to a moderately supported *C. leucogaster* subclade.

Table 3. Distribution of *Chaerephon atsinanana* cytochrome *b* and control region haplotypes at 17 localities in eastern Madagascar

Locality	Hap 1	Hap 2	Hap 3	Hap 4	Hap 5	Hap 6	Hap 7	Hap 8	No. cyt *b*	No. CR	Latitude *C. atsinanana*	Altitude (m) *C. atsinanana*
Ambatondrazaka	–	–	–	–	–	–	5 (1.00)	7 (1.00)	5	7	N	601–1000
Ampasika	10 (1.00)	13 (1.00)	–	–	–	–	–	–	10	13	N	601–1000
Anjiro	1 (0.33)	3 (1.00)	–	–	–	2 (0.67)	–	–	3	3	N	601–1000
Andasibe	–	8 (1.00)	–	–		5 (1.00)	–	–	5	8	N	601–1000
Beforona	–	1 (0.11)	–	–	3 (0.75)	1 (0.25)	8 (0.89)	–	4	9	N	101–600
Brickaville	–	–	–	–	1 (1.00)	–	1 (1.00)	–	1	1	N	601–1000
Fanandrana	–	–	–	–	3 (1.00)	1 (0.25)	3 (0.75)	–	3	4	N	0–100
Farafangana	6 (1.00)	3 (1.00)	–	–	–	–	–	–	3	6	S	0–100
Ifanadiana	–	–	3 (1.00)	9 (1.00)	–	–	–	–	3	9	S	101–600
Manakara	6 (1.00)	3 (1.00)	–	–	–	–	–	–	3	6	S	0–100
Marozevo	–	3 (1.00)	–	–	–	3 (1.00)	–	–	3	3	N	101–600
Moramanga	–	4 (1.00)	–	–	–	4 (1.00)	–	–	4	4	N	601–1000
Ranomafana Atsinanana	–	–	–	–	1 (1.00)	–	3 (1.00)	–	1	3	N	601–1000
Ranomafana Ifanadiana	–	–	1 (1.00)	8 (0.89)	1 (0.11)	–	–	–	1	9	S	101–600
Toamasina ville	–	–	6 (1.00)	7 (1.00)	–	–	–	–	7	6	N	0–100
Vangaindrano	5 (1.00)	3 (1.00)	–	–	–	–	–	–	3	5	S	0–100
Vohipeno	11 (1.00)	3 (1.00)	–	–	–	–	–	–	3	11	S	0–100
No. (cyt *b*)	11 (0.18)	12 (0.19)	4 (0.06)	7 (0.11)	8 (0.13)	15 (0.24)	5 (0.08)		62 (1.00)	1	–	–
No. (control region)	28 (0.26)	32 (0.30)	6 (0.06)	17 (0.16)	1 (0.01)	1 (0.01)	15 (0.14)	7 (0.07)		107 (1.00)	–	–

Data are reported as numbers of specimens, followed by the proportion in brackets. Latitude and altitude designations refer to the groupings used in the analysis of molecular variance. Shaded blocks, cytochrome *b*; unshaded text, control region; Hap, haplotype; N, north; S, south; CR, control region.

Population struture and historical demography

Analysis of 301 nucleotides of the control region of *C. atsinanana* yielded 66 variable characters, of which 41 were parsimony informative. There were two singletons. The 107 sequences comprised eight haplotypes; the haplotype diversity was 0.793 ± 0.026 and the nucleotide diversity 0.03448 ± 0.002. A 26-nucleotide insertion was shared by all samples exhibiting haplotype 2, and a one-nucleotide deletion was present in haplotype 3. Four haplotypes (3, 5, 6, and 8) were unique to collection localities (Toamasina, Ranomafana Ifanadiana, Fanandrana, and Ambatondrazaka, respectively). All localities contained only a single haplotype, with the exception of Ranomafana Ifanadiana (haplotypes 4 and 5, separated by one mutation), Fanandrana (haplotypes 6 and 7, separated by one mutation), and Beforona (haplotypes 2 and 7, separated by 14 mutations). HKY + G genetic distances between haplotypes ranged from 0.01 to 8.14% (mean 4.95%) (Table 1).

Analysis of 1164 nucleotides of the concatenated mitochondrial cytochrome *b* (863 nt) and control region (301 nt) of 54 *C. atsinanana* specimens from eastern Madagascar yielded seven haplotypes for which the distribution over localities was congruent with those (above) derived from the control region data set. The haplotype diversity was 0.0843 ± 0.00037 and the nucleotide diversity was 0.01102 ± 0.0000006.

The *C. atsinanana* mismatch distribution (Fig. 4A) was multimodal and significantly ragged ($P < 0.001$; Table 4), consistent with a population that has been stationary for a long time (Harpending, 1994). The *C. leucogaster* data yielded an essentially unimodal mismatch distribution (Fig. 4B), which was relatively smooth, and had a low, non-significant raggedness statistic, consistent with past population expansion.

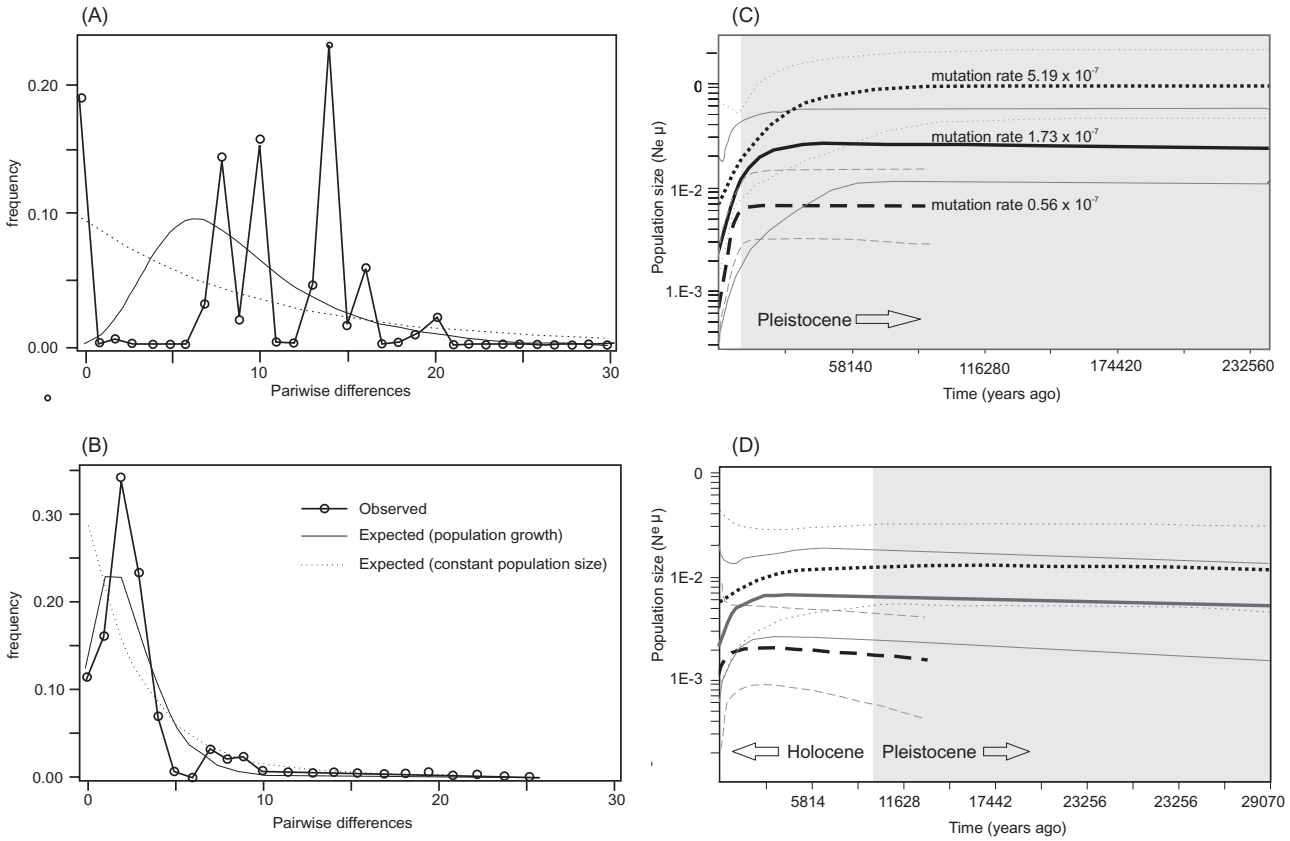

Figure 4. A, B, mismatch distributions for Malagasy populations of *Chaerephon atsinanana* and *Chaerephon leucogaster*, respectively. The observed distribution (heavy solid line) and those expected under the growth – decline (feint solid line) and constant (feint dotted line) population models are represented. C, D, Bayesian skyline estimations of past population sizes of *C. atsinanana* and *C. leucogaster*, respectively. Each plot was based on three different mutation rates: 0.56×10^{-7} (dotted lines); 1.73×10^{-7} (solid lines); and 5.19×10^{-7} (dashed lines) mutations per site per generation. For each mutation rate, the heavy line represents the median, and the feint lines bound the 95% credibility interval.

Table 4. Indices of diversity, neutrality, and historical demography based on an analysis of control region sequences, and cytochrome *b* and control region haplotype prevalence for the Malagasy molossids, *Chaerephon atsinanana* and *Chaerephon leucogaster*

Parameter	*C. atsinanana*	*C. leucogaster*	Expectation†
Nucleotide diversity per site (π)	0.03448	0.00739	Low
Haplotype diversity (h)	0.793	0.885	High
Expansion coefficient ($S\,d^{-1}$)	3.399	4.444	High
Fu & Li (1993) F*	2.02897*;	1.16927	Not significant
Fu & Li (1993) D*	1.91681*	1.38919	Not significant
Fu (1997) F_s	13.015*	–2.340 ns	Significant
Raggedness statistic	0.2246**	0.0741	Not significant
Mismatch distribution	Multimodal	Unimodal	Unimodal
Tau (τ)	4.671	1.303	–
Time since expansion (years BP)‡	–	11 141	–
FST (variance between localities)	0.994	0.792	–

Haplotypes	Cytochrome *b*	Control region	Cytochrome *b*	Control region
Sample size	62	107	39	71
Number haplotypes	7	8	6	11
Frequency of H1	0.19	0.26	0.48	0.03
Frequency of H2	0.19	0.30	0.05	0.25
Frequency of H3	0.06	0.06	0.13	0.06
Frequency of H4	0.11	0.16	0.10	0.08
Frequency of H5	0.13	0.01	0.03	0.07
Frequency of H6	0.24	0.01	0.21	0.15
Frequency of H7	0.08	0.14	–	0.08
Frequency of H8	–	0.06	–	0.08
Frequency of H9	–	–	–	0.14
Frequency of H10	–	–	–	0.03
Frequency of H11	–	–	–	0.03

*$P < 0.02$; **$P < 0.00001$.

†Expected trends for a model of demographic population expansion (Hull & Girman, 2005).

‡Value obtained from formula $\tau = 2ut$, following Rogers & Harpending (1992), where u was the product of mutation rate ($\mu = 1.73 \times 10^{-7}$) per site per generation and sequence length, and t was the time (in generations) since expansion (generation time taken as 1 year). D* and F*; departures from neutrality assessed as a deviation between estimates of nucleotide diversity derived from external branches of a phylogeny and from the total number of mutations (D*) or from the average pairwise diversity (F*) (Fu and Li, 1993). Fs; an estimate of the probability of observing a random sample with a number of alleles equal to or smaller than the observed value, given the observed level of diversity (Fu, 1997).

The shapes of the Bayesian skyline plots of the past sizes of Malagasy *Chaerephon* populations appear relatively independent of the mutation rate over the ninefold range for which they were estimated (Fig. 4A, B). In general, lower estimates of mutation rates produced higher estimates of population size. *Chaerephon atsinanana* populations (Fig. 4C) appear to have maintained a constant size over the period from *c*. 232 560 to *c*. 30 000 years before present, and to have decreased in size from *c*. 3000 years ago until the present, although the 95% credibility intervals indicate low precision for this predicted decrease, the timing of which also depends on the mutation rate used. The *C. leucogaster* population (Fig. 4D) appears to have increased very slowly in the period between 29 000 and *c*. 5000 years before present, although this increase is more pronounced when higher mutation rates are assumed. *Chaerephon leucogaster* populations appear to have decreased in size over the last *c*. 4000 years, although the confidence associated with this predicted decrease appears low and its timing depends on the mutation rate.

HAPLOTYPE NETWORKS

The *C. atsinanana* control region haplotype network (Fig. 3A) comprised eight haplotypes, and adjacent haplotypes were separated by between one and

17 steps. Individual haplotypes represented samples from between one and six sampling sites. A Mantel test for isolation by distance across all sites was significant ($P < 0.01$). The haplotype network (Fig. 3B) derived from the concatenated control region and cytochrome *b* data set comprised seven haplotypes; adjacent haplotypes were also separated by between one and 17 steps. Individual haplotypes represented samples from between one and four sampling sites. The network structure and grouping of samples from different localities into haplotypes show high congruency in both cytochrome *b* and concatenated haplotype networks (Fig. 3A, B).

AMOVA

Analyses of molecular variance of *C. atsinanana* control region data (Tables 2 and 3) were consistent with high levels of structure in all three analyses. The overall FST, with no higher-level groupings of populations, was 0.994. FST, the fixation index, is a measure of population differentiation. It is a measure of the diversity of randomly chosen alleles of a sub-population relative to that of the entire population. It is often expressed as the proportion of genetic diversity due to allele frequency differences among populations (Holsinger and Weir, 2009). When populations were divided into northern and southern latitudinal groups, there was highly significant structure among groups (27.76% of variance), among populations within groups (67.36% of variance), and within populations (4.88% of variance). There was also highly significant structure among altitude-stratified population groups (44.85% of variance), among populations within groups (54.92% of variance), and within populations (0.22% of variance).

The overall FST value for the *C. leucogaster* sample set was 0.792. No significant structure was found among latitude-stratified population groups (3.82% of variance), although there was significant structuring among populations within groups (45.02% of variance) and within populations (51.17% of variance).

Predicted distribution of *Chaerephon atsinanana*

For *C. atsinanana*, the MaxEnt algorithm converged after 260 iterations with a regularized training gain of 1.773. Model performance as assessed by AUC was very high (0.964), indicating the efficient classification of suitable versus unsuitable habitats. The variables contributing most strongly to the final model were annual precipitation (38.0%), human footprint (29.9%), and terrestrial ecosystems of the world (20.8%). Temperature seasonality contributed moderately (10.9%), whereas the contribution of mean annual temperature was very low (0.4%). An examination of response curves depicting effects of environmental variables on MaxEnt predictions indicated that highly suitable habitat was associated with high annual rainfall, highly seasonal temperature profiles, high human disturbance, and the 'Madagascar Lowlands Forests' ecoregion of Olson *et al*. (2001). The MaxEnt model revealed a rather patchy predicted distribution pattern that was limited to the Madagascar Lowland Forests ecoregion (Fig. 5A), and was strongly associated with human population centres and transport routes.

For *C. leucogaster*, the MaxEnt algorithm converged after 520 iterations with a regularized training gain of 1.369. Model performance as assessed by AUC was high (0.945), indicating efficient classification of suitable versus unsuitable habitats. The environmental variable with the highest explanatory power when used in isolation was annual precipitation. This same variable decreased the overall gain of the model most when omitted. The three variables contributing most strongly to the final model were annual precipitation (53.4%), mean annual temperature (14.7%), and temperature seasonality (14.3%). Terrestrial ecosystems of the world (9.1%) and human footprint (8.5%) contributed less significantly. An examination of response curves depicting the effects of environmental variables on MaxEnt predictions indicated that highly suitable habitat was associated with low annual rainfall, high mean annual temperature, and high human influence; there was no association with ecoregion and temperature seasonality. The MaxEnt model for this species (Fig. 5B) was almost identical to that depicted by Ratrimomanarivo *et al*. (2009) (using only bioclimatic variables and altitude as environmental variables), with a continuous area of consistently high suitability in the south west and more isolated areas of suitability in the north west.

DISCUSSION

In this study, we have used analyses of mitochondrial cytochrome *b* and D-loop sequences to examine population-genetic structuring in two congeneric Malagasy molossid bats: *C. atsinanana* and *C. leucogaster*. We have shown that these synanthropic bats, which occupy similar types of day roost spaces in human-built structures, and have similar dispersal capabilities, exhibit markedly different levels of genetic and phylogeographic structure, as well as historical demographic patterns, causing us to reject the null model of concordance in historical demographic and genetic patterns. *Chaerephon leucogaster*, which underwent a population expansion at

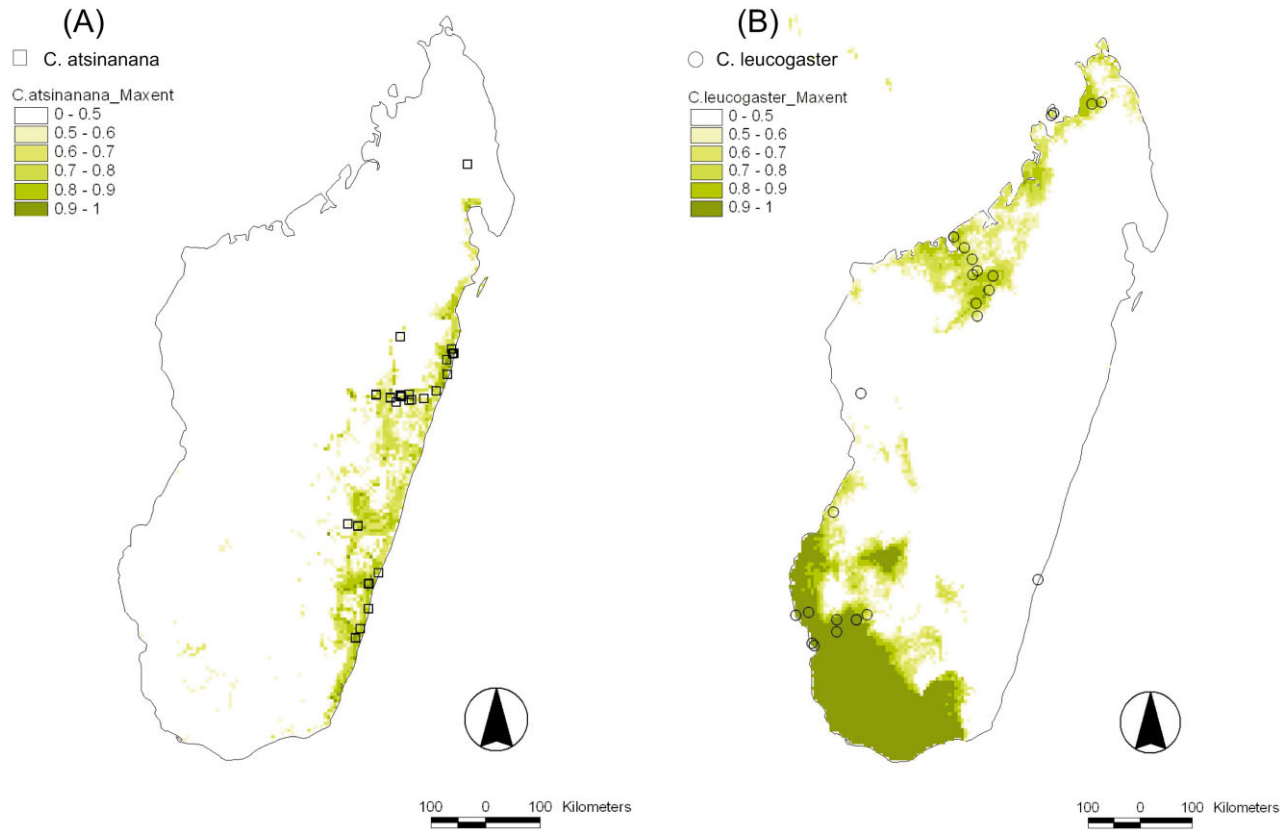

Figure 5. A, modelled potential distribution (MaxEnt) of *Chaerephon atsinanana* in Madagascar. B, modelled potential distribution (MaxEnt) of *C. leucogaster* in the Madagascar region.

the end of the last glacial period, shows markedly lower levels of structure, whereas *C. atsinanana* populations are highly structured; *C. atsinanana* appears to be an older species with a population size that was stable over much of the last *c*. 230 000 years of the Quaternary. Under the scenario that eastern and western Madagascar underwent similar levels of climatic oscillations during the Quaternary (see discussion below), the high levels of genetic and phylogeographic structure shown by this species are unlikely to be related to such vicissitudes, and most probably result at least in part from behavioural factors, such as philopatry. It is also likely that the differing demographic histories of these two essentially allopatrically distributed species reflect ecological or physiological factors resulting from adaptation to different habitats.

DISTRIBUTION

Chaerephon atsinanana is endemic to most of the breadth of eastern Madagascar, and shows a similarly confined and patchy predicted distribution range associated with nodes of human influence. *Chaerephon leucogaster* is distributed largely allopatrically over an equivalent range in the west, although the predicted distribution range is wider than the known extent of occurrence of this species. Based on phylogenetic inference, *C. leucogaster* is more closely related to *C. pusillus* from Aldabra and the Comoros, and *C. pumilus* from South Africa, than to its Malagasy congener, *C. atsinanana*, with which it last shared a common ancestor in the early Pleistocene (Lamb *et al*., 2011) (Fig. 2). During their *c*. 1.9 million years of independent evolution, these non-sister species may have evolved different behavioural mechanisms, habitat associations, and physiological adaptations; they may also have used different colonization routes, all of which could explain differences in demographic and genetic patterns. Whereas the range of *C. atsinanana* is relatively confined, that of *C. leucogaster* also includes Pemba Island off the coast of Tanzania and the Comoros Archipelago in the Mozambique Channel (Ratrimomanarivo *et al*., 2009). This species has not yet been genetically identified on the African mainland, although specimens from there have been referred to it. In common with the molossid *Mops midas* (Ratrimomanarivo *et al*., 2007), *C. leuco-*

gaster is capable of traversing the several hundred kilometres of open water in the Mozambique Channel between Madagascar, African off-shore islands, and the Comoros.

Although *C. leucogaster* is primarily a lowland species in the dry west, *C. atsinanana* is found in the mesic east at elevations of up to 1100 m a.s.l. As both species are capable of sustained high and strong flight, they are probably able to traverse passes within the mountainous divide separating their distributional ranges, as has been shown by the recovery of a single *C. leucogaster* individual from Manakara (Fig. 5B), on the east coast, and sympatric with *C. atsinanana*. It therefore appears that the genetic break between these taxa is unlikely to be a result of vicariance.

In the MaxEnt analyses, the annual precipitation was most strongly predictive of the distribution of both *C. atsinanana* (found in mesic eastern regions) and *C. leucogaster* (found in dry western regions). The strong contribution of ecoregion (Madagascar Lowland Forest) to the MaxEnt-predicted distribution of *C. atsinanana* appears to reflect habitat associations. The comparatively much reduced effect of ecoregion on the predicted distribution of *C. leucogaster* in relation to climate (annual precipitation) suggests that physiological tolerance rather than habitat associations may be an important factor in determining the distribution of *C. leucogaster* in the drier western region of Madagascar. Such differences in factors underlying the distribution of modern species may be central to understanding the divergent responses of these two species to historical climatic regimes, as reflected in their radically different population genetic profiles.

There are many difficulties inherent in using presence-only data (such as museum records) for estimating the predicted distributions of species. We note that the modelled potential distributions of *C. atsinanana* and *C. leucogaster* (Fig. 5) appear not to include all the points in the respective models, or to include these with low probability. A potential problem with modelling the distributions of highly mobile organisms, such as molossid bats, is that they may be sampled outside their most common range. Costa *et al*. (2010), in their evaluation of sampling bias in ecological niche modelling, report that the MaxEnt model seldom fails to predict species collected, and that where this occurs, the numbers of sampled localities are ususally low (i.e. fewer than five), which is not the case in this study. Although MaxEnt tends to be more sensitive to sampling bias than other models (Costa *et al*., 2010), it has been shown to function well with presence-only data, and to perform generally better than alternative 'climatic envelope' models such as GARP and BIOCLIM (Elith *et al*., 2006).

Historical demography

Episodes of population growth and decline leave characteristic signatures in a mismatch distribution (Rogers & Harpending, 1992). Our population genetic analyses are consistent with a *C. atsinanana* population that has been stationary for a relatively long period. Diversity and neutrality statistics are incompatible with a model of demographic population expansion (Hull & Girman, 2005) (Table 4), and the mismatch distribution is multimodal and significantly ragged (Fig. 4A; Table 4): characteristic of a population at demographic equilibrium (Slatkin & Hudson, 1991; Rogers & Harpending, 1992; Schneider & Excoffier, 1999). Furthermore, there are very few singletons (zero and two in the cytochrome *b* and control region data sets, respectively), and the haplotype networks do not exhibit the star shape associated with expanding populations (see Excoffier *et al*., 2009). Bayesian skyline analysis plots indicate similar patterns over a nine-fold variation in estimated mutation rate (Fig. 4C), and provide no evidence that *C. atsinanana* underwent cycles of population contraction and expansion, corresponding with glacial and interglacial periods, in the latter part of the Pleistocene.

Our analyses are, however, consistent with a period of population expansion in *C. leucogaster* at the end of the last Pleistocene glacial cycle. Mismatch distributions indicate that approximately 11 000 years have elapsed since the period of expansion. The Bayesian skyline plot (Fig. 4D) is consistent with a relatively small increase in population size in the period between *c.* 29 000 and *c.* 5000 years before present (Fig. 4D), which is more pronounced at higher assumed mutation rates. Such an expansion may account for the markedly lower levels of genetic and phylogeographic structure observed in *C. leucogaster* relative to *C. atsinanana*.

In Madagascar, the climate was cooler and drier in glacial periods, with consequent habitat shifts (Straka, 1995; Burney *et al*., 2004; Wilmé, Goodman & Ganzhorn, 2006). Data from late Quaternary fossil deposits on Madagascar, particularly pollen cores, provide insight into the levels of climatic change during this period. These vicissitudes, which occurred before the first colonization of the island by humans some 2300 years ago (Burney *et al*., 2004), had a direct impact on vegetation structure, which in turn would have caused shifts in the distribution of certain animal species. The western portion of the island, which today has a native vegetation of dry deciduous forest in the north west, grading into dry spiny bush towards the south west, underwent a drastic change during the period from the Late Pleistocene to the Holocene. On the basis of cores falling along a north–

south cline in the western lowlands, shifts in plant communities were notably more drastic towards the south (Burney, 1993, 1999; Matsumoto & Burney, 1994). During periods of drier conditions, locally occurring species of animals, which still exist today, would have been pushed into geographically limited zones of ecological refuge, creating genetic bottlenecks, as seen in the low levels of geographical structure amongst existing populations of *C. leucogaster*. Although few pollen cores from a comparable geological period are available from the humid forest lowlands of eastern Madagascar, there is good evidence that this zone was distinctly more stable (Virah-Sawmy, Gillson & Willis, 2009a; Virah-Sawmy, Willis & Gillson, 2009b), and that certain locally occurring animal populations did not experience the same shifts in climatic regimes, and were able to maintain aspects of genetic variation, as demonstrated by the phylogeographical structure of *C. atsinanana*.

Accordingly, a mobile bat such as *C. leucogaster* may have retreated into refugia on the island, from which such populations may have subsequently expanded during more mesic times (Vences *et al*., 2009). Nested clade analysis has revealed some significant latitudinal phylogeographic associations between north versus central and south versus central *C. leucogaster* haplotype groups (Ratrimomanarivo *et al*., 2009), and provides an inference of past fragmentation followed by range expansion. Such latitudinal stratification has also been reported for the widespread endemic Malagasy bat, *Myotis goudoti* (Weyeneth *et al*., 2011). These authors report a zone of admixture between southern and central northern lineages in a broad central band (from 20° to 23°S), located further south than the central *C. leucogaster* haplotypes (from 15° to 17°S). The probable timing of the population expansions of *C. leucogaster* and *Myotis goudoti* differ, making it unlikely that they were caused by the same historical climate shift processes. It is also possible that latitudinal genetic differentiation in *C. leucogaster* is a result of the process of demographic expansion across a spatially and temporally heterogeneous environment, and was not primarily dependent on long-term isolation in glacial refugia (Knowles & Alvarado-Serrano, 2010).

Although Bayesian skyline plots for both species of *Chaerephon* indicate the possibility of a relatively recent decline in population size, this should be regarded with caution, as the confidence limits on this part of the plot are wide. Both species adapted their roosting behaviour to take advantage of new sites afforded by man-made structures in the period following the arrival of humans on Madagascar. The high levels of human population pressure experienced on Madagascar in modern times have led to the large-scale destruction of natural forests to create agricultural and residential landscapes (Harper *et al*., 2007). The construction of buildings is likely to have led to a population expansion to fill these new roost sites, which may previously have been a limiting factor, as compared with foraging habitat *per se*. If indeed the case, this would explain why these two bat species have adapted to the dramatic transformation of the natural habitats of the island, and are locally abundant (Goodman *et al*., 2010; Goodman, 2011). Neither mismatch distribution analysis nor the Bayesian skyline plots reflect the massive population expansion of these molossid bats over the last 100 generations, possibly indicative of a limitation in their applicability at certain time scales.

Genetic structure

The genetic distances between *C. atsinanana* cytochrome *b* haplotypes ranged from 0.14 to 1.01% (mean 0.54%), which is consistent with intraspecific variation (Baker & Bradley, 2006), although the high levels of structure between subpopulations may be a snapshot of populations in the process of speciation.

Analysis of molecular variance reveals significant levels of genetic structure in both *C. atsinanana* and *C. leucogaster*, although structuring in *C. atsinanana* is especially high (overall FST 0.994 versus 0.792). In *C. atsinanana*, 44.85% of the variance occurs among three altitudinal groups ranging from sea level to ~ 1000 m a.s.l., the upper level of which is close to certain passes leading to the western side of the island. Additionally, 27.76% of the variance occurs among north–south population groups. Only 0.22% (altitude) and 4.88% (latitude) occurs within populations (Table 2). In contrast, AMOVA of *C. leucogaster* control region data shows non-significant structuring among latitudinal groups (3.82% of the variance), with most of the variance (51.10%) occurring among populations.

Most locales exhibit a single *C. atsinanana* haplotype, and haplotypes, if shared, tend to be common to adjacent localities (Fig. 3). The major *C. atsinanana* control region haplotypes are separated by high numbers of mutational steps (Fig. 3), consistent with a mean divergence between haplotypes of 4.95%, with some haplotypes differing by up to 8.14%. Thus, *C. atsinanana* comprises a set of highly diverse, structured subpopulations. Such levels of genetic differentiation could be caused by isolation and genetic drift (Atartouch *et al*., 2006), which begs the question as to the cause of the isolation: up to 45% of the variance between groups can be explained by ecological variables such as altitude and latitude. However, the variance among populations is extremely low (as low as 0.22%), making it likely that other factors are contributing to the high level of genetic structuring in this species.

Interestingly, the control region genetic distances between populations of *C. atsinanana* are similar to those reported for another species of endemic Malagasy bat, *Myotis goudoti* (mean 4.2%; maximum 9%; Weyeneth *et al*., 2011) and the relatively less mobile Australian ghost bat, *Macroderma gigas* (Dobson, 1887) (control region divergence up to 6%, 87% of variance among populations; Worthington-Wilmer *et al*., 1994). As is the case for *Macroderma gigas*, *C. atsinanana* fits Avise *et al*.'s (1987) category 1, in which the major haplotypes of deep gene trees are restricted to particular areas, indicating populations that have been isolated for a long period. Worthington-Wilmer *et al*. (1994) proposed this extreme structuring to be a consequence of long-term isolation of subpopulations, accentuated by female philopatry. Furthermore, in the case of *C. atsinanana*, relatively stable climatic periods during the Quaternary of eastern Madagascar did not result in genetic bottlenecks, and allowed this taxon to retain phylogeographic structure.

A significant degree of isolation by distance in *C. atsinanana* further indicates the limitation of female-mediated gene flow between localities. As *C. atsinanana* presumably has the capacity to be a highly mobile bat, and occupies a considerable swath of eastern Madagascar that contains no significant geographic barriers, particularly mountain ranges, the low level of gene flow between subpopulations is likely to be maintained by behavioural aspects associated with dispersal. An excellent example is female philopatry, consistent with our analyses of a maternally inherited marker reflecting movements of females (Ballard & Whitlock, 2004). Future work will focus on the sequencing of nuclear genes and analysis of microsatellites in order to obtain a finer-scale picture of population genetic structuring in *C. atsinanana*, and to assess the male contribution to gene flow. Use of a multilocus approach may also address the possibility that the patterns observed here reflect selection rather than population history, as mutation, drift, and selection operate independently on unlinked loci (Knowles, 2004).

We conclude that the Malagasy molossid bats *C. atsinanana* and *C. leucogaster* show markedly different demographic patterns, as well as different levels of population genetic and phylogeographic structuring. The lower levels of structure in *C. leucogaster* may at least partly reflect a late-Pleistocene population expansion, subsequent to a genetic bottleneck. The high levels of population structure in *C. atsinanana* are likely to reflect ancient genetic subdivisions, possibly stemming from low levels of gene flow caused by female philopatry, and maintained by the climatic stability of eastern Madagascar during Quaternary glacial cycling. Furthermore, differing demographic histories of the two species may have been shaped by differing climate or habitat preferences. MaxEnt ecological niche modelling predicts the occurrence of *C. atsinanana* in areas of high rainfall, covered with lowland forest and associated with high human influence. In contrast, *C. leucogaster* is most strongly associated with areas of low rainfall, suggesting that physiological tolerance is an important determinant of its distribution.

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APPENDIX

Chaerephon atsinanana, *Chaerephon leucogaster*, and out-group taxa included in this study. GenBank accession numbers are listed for samples used in genetic analyses. The MaxEnt ecological niche modelling of the predicted distributions of *C. atsinanana* and *C. leucogaster* was based on the sample localities listed here. FMNH, Field Museum of Natural History; UADBA, Université d'Antananarivo, Département de Biologie Animale; NA, not available; hap., haplotype; SMG, Steven Goodman; *field collection number.

Name	Museum number	Locality in Madagascar	Latitude	Longitude	Hap Cyt *b*	Hap Control region	GenBank no. cytochrome *b*	Genbank no. control region
C. atsinanana	UADBA 43912	Ampasika	–19.01915	48.34871	1	2	JN867794	JN867871
C. atsinanana	SMG 16901*	Ampasika	–19.01915	48.34871	1	2	JN867795	JN867872
C. atsinanana	SMG 16902*	Ampasika	–19.01915	48.34871	1	2	JN867796	JN867873
C. atsinanana	SMG 16903*	Ampasika	–19.01915	48.34871	1	2	JN867797	JN867874
C. atsinanana	UADBA 43913	Ampasika	–19.01915	48.34871	1	2	JN867798	JN867875
C. atsinanana	UADBA 43914	Ampasika	–19.01915	48.34871	–	2	–	JN867876
C. atsinanana	UADBA 43915	Ampasika	–19.01915	48.34871	1	2	JN867799	JN867877
C. atsinanana	UADBA 43916	Ampasika	–19.01915	48.34871	1	2	JN867800	JN867878
C. atsinanana	UADBA 43917	Ampasika	–19.01915	48.34871	1	2	JN867801	JN867879
C. atsinanana	UADBA 43918	Ampasika	–19.01915	48.34871	1	2	JN867802	JN867880
C. atsinanana	UADBA 43919	Ampasika	–19.01915	48.34871	1	2	JN867803	JN867881
C. atsinanana	UADBA 43921	Ampasika	–19.01915	48.34871	–	2	–	JN867882
C. atsinanana	SMG 16914*	Ampasika	–19.01915	48.34871	–	2	–	JN867883
C. atsinanana	FMNH 184677	Anjiro	–18.88240	47.97075	–	2	–	JN867884
C. atsinanana	FMNH 184678	Anjiro	–18.88240	47.97075	1	2	GQ489177	JN867885
C. atsinanana	FMNH 184680	Anjiro	–18.88240	47.97075	–	2	–	JN867886
C. atsinanana	FMNH 184681	Anjiro	–18.88240	47.97075	6	–	JN867826	–
C. atsinanana	FMNH 184682	Anjiro	–18.88240	47.97075	6	–	JN867827	–
C. atsinanana	FMNH 184491	Andasibe	–18.89500	48.41511	6	2	JN867828	JN867887
C. atsinanana	FMNH 184492	Andasibe	–18.89500	48.41511	6	2	JN867829	JN867888
C. atsinanana	FMNH 184493	Andasibe	–18.89500	48.41511	6	2	JN867830	JN867889
C. atsinanana	FMNH 184494	Andasibe	–18.89500	48.41511	6	2	JN867831	JN867890
C. atsinanana	FMNH 184495	Andasibe	–18.89500	48.41511	6	2	JN867832	JN867891
C. atsinanana	FMNH 184496	Andasibe	–18.92333	48.42076	–	2	–	JN867892
C. atsinanana	FMNH 184499	Andasibe	–18.92333	48.42076	–	2	–	JN867893
C. atsinanana	FMNH 184500	Andasibe	–18.92333	48.42076	–	2	–	JN867894
C. atsinanana	FMNH 184509	Beforona	–18.88915	48.57755	5	7	JN867823	JN867924
C. atsinanana	FMNH 184510	Beforona	–18.88915	48.57755	6	2	JN867833	JN867895
C. atsinanana	FMNH 184511	Beforona	–18.88915	48.57755	–	7	–	JN867925
C. atsinanana	FMNH 184512	Beforona	–18.88915	48.57755	5	7	JN867824	JN867926
C. atsinanana	FMNH 184513	Beforona	–18.88915	48.57755	5	7	JN867825	JN867927
C. atsinanana	FMNH 184514	Beforona	–18.88915	48.57755	–	7	–	JN867928
C. atsinanana	FMNH 184515	Beforona	–18.88915	48.57755	–	7	–	JN867929
C. atsinanana	FMNH 184516	Beforona	–18.88915	48.57755	–	7	–	JN867930
C. atsinanana	FMNH 184522	Beforona	–18.88866	48.57852	–	7	–	JN867931
C. atsinanana	FMNH 188142	Marozevo	–18.98300	48.61700	6	2	JN867834	JN867896
C. atsinanana	FMNH 188143	Marozevo	–18.98300	48.61700	6	2	JN867835	JN867897
C. atsinanana	FMNH 188144	Marozevo	–18.98300	48.61700	6	2	JN867836	JN867898
C. atsinanana	FMNH 188113	Moramanga	–18.93330	48.20000	6	2	JN867837	JN867899
C. atsinanana	FMNH 188114	Moramanga	–18.93330	48.20000	–	2	–	JN867900
C. atsinanana	FMNH 188115	Moramanga	–18.93331	48.19999	6		JN867840	
C. atsinanana	FMNH 188116	Moramanga	–18.93329	48.20000	6	2	JN867838	JN867901
C. atsinanana	FMNH 188117	Moramanga	–18.93330	48.20000	6	2	JN867839	JN867902
C. atsinanana	FMNH 185229	Vangaindrano	–23.35500	47.59605	–	1	–	JN867846
C. atsinanana	FMNH 185230	Vangaindrano	–23.35500	47.59605	2	1	GQ489168	JN867849
C. atsinanana	FMNH 185231	Vangaindrano	–23.35500	47.59605	–	1	–	JN867848
C. atsinanana	FMNH 185232	Vangaindrano	–23.35500	47.59605	–	1	–	JN867849
C. atsinanana	FMNH 185233	Vangaindrano	–23.35500	47.59605	2	1	JN867804	JN867850
C. atsinanana	FMNH 185235	Vangaindrano	–23.35500	47.59605	2	–	JN867805	–
C. atsinanana	FMNH 185259	Farafangana	–22.82125	47.83100	2	1	GQ489169	JN867851
C. atsinanana	FMNH 185260	Farafangana	–22.82125	47.83100	–	1	–	GQ489119
C. atsinanana	FMNH 185261	Farafangana	–22.82125	47.83100	–	1	–	JN867852

APPENDIX *Continued*

Name	Museum number	Locality in Madagascar	Latitude	Longitude	Hap Cyt *b*	Hap Control region	GenBank no. cytochrome *b*	Genbank no. control region
C. atsinanana	FMNH 185262	Farafangana	–22.82125	47.83100	–	1	–	JN867853
C. atsinanana	FMNH 185263	Farafangana	–22.82125	47.83100	2	1	JN867806	JN867854
C. atsinanana	FMNH 185265	Farafangana	–22.82125	47.83100	2	1	JN867807	JN867855
C. atsinanana	FMNH 185283	Vohipeno	–22.36661	47.83676	–	1	–	JN867856
C. atsinanana	FMNH 185284	Vohipeno	–22.36661	47.83676	–	1	–	JN867857
C. atsinanana	FMNH 185285	Vohipeno	–22.36661	47.83676	–	1	–	JN867858
C. atsinanana	FMNH 185286	Vohipeno	–22.36661	47.83676	2	1	GQ489170	GQ489120
C. atsinanana	FMNH 185287	Vohipeno	–22.36661	47.83676	2	1	JN867808	JN867859
C. atsinanana	FMNH 185288	Vohipeno	–22.36661	47.83676	2	1	JN867809	JN867860
C. atsinanana	FMNH 185290	Vohipeno	–22.36661	47.83676	–	1	–	JN867861
C. atsinanana	FMNH 185291	Vohipeno	–22.36661	47.83676	–	1	–	JN867862
C. atsinanana	FMNH 185292	Vohipeno	–22.36661	47.83676	–	1	–	JN867863
C. atsinanana	FMNH 185295	Vohipeno	–22.36661	47.83676	–	1	–	JN867864
C. atsinanana	FMNH 185307	Vohipeno	–22.36661	47.83676	–	1	–	JN867865
C. atsinanana	FMNH 185313	Manakara	–22.15696	48.01681	–	1	–	JN867866
C. atsinanana	FMNH 185314	Manakara	–22.15696	48.01681	2	1	GQ489171	JN867867
C. atsinanana	FMNH 185315	Manakara	–22.15696	48.01681	–	1	–	GQ489121
C. atsinanana	FMNH 185316	Manakara	–22.15696	48.01681	–	1	–	JN867868
C. atsinanana	FMNH 185317	Manakara	–22.15696	48.01681	2	1	JN867810	JN867869
C. atsinanana	FMNH 185318	Manakara	–22.15696	48.01681	2	1	JN867811	JN867870
C. atsinanana	FMNH 185319	Ifanadiana	–21.30656	47.63573	3	4	JN867812	JN867907
C. atsinanana	FMNH 185320	Ifanadiana	–21.30656	47.63573	–	4	–	JN867908
C. atsinanana	FMNH 185321	Ifanadiana	–21.30656	47.63573	–	4	–	JN867909
C. atsinanana	FMNH 185322	Ifanadiana	–21.30656	47.63573	3	4	GQ489175	JN867910
C. atsinanana	FMNH 185323	Ifanadiana	–21.30656	47.63573	–	4	–	JN867911
C. atsinanana	FMNH 185324	Ifanadiana	–21.30656	47.63573	3	4	JN867813	JN867912
C. atsinanana	FMNH 185326	Ifanadiana	–21.30656	47.63573	–	4	–	JN867913
C. atsinanana	FMNH 185335	Ifanadiana	–21.29800	47.63773	–	4	–	JN867914
C. atsinanana	FMNH 185336	Ifanadiana	–21.29800	47.63773	–	4	–	JN867915
C. atsinanana	FMNH 187797	Toamasina	–18.14068	49.37783	–	3	–	GQ489122
C. atsinanana	FMNH 187798	Toamasina	–18.14068	49.37783	4		GQ489172	
C. atsinanana	FMNH 187799	Toamasina	–18.14068	49.37783	–	3	–	GQ489123
C. atsinanana	FMNH 187801	Toamasina	–18.14068	49.37783	4	3	JN867814	JN867903
C. atsinanana	FMNH 187803	Toamasina	–18.14068	49.37783	4	3	JN867815	JN867904
C. atsinanana	FMNH 187804	Toamasina	–18.14068	49.37783	4	3	JN867816	JN867905
C. atsinanana	FMNH 187805	Toamasina	–18.14068	49.37783	4	3	JN867817	JN867906
C. atsinanana	FMNH 187806	Toamasina	–18.12615	49.40213	4	–	JN867818	–
C. atsinanana	FMNH 187807	Toamasina	–18.12615	49.40213	4	–	JN867819	–
C. atsinanana	FMNH 187816	Fanandrana	–18.25203	49.26778	5	7	JN867820	JN867932
C. atsinanana	FMNH 187817	Fanandrana	–18.25203	49.26778	–	7	–	JN867933
C. atsinanana	FMNH 187820	Fanandrana	–18.25203	49.26778	–	6	–	JN867923
C. atsinanana	FMNH 187822	Fanandrana	–18.25203	49.26778	5	7	JN867821	JN867934
C. atsinanana	FMNH 187931	Fanandrana	–18.25203	49.26778	5	–	JN867822	–
C. atsinanana	FMNH 187823	Brickaville	–18.82195	49.07238	5	7	GQ489173	JN867935
C. atsinanana	FMNH 187834	Ranomafana Atsinanana	–18.96060	48.84741	5	–	GQ489174	–
C. atsinanana	FMNH 187835	Ranomafana Atsinanana	–18.96060	48.84741	–	7	–	JN867936
C. atsinanana	FMNH 187836	Ranomafana Atsinanana	–18.96060	48.84741	–	7	–	GQ489125
C. atsinanana	FMNH 187837	Ranomafana Atsinanana	–18.96060	48.84741	–	7	–	JN867937
C. atsinanana	FMNH 188082	Ranomafana Ifanadiana	–21.25760	47.45591	–	4	–	JN867916
C. atsinanana	FMNH 188083	Ranomafana Ifanadiana	–21.25760	47.45591	–	4	–	JN867917
C. atsinanana	FMNH 188084	Ranomafana Ifanadiana	–21.25760	47.45591	–	4	–	JN867918

APPENDIX *Continued*

Name	Museum number	Locality in Madagascar	Latitude	Longitude	Hap Cyt *b*	Hap Control region	GenBank no. cytochrome *b*	Genbank no. control region
C. atsinanana	FMNH 188085	Ranomafana Ifanadiana	–21.25760	47.45591	–	4	–	JN867919
C. atsinanana	FMNH 188086	Ranomafana Ifanadiana	–21.25760	47.45591	–	4	–	JN867920
C. atsinanana	FMNH 188088	Ranomafana Ifanadiana	–21.25760	47.45591	–	4		GQ489126
C. atsinanana	FMNH 188089	Ranomafana Ifanadiana	–21.25760	47.45591	3	4	GQ489176	GQ489127
C. atsinanana	FMNH 188090	Ranomafana Ifanadiana	–21.25760	47.45591	–	4	–	JN867921
C. atsinanana	FMNH 188091	Ranomafana Ifanadiana	–21.25760	47.45591	–	5	–	JN867922
C. atsinanana	FMNH 184651	Ambatondrazaka	–17.90000	48.48300	7	8	JN867841	JN867938
C. atsinanana	FMNH 184652	Ambatondrazaka	–17.90000	48.48300	7	8	JN867842	JN867939
C. atsinanana	FMNH 184653	Ambatondrazaka	–17.90000	48.48300	7	8	JN867843	JN867940
C. atsinanana	FMNH 184654	Ambatondrazaka	–17.90000	48.48300	7	8	JN867844	JN867941
C. atsinanana	FMNH 184655	Ambatondrazaka	–17.90000	48.48300	7	8	JN867845	JN867942
C. atsinanana	FMNH 184659	Ambatondrazaka	–17.90000	48.48300	–	8	–	JN867943
C. leucogaster	FMNH 184922	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727502.1
C. leucogaster	FMNH 184923	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727503.1
C. leucogaster	FMNH 184924	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727504.1
C. leucogaster	FMNH 184925	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727505.1
C. leucogaster	FMNH 184926	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727506.1
C. leucogaster	FMNH 184955	Andranofasika	–16.33715	46.84657	–	–	–	EU727512.1
C. leucogaster	FMNH 184956	Andranofasika	–16.33715	46.84657	–	–	–	EU727513.1
C. leucogaster	FMNH 184957	Andranofasika	–16.33715	46.84657	–	–	–	EU727514.1
C. leucogaster	FMNH 184958	Andranofasika	–16.33715	46.84657	–	–	–	EU727515.1
C. leucogaster	FMNH 184959	Andranofasika	–16.33715	46.84657	–	–	–	EU727516.1
C. leucogaster	FMNH 184950	Ambondramamy	–16.43622	47.15548	–	–	–	EU727507.1
C. leucogaster	FMNH 184951	Ambondramamy	–16.43622	47.15548	–	–	–	EU727508.1
C. leucogaster	FMNH 184952	Ambondramamy	–16.43622	47.15548	–	–	–	EU727509.1
C. leucogaster	FMNH 184953	Ambondramamy	–16.43622	47.15548	–	–	–	EU727510.1
C. leucogaster	FMNH 184954	Ambondramamy	–16.43622	47.15548	–	–	–	EU727511.1
C. leucogaster	FMNH 184896	Antanimbary	–17.18507	46.85510	–	–	–	EU727489.1
C. leucogaster	FMNH 184897	Antanimbary	–17.18507	46.85510	–	–	–	EU727490.1
C. leucogaster	FMNH 184898	Antanimbary	–17.18507	46.85510	–	–	–	EU727491.1
C. leucogaster	FMNH 184899	Antanimbary	–17.18507	46.85510	–	–	–	EU727492.1
C. leucogaster	FMNH 184900	Antanimbary	–17.18507	46.85510	–	–	–	EU727493.1
C. leucogaster	FMNH 184901	Antanimbary	–17.18507	46.85510	–	–	–	EU727494.1
C. leucogaster	FMNH 184902	Antanimbary	–17.18507	46.85510	–	–	–	EU727495.1
C. leucogaster	FMNH 184915	Maevatanana	–16.95753	46.82388	–	–	–	EU727496.1
C. leucogaster	FMNH 184916	Maevatanana	–16.95753	46.82388	–	–	–	EU727497.1
C. leucogaster	FMNH 184917	Maevatanana	–16.95753	46.82388	–	–	–	EU727498.1
C. leucogaster	FMNH 184919	Maevatanana	–16.95753	46.82388	–	–	–	EU727499.1
C. leucogaster	FMNH 184920	Maevatanana	–16.95753	46.82388	–	–	–	EU727500.1
C. leucogaster	FMNH 184975	Ankazomborona	–16.11602	46.75667	–	–	–	EU727519.1
C. leucogaster	FMNH 184976	Ankazomborona	–16.11602	46.75667	–	–	–	EU727521.1
C. leucogaster	FMNH 184977	Ankazomborona	–16.11602	46.75667	–	–	–	EU727520.1
C. leucogaster	FMNH 184978	Ankazomborona	–16.11602	46.75667	–	–	–	EU727522.1
C. leucogaster	FMNH 184979	Ankazomborona	–16.11602	46.75667	–	–	–	EU727523.1
C. leucogaster	FMNH 184604	Mahajanga	–15.71297	46.31253	–	–	–	EU727484.1
C. leucogaster	FMNH 184605	Mahajanga	–15.71297	46.31253	–	–	–	EU727485.1
C. leucogaster	FMNH 184606	Mahajanga	–15.71297	46.31253	–	–	–	EU727486.1
C. leucogaster	FMNH 184607	Mahajanga	–15.71297	46.31253	–	–	–	EU727487.1
C. leucogaster	FMNH 184608	Mahajanga	–15.71297	46.31253	–	–	–	EU727488.1
C. leucogaster	FMNH 184973	Ankijibe	–16.41345	46.76460	–	–	–	EU727517.1
C. leucogaster	FMNH 184974	Ankijibe	–16.41345	46.76460	–	–	–	EU727518.1
C. leucogaster	FMNH 185020	Berivotra	–15.90408	46.59788	–	–	–	EU727524.1
C. leucogaster	FMNH 185021	Berivotra	–15.90408	46.59788	–	–	–	EU727525.1
C. leucogaster	FMNH 185022	Berivotra	–15.90408	46.59788	–	–	–	EU727526.1
C. leucogaster	FMNH 185027	Berivotra	–15.90408	46.59788	–	–	–	EU727527.1
C. leucogaster	FMNH 185028	Berivotra	–15.90408	46.59788	–	–	–	EU727528.1
C. leucogaster	FMNH 185029	Berivotra	–15.90408	46.59788	–	–	–	EU727529.1
C. leucogaster	FMNH 185030	Berivotra	–15.90408	46.59788	–	–	–	EU727530.1
C. leucogaster	FMNH 184259	Sakaraha	–22.90910	44.52623	–	–	–	EU727532.1

APPENDIX *Continued*

Name	Museum number	Locality in Madagascar	Latitude	Longitude	Hap Cyt *b*	Hap Control region	GenBank no. cytochrome *b*	Genbank no. control region
C. leucogaster	FMNH 184263	Sakaraha	–22.90910	44.52623	–	–	–	EU727531.1
C. leucogaster	FMNH 184264	Sakaraha	–22.90910	44.52623	–	–	–	EU727461.1
C. leucogaster	FMNH 184237	Toliara	–23.39507	43.72032	–	–	–	EU727470.1
C. leucogaster	FMNH 184238	Toliara	–23.39507	43.72032	–	–	–	EU727462.1
C. leucogaster	FMNH 184239	Toliara	–23.39507	43.72032	–	–	–	EU727471.1
C. leucogaster	FMNH 184240	Toliara	–23.39507	43.72032	–	–	–	EU727483.1
C. leucogaster	FMNH 188497	Dzamandzar	–13.35158	48.18845	–	–	–	EU727474.1
C. leucogaster	FMNH 188498	Dzamandzar	–13.35158	48.18845	–	–	–	EU727475.1
C. leucogaster	FMNH 188499	Dzamandzar	–13.35158	48.18845	–	–	–	EU727476.1
C. leucogaster	FMNH 188500	Dzamandzar	–13.35158	48.18845	–	–	–	EU727477.1
C. leucogaster	FMNH 187750	near Hell–ville	13.40513	48.30335	–	–	–	EU727463.1
C. leucogaster	FMNH 187751	near Hell–ville	13.40513	48.30335	–	–	–	EU727464.1
C. leucogaster	FMNH 188640	Nosy Komba	–13.44270	48.34790	–	–	–	EU727478.1
C. leucogaster	FMNH 188641	Nosy Komba	–13.44270	48.34790	–	–	–	EU727479.1
C. leucogaster	FMNH 188642	Nosy Komba	–13.44270	48.34790	–	–	–	EU727480.1
C. leucogaster	FMNH 188643	Nosy Komba	–13.44270	48.34790	–	–	–	EU727481.1
C. leucogaster	FMNH 188644	Nosy Komba	–13.44270	48.34790	–	–	–	EU727482.1
C. leucogaster	FMNH 188495	Hell–ville	–13.40513	48.30335	–	–	–	EU727472.1
C. leucogaster	FMNH 188496	Hell–ville	–13.40513	48.30335	–	–	–	EU727473.1
C. leucogaster	FMNH 187752	Ambatozazavy	–13.36687	48.31545	–	–	–	EU727465.1
C. leucogaster	FMNH 187753	Ambatozazavy	–13.40513	48.30335	–	–	–	EU727466.1
C. leucogaster	FMNH 187754	Ambatozazavy	–13.40513	48.30335	–	–	–	EU727467.1
C. leucogaster	FMNH 187755	Ambatozazavy	–13.40513	48.30335	–	–	–	EU727468.1
C. leucogaster	FMNH 187756	Ambatozazavy	–13.40513	48.30335	–	–	–	EU727469.1
C. leucogaster	FMNH 184922	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727502.1
C. leucogaster	FMNH 184923	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727503.1
C. leucogaster	FMNH 184924	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727504.1
C. leucogaster	FMNH 184925	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727505.1
C. leucogaster	FMNH 184926	Ambalanjanakomby	–16.70103	46.07173	–	–	–	EU727506.1
C. leucogaster	FMNH 184955	Andranofasika	–16.33715	46.84657	–	–	–	EU727512.1
C. leucogaster	FMNH 184956	Andranofasika	–16.33715	46.84657	–	–	–	EU727513.1
C. leucogaster	FMNH 184957	Andranofasika	–16.33715	46.84657	–	–	–	EU727514.1
C. leucogaster	FMNH 184958	Andranofasika	–16.33715	46.84657	–	–	–	EU727515.1
C. leucogaster	FMNH 184959	Andranofasika	–16.33715	46.84657	–	–	–	EU727516.1
C. leucogaster	FMNH 184950	Ambondramamy	–16.43622	47.15548	–	–	–	EU727507.1
C. leucogaster	FMNH 184951	Ambondramamy	–16.43622	47.15548	–	–	–	EU727508.1
C. leucogaster	FMNH 184952	Ambondramamy	–16.43622	47.15548	–	–	–	EU727509.1
C. leucogaster	FMNH 184953	Ambondramamy	–16.43622	47.15548	–	–	–	EU727510.1
C. leucogaster	FMNH 184954	Ambondramamy	–16.43622	47.15548	–	–	–	EU727511.1
C. leucogaster	FMNH 184896	Antanimbary	–17.18507	46.85510	–	–	–	EU727489.1
C. leucogaster	FMNH 184897	Antanimbary	–17.18507	46.85510	–	–	–	EU727490.1
C. leucogaster	FMNH 184898	Antanimbary	–17.18507	46.85510	–	–	–	EU727491.1
C. leucogaster	FMNH 184899	Antanimbary	–17.18507	46.85510	–	–	–	EU727492.1
C. leucogaster	FMNH 184900	Antanimbary	–17.18507	46.85510	–	–	–	EU727493.1
C. leucogaster	FMNH 184901	Antanimbary	–17.18507	46.85510	–	–	–	EU727494.1
C. leucogaster	FMNH 184902	Antanimbary	–17.18507	46.85510	–	–	–	EU727495.1
C. leucogaster	FMNH 184915	Maevatanana	–16.95753	46.82388	–	–	–	EU727496.1
C. leucogaster	FMNH 184916	Maevatanana	–16.95753	46.82388	–	–	–	EU727497.1
C. leucogaster	FMNH 184917	Maevatanana	–16.95753	46.82388	–	–	–	EU727498.1
C. leucogaster	FMNH 184919	Maevatanana	–16.95753	46.82388	–	–	–	EU727499.1
C. leucogaster	FMNH 184920	Maevatanana	–16.95753	46.82388	–	–	–	EU727500.1
C. leucogaster	FMNH 184975	Ankazomborona	–16.11602	46.75667	–	–	–	EU727519.1
C. leucogaster	FMNH 184976	Ankazomborona	–16.11602	46.75667	–	–	–	EU727521.1
C. leucogaster	FMNH 184977	Ankazomborona	–16.11602	46.75667	–	–	–	EU727520.1
C. leucogaster	FMNH 184978	Ankazomborona	–16.11602	46.75667	–	–	–	EU727522.1
C. leucogaster	FMNH 184979	Ankazomborona	–16.11602	46.75667	–	–	–	EU727523.1
C. leucogaster	FMNH 184604	Mahajanga	–15.71297	46.31253	–	–	–	EU727484.1
C. leucogaster	FMNH 184605	Mahajanga	–15.71297	46.31253	–	–	–	EU727485.1
C. leucogaster	FMNH 184606	Mahajanga	–15.71297	46.31253	–	–	–	EU727486.1

APPENDIX *Continued*

Name	Museum number	Locality in Madagascar	Latitude	Longitude	Hap Cyt *b*	Hap Control region	GenBank no. cytochrome *b*	Genbank no. control region
C. leucogaster	FMNH 184607	Mahajanga	–15.71297	46.31253	–	–	–	EU727487.1
C. leucogaster	FMNH 184608	Mahajanga	–15.71297	46.31253	–	–	–	EU727488.1
C. leucogaster	FMNH 184973	Ankijibe	–16.41345	46.76460	–	–	–	EU727517.1
C. leucogaster	FMNH 184974	Ankijibe	–16.41345	46.76460	–	–	–	EU727518.1
C. leucogaster	FMNH 185020	Berivotra	–15.90408	46.59788	–	–	–	EU727524.1
C. leucogaster	FMNH 185021	Berivotra	–15.90408	46.59788	–	–	–	EU727525.1
C. leucogaster	FMNH 185022	Berivotra	–15.90408	46.59788	–	–	–	EU727526.1
C. leucogaster	FMNH 185027	Berivotra	–15.90408	46.59788	–	–	–	EU727527.1
C. leucogaster	FMNH 185028	Berivotra	–15.90408	46.59788	–	–	–	EU727528.1
C. leucogaster	FMNH 185029	Berivotra	–15.90408	46.59788	–	–	–	EU727529.1
C. leucogaster	FMNH 185030	Berivotra	–15.90408	46.59788	–	–	–	EU727530.1
C. leucogaster	FMNH 184259	Sakaraha	–22.90910	44.52623	–	–	–	EU727532.1
C. leucogaster	FMNH 184263	Sakaraha	–22.90910	44.52623	–	–	–	EU727531.1
C. leucogaster	FMNH 184264	Sakaraha	–22.90910	44.52623	–	–	–	EU727461.1
C. leucogaster	FMNH 184237	Toliara	–23.39507	43.72032	–	–	–	EU727470.1
C. leucogaster	FMNH 184238	Toliara	–23.39507	43.72032	–	–	–	EU727462.1
C. leucogaster	FMNH 184239	Toliara	–23.39507	43.72032	–	–	–	EU727471.1
C. leucogaster	FMNH 184240	Toliara	–23.39507	43.72032	–	–	–	EU727483.1
C. leucogaster	FMNH 188497	Dzamandzar	–13.35158	48.18845	–	–	–	EU727474.1
C. leucogaster	FMNH 188498	Dzamandzar	–13.35158	48.18845	–	–	–	EU727475.1
C. leucogaster	FMNH 188499	Dzamandzar	–13.35158	48.18845	–	–	–	EU727476.1
C. leucogaster	FMNH 188500	Dzamandzar	–13.35158	48.18845	–	–	–	EU727477.1
C. leucogaster	FMNH 187750	near Hell–ville	13.40513	48.30335	–	–	–	EU727463.1
C. leucogaster	FMNH 187751	near Hell–ville	13.40513	48.30335	–	–	–	EU727464.1
C. leucogaster	FMNH 188640	Nosy Komba	–13.44270	48.34790	–	–	–	EU727478.1
C. leucogaster	FMNH 188641	Nosy Komba	–13.44270	48.34790	–	–	–	EU727479.1
C. leucogaster	FMNH 188642	Nosy Komba	–13.44270	48.34790	–	–	–	EU727480.1
C. leucogaster	FMNH 188643	Nosy Komba	–13.44270	48.34790	–	–	–	EU727481.1
C. leucogaster	FMNH 188644	Nosy Komba	–13.44270	48.34790	–	–	–	EU727482.1
C. leucogaster	FMNH 188495	Hell – ville	–13.40513	48.30335	–	–	–	EU727472.1
C. leucogaster	FMNH 188496	Hell – ville	–13.40513	48.30335	–	–	–	EU727473.1
C. leucogaster	FMNH 187752	Ambatozazavy	–13.36687	48.31545	–	–	–	EU727465.1
C. leucogaster	FMNH 187753	Ambatozazavy	–13.40513	48.30335	–	–	–	EU727466.1
C. leucogaster	FMNH 187754	Ambatozazavy	–13.40513	48.30335	–	–	–	EU727467.1
C. leucogaster	FMNH 187755	Ambatozazavy	–13.40513	48.30335	–	–	–	EU727468.1
C. leucogaster	FMNH 187756	Ambatozazavy	–13.40513	48.30335	–	–	–	EU727469.1
C. leucogaster	FMNH 185228	Manakara	–22.15697	48.01682	–	–	–	EU727501.1
C. leucogaster	FMNH 176332	Ambilobe	–13.19167	49.05833	–	–	–	–
C. leucogaster	FMNH 179381	Betsiaka	–13.15702	49.23650	–	–	–	–
C. leucogaster	FMNH 169671	Antsalova	–18.30667	44.73950	–	–	–	–
C. leucogaster	FMNH 175889	Katsepy	–15.76342	46.24492	–	–	–	–
C. leucogaster	FMNH 184476	Andranovory	–23.14135	44.14615	–	–	–	–
C. leucogaster	FMNH 176130	Belo sur Mer	–20.73565	44.00443	–	–	–	–
C. leucogaster	FMNH 151946	Forêt de Zombitse	–22.81783	44.73350	–	–	–	–
C. leucogaster	FMNH 176111	Kirindy – Mite	–20.88667	44.08000	–	–	–	–
C. leucogaster	FMNH 176175	Morombe	–21.74028	43.37222	–	–	–	–
Out-groups (Fig. 2)								
C. leucogaster	–	–	–	–	–	–	EU716039.1	–
C. leucogaster	–	–	–	–	–	–	EU716013.1	–
C. leucogaster	–	–	–	–	–	–	EU716023.1	–
C. pusillus	–	–	–	–	–	–	GQ489152	–
Mops condylurus	–	–	–	–	–	–	EF474030.1	–
Mops midas	–	–	–	–	–	–	EF474048.1	–
Mops leucostigma	–	–	–	–	–	–	FJ546257.1	–