PATTERNS OF GENETIC VARIATION IN *MOPS LEUCOSTIGMA* (MOLOSSIDAE) FROM MADAGASCAR AND THE COMOROS

By

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ABSTRACT

The synanthropic molossid bat, *Mops leucostigma* (Allen 1918), is widely distributed across Madagascar and has recently been described from the Comoros. *M. leucostigma* individuals from eastern Malagasy populations are markedly larger than those from the west, and *Mops leucostigma* populations from Madagascar are morphologically distinct from populations of its putative sister species, *Mops condylurus* from mainland Africa (Ratrimomanarivo *et al.* in press, a).

Genetic diversity was assessed by sequencing the mitochondrial cytochrome *b* (n = 56) and displacement loop (D-loop) (n = 64) regions of *Mops leucostigma* individuals from a broad range of locations across Madagascar, and Mohéli and Anjouan in the Comoros. Specimens of *Mops condylurus* (n =3), *Mops midas* (n =3) and *Otomops martiensseni* (n = 1) were included in the study for comparative purposes as outgroups. Phenetic and cladistic analysis of cytochrome *b* and D-loop sequences strongly supported the reciprocally-monophyletic status of *Mops condylurus* and *M. leucostigma*. Comorian (Mohéli and Anjouan) and Malagasy *M. leucostigma* samples formed a monophyletic *Mops leucostigma* group, within which Comorian samples formed a poorly-supported subclade in the cytochrome *b* analysis only. Cytochrome *b* genetic distances of 13.8 % separated *M. midas* from *M. condylurus* and *M. leucostigma*, which formed reciprocally-monophyletic sister groups separated by genetic distances of 2.5 % for cytochrome *b* and 13 % for the D-loop. 49 *M. leucostigma* cytochrome *b* sequences yielded seven haplotypes, two of which were exclusive to the Comoros. D-loop haplotype analysis did not support the distinctiveness of the Comorian samples. Genetic distances within *M. leucostigma* samples were low (0.22 % for cytochrome *b* and 1.91 % for the D-loop). Comorian samples were found to be genetically attributable to *M. leucostigma*. Clear phylogenetic separation between *M. condylurus* and *M. leucostigma* was found in all analyses, consistent with their status as phylogenetic species within the genus *Mops*. There was no clear correlation between haplotype distribution and aspect (east/west-facing slopes), elevation or gender. Low mtDNA variation (cytochrome *b* and D-loop) and lack of phylogeographic concordance indicates that the observed morphometric variation between eastern and western *Mops leucostigma* populations may possibly be explained in terms of adaptation to local environmental conditions.

PREFACE

The experimental work described in this thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, from January 2006 to December 2008, under the supervision of Dr. Jennifer Lamb.

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

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I, Nikhat Hoosen, declare that:

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DECLARATION 2 – PUBLICATIONS

Ratrimomanarivo, F.H., Goodman, S.M., Hoosen, N., Taylor, P. J. and Lamb, J. (in press, a) Morphological and molecular variation in *Mops leucostigma* (Chiroptera: Molossidae) of Madagascar and the Comoros: phylogeny, phylogeography and geographic variation. *Mitteilungen aus dem Hamburgischen Zoologischen Museum*.

Ratrimomanarivo, F. H. and Goodman, S.M.: Field inventories, sample collection and morphological analysis

Taylor, P.J.: Ecological and morphological analysis

Lamb, J. and Hoosen, N.: Molecular laboratory work and analysis

Signed:…….

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Firstly I would like to acknowledge my mother, the biggest support system in my life, who helped keep me going when the going got really tough and prop me up when I felt hope was nowhere in sight. I wish to next extend my sincerest thanks to my supervisor, Dr. Jennifer Lamb, and co supervisor Dr. Peter J. Taylor, who have both worked so hard and put in so much time and effort to guide, assist and support me with such patience. I would also like to thank all my friends without whom the many frustrating times during this year would have been unbearable. Thank you to all of you.

ABBREVIATIONS

µl - microlitre

D-loop - displacement loop

g - grams

Iss – index of substitution saturation

Iss.c.Sym - critical value of index of substitution saturation

m - meter

ML - Maximum Likelihood

mm - millimetre

MP - Maximum Parsimony

mtDNA - mitochondrial DNA

ncDNA - nuclear DNA

ng ml^{-1} - nanogram per millilitre

ng ul^{-1} - nanogram per microlitre

NJ – Neighbour Joining

pp – posterior probability

1 INTRODUCTION

Bats (Chiroptera) are the second-most diverse mammalian order, occurring globally in all major unglaciated landmasses except for a few extremely isolated oceanic islands. As the only mammals capable of true powered flight, their capacity for long-distance dispersal is a key factor in their global distribution, making them of particular interest to evolutionary biologists (Garbutt 1999, Ditchfield 2000, Burland and Wilmer 2001, Myers 2001, Mickleburgh *et al.* 2002, Aspetsberger *et al.* 2003, Fenton 2003, Walker and Molur 2003, Stadelmann *et al.* 2004a, Carvajal and Adler 2005, Eick *et al.* 2005, Proches 2005, Jones and Teeling 2006, Moreira and Morielle-Versute 2006). Due to their capacity for flight, phylogeographic and population genetic variation may be strikingly different between this group and other small, flightless mammals (Ditchfield 2000, Miller-Butterworth *et al.* 2003, Moreira and Morielle-Versute 2006).

Madagascar, the world's fourth largest island, measuring 1570 km from north to south and at its widest point, about 560 km from east to west (Grubb 2003), is situated ~ 400 km to the east of mainland Africa at the narrowest point of the Mozambique Channel (Tveter 2002, Yoder *et al*. 2005, Rabinowitz and Woods 2006). It has been described by Hutcheon (1994) as a zoogeographical composite, its diverse faunal groups existing in an environment that can only be described as unique. Due to its isolation from Africa for nearly 160 million years, and other significant landmasses, such as India, for 88 million years, (Yoder *et al.* 2005) species on the island have evolved separately (Tveter 2002). Consequently Madagascan faunal diversity is extraordinary, and unparalleled levels of taxonomic endemism exist (Garbutt 1999, Yoder *et al*. 2000, Tveter 2002, Goodman and Benstead 2005, Teeling *et al.* 2005, Yoder *et al.* 2005). According to Goodman *et al*. (2005), all 101 native Malagasy land mammal species are endemic to the island. This has significant implications for the implementation of conservation strategies, especially in the face of human-mediated destruction through deforestation (Agarwal *et al.* 2005, Goodman *et al.* 2005). Patterns of micro-endemism further complicate the threat to these species (Goodman *et al.* 2005). The evolutionary history of the country is, surprisingly, still not well understood (Hutcheon 1994, Russ *et al.* 1998, Yoder *et al*. 2005), and several of the most well-known floral and faunal species are listed as globally threatened by the International Union for the Conservation of Nature (IUCN) (Agarwal *et al.* 2005).

Conservation biologists have recently taken steps to ensure the survival of Malagasy mammals by initiating studies on their distribution, status and natural history (Goodman *et al.* 2005). Although poorly understood and an important part of Malagasy fauna, bats (particularly microchiropterans – now classified as Vespertilioniformes, see 1.1.3 for further details) received little consideration until about a decade ago (Russ *et al*. 1998, Garbutt 1999, Goodman and Cardiff 2004, Goodman *et al.* 2005, Goodman *et al.* 2007a,b). Consequently taxonomic and biogeographic information has been lacking (Hutcheon 1994, Russ *et al*. 1998, Goodman and Cardiff 2004, Goodman *et al.* 2005). Without a functioning understanding of the taxonomy, distribution and habitats of particular species it is not feasible to develop conservation strategies and select groups which require protection to safeguard the diversity of gene pools and genetic diversity (Russ *et al.* 1998). Additionally, insufficient molecular datum exists to clarify existing uncertain taxonomic relationships (Russ *et al.* 1998). This has led to the classification of families, genera and species becoming a subject of conjecture among authorities (Russ *et al*. 1998, Garbutt 1999, Goodman *et al*. 2005).

1.1 Study species *Mops leucostigma*

Mops leucostigma is a hitherto poorly studied insectivorous molossid bat, previously considered endemic to Madagascar. The species has been listed by the IUCN as data-deficient (Hilton-Taylor 2000, IUCN 2007), indicating that not enough information exists to make an assessment on the species' extinction risk. However recently this has been changed to Least Concern (Andriafidison *et al.* 2008). Recent field inventories uncovered the existence of this species in the Comoros, which revokes its previous status as a Malagasy-endemic (Racey *et. al.* in press).

1.1.1 *Description*

Mops leucostigma, commonly known as the Madagascan white-bellied free-tailed bat is a medium-sized, heavy-bodied species closely related to *Mops condylurus* (Russ 2001, Simmons 2005, Wilson and Reeder 2005, African Chiroptera Report 2006). It weighs approximately 35 g, has an estimated total body length of 110 - 120 mm and a forearm length of 41 - 47 mm (Peterson *et al*. 1995, Russ *et al*. 1998, Garbutt 1999). The pelage is short and variable in colour, generally greyish brown to brownish and grizzled, sometimes red on top and more or less white below. The ears join at a short tuft of hair in both sexes and the species has a relatively small tragus and large anti-tragus. The skull is heavy, with a strong sagittal crest.

There is no indentation in the palate. The anterior premolar is small, absent in adults and situated outside the dental row (Peterson *et al.* 1995, Russ *et al*. 1998).

Ratrimomanarivo *et al.* (in press, a) showed that populations of *M. leucostigma* from eastern and western slopes of the central highlands in Madagascar differed substantially in external morphology and craniodental traits, with eastern populations being larger than those from the west. Populations from the Comoros were morphometrically more similar to western than eastern populations.

Variation in pelage polychromatism was also observed between eastern and western populations of *Mops leucostigma*. In the east, some individuals possessed a greyish-brown or brown dorsum and a white venter, while in others the dorsum was slightly red to rusty, and the venter creamy beige. Western *M. leucostigma* populations did not exhibit this type of variation. Ratrimomanarivo *et al.* (in press, a) found no prominent variation in the soft parts of the head, except that some individuals from the east possessed a slightly rounded muzzle, which was relatively large at the base, whilst those from the west had a slightly pointed muzzle which was narrower at the base.

The genetic data obtained in this study will be used to test the hypothesis, based on morphological findings, that eastern and western Malagasy populations are distinct and that Comoros populations vary from Malagasy populations although they are more closely affiliated to western Madagascan populations.

1.1.2 *Distribution, behaviour and ecology*

Mops leucostigma has a wide distribution, and has been documented in all areas of Madagascar except the extreme south (Peterson *et al*. 1995, Russ *et al.* 1998, Garbutt 1999). It is one of the most common synanthropic Malagasy bat species, occupying a variety of elevations and bioclimatic zones, from humid to comparatively dry (Russ *et al.* 2001, Goodman and Cardiff 2004, Goodman *et al*. 2005, Andrianarivelo *et al*. 2006, Andriafidison *et al.* 2006, Rakotonandrasana and Goodman 2007). The behaviour and ecology of this species still remain unknown (Garbutt 1999). *Mops leucostigma* shows a preference for high temperatures. Its original habitat was likely to have been in trees rather than in caves (Peterson *et al*. 2005). Goodman *et al.* (2005) observed that many bat species occurring in the drier western region of Madagascar (including *M. leucostigma*) did not rely on forest cover for roosting and foraging.

The species is recorded as occurring in public buildings and especially houses, and is considered largely synanthropic, forming colonies comprising several hundred individuals (Eger and Mitchell 2003, Goodman and Cardiff 2004, Goodman *et al.* 2005, Andrianarivelo *et al*. 2006, Randrianandrianina *et al.* 2006, Rakotonandrasana and Goodman 2007).

Ratrimomanarivo *et al*. (in press, a) mentioned remarkably few records of the species from natural day roosts; such records included roosts under the leaves of palms and the bark of standing dead trees. According to current information, *Mops leucostigma* has not been definitively found roosting in a cave (see Goodman *et al.* 2005, Table 2, footnote 21). The prevalence of synanthropic roosting sites leads to speculation as to whether the species expanded its geographical range subsequent to human colonization and construction of buildiungs in Madagascar, and why *M. leucostigma* has abandoned natural day roost sites for synanthropic sites.

Day roost sites were found almost exclusively in attics of brick buildings, many of which possessed roofs of metal sheeting and aeration holes in the walls. During the day *M. leucostigma* was most often found roosting within the attic space either in holes within brick walls or hanging from the walls or in narrow spaces between roof beams and metal sheeting. *M*ops *leucostigma* can usually be found co-existing in the same day roost sites as other Molossidae such as *M. midas*, *Chaerephon pumilus*, *C. leucogaster* and *Mormopterus jugularis*, though always somewhat isolated in a different space within the attic (Ratrimomanarivo *et al.*, in press a).

Recent field inventories in the Comoros Islands uncovered the existence of synanthropic populations of bats identifiable as *Mops leucostigma* (Racey *et al.* in press), in contrast to the finding of Louette (2004), who had previously failed to record the presence of *Mops* species in the Comoros. *M. leucostigma* cannot thus be considered a Malagasy endemic, but is rather a regional one. Surprisingly no indication of *Mops* was found in a survey of synanthropic bat species on Mayotte Island (Comoros) by S.M. Goodman in 2007, even though Mayotte is the closest island of the Comoros to Madagascar, and despite the occurrence of *M. leucostigma* on Mohéli and Anjouan. A possible explanation for this absence could be the substantial levels of new construction on Mayotte over the past decades, which have left very few old buildings in existence on the island.

1.1.3 *Taxonomy*

Mops leucostigma is a member of the family Molossidae (free-tailed bats) which was previously placed in suborder Microchiroptera. However molecular evidence supports an alternative classification recognising two suborders, Vespertilioniformes (including Molossidae as well as Emballonuridae, Nycteridae, Noctilionidae, Mormoopidae, Phyllostomidae, Natalidae, Furipteridae, Thyropteridae, Myzopodidae, Vespertilionidae, Mystacinidae and Miniopteridae) and Pteropodiformes (Pteropodidae, Rhinolophidae, Hipposideridae, Megadermatidae, and Rhinopomatidae) (Hutcheon & Kirsch 2004, Hutcheon & Kirsch 2006, Eick *et al.* 2005). Hence *M. leucostigma* is now classified within the suborder Vespertilioniformes.

Mops leucostigma was originally described by Allen (1918) as *Chaerephon leucostigma* and later regarded by Koopman (1994: 236) as a subspecies of the mainland African taxon *Tadarida* (*Mops) condylurus* (Garbutt 1999, African Chiroptera Report 2006).

The species is apart of the genus *Mops* (*Mops* Lesson 1842) which was regarded as a full genus by Freeman (1981), Honacki *et al.* (1982), and Koopman (1984), but only as a subgenus of *Tadarida* by Corbet and Hill (1986), Legendre (1984) and Meester *et al.* (1986). Type species of the genus is *Mops indicus* (Lesson 1842) and synonyms include *Allomops* (Allen 1917), *Philippinopterus* (Taylor, 1934), *Xiphonycteris* (Dollman 1911) (Simmons 2005:441). The genus *Mops* contain two subgenera and 12 species that can be identified according to the key provided by Dunlop (1999) (modified from Corbet and Hill (1992), El-Rayah (1981) Hayman and Hill (1971) and Koopman (1994)). *Mops* is distinguished from *Chaerephon* and *Tadarida* by more developed sagittal and lambdoidal crests in the skull, a generally thicker dentary with a higher coronoid process, the absence of palatal emargination and reduced dentition (Nowak 1994).

Mops leucostigma was considered a valid species by Peterson *et al.* (1995: 152), Russ *et al.* (2001), Hutson *et al.* (2001: 34), Goodman and Cardiff (2004: 227) and Simmons (2005). Inititally it was suggested by Hayman and Hill (1971) that *M. leucostigma* was allied to the southern African *M. niveiventer* (Cabrera and Ruxton 1926). *Mops leucostigma* was also previously considered to be a subspecies of *M. condylurus* (Koopman 1994) or even in some cases a synonym of it (Honacki *et al.* 1982). Although work undertaken by Jones *et al.* (2002) was unable to provide clarity on this phylogenetic relationship, *M. condylurus* (Africa) and *M. leucostigma* (Madagascar) are currently considered sister species based on their biogeographical separation.

1.1.4 A morphological comparison of *Mops leucostigma* and *Mops condylurus*

Chaerephon species possess a tuft of hair on their head, a feature which *Mops leucostigma* also possesses, but which is lacking in its sister species *Mops condylurus* (Peterson *et al.* 1995). This shared morphological feature indicates that *M. leucostigma* may exhibit a closer relationship to *Chaerephon*. Koopman (1966) and Ansell (1967) (in Meester and Setzer 1971) mention that *M. condylurus* shows a degree of reduction of the posterior commissure of M^3 relative to *M. leucostigma*.

Ratrimomanarivo *et al.* (in press, a) reported that both *Mops condylurus* and *M. leucostigma* showed distinct sexual dimorphism in the soft head anatomy of adults. In males, the nostrils tended to be more elongated and flared than in the more blunt-nosed females. The ears of males were joined by a band of skin larger and more inflated than that found in females. Sexual dimorphism in cranial variables was more evident (e.g. pronounced sagittal and occipital crests were found in males) in *M. leucostigma* than in *M. condylurus*, although sample sizes of the latter were distinctly smaller.

Mops leucostigma and *M. condylurus* varied markedly from each other in a variety of external, cranial and dental measurements (Ratrimomanarivo *et al.* in press, a). Notable differences were found in the soft part anatomy. Comparatively, from the dorsal view, the rostrum of the male *M. leucostigma* is considerably blunter with more flaring nostrils than that of the male *M. condylurus*. *M. condylurus* was also found to possess a broader and more elongate fleshy portion between the ears. *Mops leucostigma* males, in profile, were inclined to have a larger antitragus, and slightly broader less-wrinkled lips than male *M. condylurus*. These same general patterns were seen to occur for female *M. leucostigma* and *M. condylurus,* which were liable to be smaller than their male counterparts.

1.2 Conservation

1.2.1 *Genetics and conservation*

Molecular analysis can provide conservationists with precise species genetic parameters, which are crucial for decision-making (DeSalle and Amato 2004). In most situations, molecular genetic evaluations are emphasised and are an essential and important guide to descriptions of biotic diversity, reforming the way in which relationships among species, population structure and individuals are delineated, and updated taxonomies that include input from molecular

genetics should provide a firm foundation for the proper recognition and hence management of biodiversity (Avise 1989, Haig 1998, Burland *et al.* 1999, Zhang *et al.* 2002).

Biodiversity conservation is aimed at maintaining species variety and genetic resources. It may be measured in terms of genetic variation at the intra- and inter-species level. Insufficient data on the distribution of genetic variation in a species can result in loss of genetic diversity. Accurate estimation of biodiversity depends upon the accurate identification of species. It is necessary to use non-morphological criteria, which include molecular genetic techniques, to elucidate the boundaries of cryptic species (Ehrlich and Wilson 1991, Erwin 1991, Savage 1995, Centre for Biodiversity and Conservation Biology 1996, Funk *et al.* 2002, Morrison 2005).

1.2.2 *Conservation of bats*

Bats comprise more than 20 % of all mammal species and have a widespread planetary distribution. Numbers of bat species are usually considered underestimates due to incomplete systematic studies (Mickleburgh *et al.* 2002). One of the main issues in bat conservation is concern for endemic bat species from relatively species-poor areas such as Australia, Madagascar and Japan. A major proportion of the most threatened bats globally occur on islands, where the threat to them is greatest, as species may be subject to different selective pressures, due to their highly restricted ranges, small gene pools and populations sizes and inbreeding leading to fixation of deleterious alleles (Mickleburgh *et al*. 2002, Nielson 2004).

Several of the major threats facing bats come from humans and involve habitat destruction and degradation, roost loss or disturbance, persecution, and exploitation as food (Mickleburgh *et al.* 2002, Goodman 2006). Lack of information and taxonomic uncertainties render population assessments difficult and hence hinder the formation of suitable conservation strategies (Mickleburgh *et al.* 2002, Goodman *et al*. 2005).

1.2.3 *Conservation of Madagascan fauna*

Until about 10 years ago Malagasy bats were largely unstudied (Goodman and Cardiff 2004, Goodman *et al.* 2005, Goodman *et al.* 2007a). Information necessary for suitable management was absent, including taxonomic studies, natural history information, measures of species richness and precise documentation of distributions (Goodman *et al*. 2005), therefore preventing recognition of those species in greatest need of protection.

At the beginning of this study, *Mops leucostigma* has been listed by the IUCN as data deficient (IUCN 2007), indicating that not enough information exists to make an assessment on the species extinction risk. One of the goals of this study is to provide genetic information to complement current morphological studies aimed at revealing if the species is under any threat or requires immediate conservation attention.

Bat surveys were conducted by Goodman *et al.* (2005) over a period of several years in the dry forests of Madagascar, and have helped provide prevalence information on several unknown or poorly known. Twenty-four microchiropteran (Vespertilioniformes) species were recorded during these surveys, including *Mops leucostigma*.

Goodman *et al.* (2005) state that caves are among the most susceptible ecosystems in the world and are not included in standard protected area networks where anthropogenic use of caves, might cause a negative impact on animal populations living there. The authors conclude that the human use of caves poses the greatest threat to the Malagasy bat species in the dry region of the island, which should direct conservationists to protect such sites in order to protect bat species. *Mops leucostigma*, however, has not been definitely found roosting in a cave (Goodman *et al.* 2005).

In south western Madagascar hunting poses a great threat to bats (Goodman 2006). Though the focus of hunting mainly falls on the species *Hipposideros commersoni*, the incidental capture of smaller microchiropterans (Vespertilioniformes) does occur. Goodman (2006) estimates that 70,000 - 140,000 microchiropterans (Vespertilioniformes) may be collected annually in the region, which may threaten the continued existence of local populations.

According to Russ *et al.* (1998), the taxonomy of many microchiropteran (Vespertilioniformes) bats requires revision since the relationship between Malagasy and African species remains vague; they predicted that molecular studies would confirm the existence of additional previously unknown bat species. This has proven to be the case, as recent molecular and morphometric work by Goodman and co-authors has resulted in the description of several new species of endemic bats from Madagascar and other areas, including some which were formerly thought to be conspecific with African species (*Chaerephon jobimena*, Goodman and Cardiff 2004; *Pipistrellus raceyi*, Bates *et al.* 2006; *Emballonura tiavato*, Goodman *et al.* 2006; *Chaerephon pusillus*, Goodman and Ratrimomanarivo 2007; *Myzopoda schliemanni*, Goodman *et al.* 2007a; *Miniopterus sororculus*, Goodman *et al.* 2007b, *Miniopterus petersoni*, Goodman

et al. 2008a; *Mormopterus francoismoutoui*, Goodman *et al.* 2008b). The discovery of new endemic species necessitates consideration of new conservation strategies or reviews of existing ones for these bats.

This study of *Mops leucostigma* is part of larger comparative study of Molossidae from Madagascar and the western Indian Ocean islands.

1.2.4 *Conservation units*

1.2.4.1 Species

Species have been described as the currency of biology (Agapow *et al.* 2004, Sites and Marshall 2004) and are the basic units in biogeography, ecology, evolutionary systematics and conservation biology (Sites and Marshall 2004). However, this 'currency' has always been surrounded by controversy and conflicting concepts, and there is no universally-accepted definition of a species. At least twenty different concepts are currently in use (Mallet 1995, Bradley and Baker 2001, Templeton 2001, Agapow *et al.* 2004, Sites and Marshall 2004, Freeland 2005c). A few of the more commonly used and applicable concepts are discussed below.

The Biological Species Concept (BSC) was, until recently, the most widely accepted of all the species concepts. According to this a species is defined as "a group of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups" (Mayr 1942). One difficulty associated with this concept is that it is not always easy to establish the degree of reproductive isolation of wild populations (Taylor 2004). Another difficulty is that it excludes asexual, parthenogenetic, self-fertilising and habitually-interbreeding forms, as well as hybridizing forms (e.g. in plants) (Donoghue 1985, Mallet 1995, Frankham *et al.* 2002, Agapow *et al* 2004, Taylor 2004, Freeland 2005c). The BSC is difficult to apply to high flying Molossid bats, which are seldom caught in flight.

The Morphological Species Concept (MSC) recognizes species by grouping them on the basis of morphological or phenotypic similarity and classifies species according to the presence or absence of specific characters (Mayr 2000). This method of defining species fails to identify cryptic or sibling species and is difficult to apply in cases of sexual dimorphism (as encountered in *M. leucostigma*). For these reasons it is good practice to complement morphological studies with genetic data (Chambers 1983, Taylor 2004).

According to the Genetic Species Concept (GSC) (Bradley and Baker 2001), measurement of genetic differences is used to infer reproductive isolation and evolutionary independence. In terms of this concept a species is "a group of genetically-compatible interbreeding natural populations that is genetically isolated from other such groups" (Baker and Bradley 2006). The focal point of the GSC in comparison to the BSC lies in the emphasis on genetic isolation rather than reproductive isolation (Baker and Bradley 2006). One of the main criticisms of this approach lies in the uncertainty regarding the magnitude of genetic variation that would be required to differentiate between two supposed species, as well as the inability of the GSC to establish distinct ranges of genetic variation for different taxonomic level (Bradley and Baker 2001). In recent years, the increase in molecular studies, and hence availability of genetic data, has made it possible for this concept to be critically tested. This GSC is particularly applicable to studies such as this one, which make use of DNA sequence data.

The Phylogenetic Species Concept (PSC) defines species as groups of individuals that share at least one uniquely-derived characteristic and is often interpreted to mean that a species is the smallest identifiable monophyletic group of organisms within which there is a shared pattern of ancestry and descent (Cracraft 1983, Freeland 2005c). The PSC is readily applicable to molecular sequence data. Agapow *et al.* (2004) mention that the PSC could possibly reveal morphologically- or genetically-unremarkable but nonetheless significant populations and may be a good indicator of biodiversity and the conservation worth of a population relative to other measures. A problem with the PSC is that subspecies and even individual organisms could be classified as species within this concept, as there is no level that corresponds to a species other than "the smallest aggregate" (Agapow *et al.* 2004). Strict application of this concept could lead to an increase in the number of and a more restricted geographic range of species (Agapow *et al.* 2004, Freeland 2005c).

1.2.4.2 Evolutionary Significant Units

A prominent objective of conservation efforts in recent years has been the protection of genetically differentiated populations within species (Frankham *et al.* 2002, Honjo *et al*. 2004). As mentioned by Green (2005), species ranges are genetically, demographically, spatially and ecologically heterogeneous in ways that current taxonomy may or may not capture. One of the challenges faced by conservation biologists and ecologists is classifying what constitutes the minimal unit of conservation (Vogler and DeSalle 1994). The Evolutionary Significant Unit (ESU) was created to objectively prioritise unambiguous units for conservation below the

taxonomic level of the species (Fraser and Bernatchez 2001, Frankham *et al.* 2002). This approach was developed with the idea that existing taxonomy may not completely reflect underlying genetic diversity (Avise 1989, Fraser and Bernatchez 2001).

The term Evolutionary Significant Unit was coined by Ryder (1986) 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". This classification, based on ecological and genetic data (Bottin *et al.* 2007), was limited in its lack of guidelines for operational applications as well as in finding concordance among different information types (ecological, genetic and physiological) (Fraser and Bernatchez 2001, Young 2001, Green 2005).

Moritz (1994) defined an ESU as a population that is 'reciprocally monophyletic for mtDNA alleles' and 'shows significant divergence of allele frequency at nuclear loci'. This definition takes into consideration the growing availability of molecular data, and leads to criteria based exclusively on molecular phylogenies (Bottin 2007). Vogler and DeSalle (1994) used the PSC to suggest conservation units on the basis of characters that cluster groups, individuals or populations, to the exclusion of other such clusters. The method is stringent, and also applicable to molecular datasets.

Fraser and Bernatchez (2001) defined the ESU under a unified concept of adaptive evolutionary conservation (AEC) as 'a lineage demonstrating highly restricted gene flow from other such lineages within the higher organisational level/lineage of the species'. This definition is general and also compatible with molecular data.

1.2.4.3 Management Units

The Management Unit (MU), intended to be a conservation unit level below the ESU, was proposed by Moritz (1994). An MU is defined as any population that exchanges so few migrants with others as to be genetically distinct from them (Avise 2000) and represents sets of populations that are currently demographically independent (Moritz 1995), and can be identified on the basis of significant differences in allele frequencies at multiple neutral loci (Fraser and Bernatchez 2001, Freeland 2005c).

Preservation of distinctive ESUs and MUs is advantageous as each unit contributes to species' genetic diversity (Freeland 2005c). The molecular findings of this study could therefore

potentially identify ESUs or MUs existing within *Mops leucostigma*, which could in turn assist in conservation, if necessary, of the species and its genetic diversity, by highlighting populations or groups in need of protection. For the purposes of this study the PSC, GSC and AEC will be used to define ESUs, where appropriate.

1.3 Methods and techniques used in the study of genetic diversity

1.3.1 *Molecular markers*

The use of molecular markers in the past decade has transformed the study of species at all levels, from global population structure to within-social-group relationships. These markers include DNA sequences of coding and non-coding regions of nuclear and organellar (mitochondrial and chloroplast) DNA as well as information from a variety of genotyping techniques, including; microsatellites or Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSRs), Amplified Fragment Length Polymorphism (AFLPS), Random Amplified Polymorphic DNA (RAPDs), Variable Number of Tandem Repeats (VNTR fingerprinting), allozymes and Restriction Fragment Length Polymorphism (RFLPs) (Karp *et al.* 1997, Narain 2000, Bornet and Branchard 2001, Burland and Wilmer 2001, Liu and Wendel 2001, Zhang and Hewitt 2003, Aitken *et al.* 2004, Freeland 2005a, Garant and Kruuk 2005, Gort *et al.* 2006, Selkoe and Toonen 2006).

1.3.2 *Mitochondrial versus nuclear DNA*

Mitochondrial DNA (mtDNA) sequences have been a standard molecular marker utilised in reconstruction of species phylogenies of animals and are a natural starting point and a powerful tool in phylogenetic, evolutionary and phylogeographic studies (Gülbitti Onarici and Sümer 2003, Wang *et al*. 2003, Zhang and Hewitt 2003, Ruedi and McCracken 2005, Bensch *et al*. 2006).

The mitochondrial genome of mammals is a double-stranded, circular DNA molecule, 15000 - 17000 base pairs in length, which encodes 37 genes, 22 tRNAS and 2 rRNAs (Ballard and Whitlock 2004). Advantages associated with using mtDNA sequences in phylogenetic studies include the well studied structure and small size of mtDNA (Gülbitti Onarici and Sümer 2003), as well as its existence in many copies within each cell, which allows for easier amplification of target sequences than would be possible if using the single copy nuclear DNA (ncDNA) (Ruedi and McCracken 2005). MtDNA usually does not recombine and is transmitted clonally across

generations. This avoids problems with reticulate evolution (Freeland 2005a,b, Ruedi and McCracken 2005). The arrangement of mitochondrial genes is conserved, but they have a higher overall mutation rate than nuclear genes and coalesce faster (Gülbitti Onarici and Sümer 2003, Ballard and Whitlock 2004, Freeland 2005a, Ruedi and McCracken 2005). The existence of universal primers allows acquisition of mtDNA sequence data from a wide variety of vertebrate and invertebrate species without previous knowledge being required (Freeland 2005a, Ruedi and McCracken 2005).

Use of mtDNA sequences is not, however without disadvantages. MtDNA is maternally-inherited, rather than biparentally, as in the case of ncDNA (Castella *et al*. 2001, Frankham *et al.* 2002, Gülbitti Onarici and Sümer 2003, Zhang and Hewitt 2003, Aitken *et al.* 2004, Freeland 2005a, Ruedi and McCracken 2005, Bensch *et al.* 2006) and hence results in species and population inferences being biased (Zhang and Hewitt 2003). Effective population size of mtDNA is a quarter of that of nuclear autosomal sequences, and mtDNA lineages will consequently possess a faster lineage-sorting rate and a high allele extinction rate, which can be advantages for phylogenetic analysis (Zhang and Hewitt 2003, Freeland 2005a). Additionally, mtDNA fragments can be copied into the nuclear genome and can form unexpressed pseudogenes which are not homologous to the true target gene, potentially confounding analyses if nuclear copies of mtDNA are amplified (Zhang and Hewitt 2003, Ruedi and McCracken 2005).

Mitochondrial DNA markers, as with all DNA markers, are used to reveal the genetic structure, evolutionary history and evolutionary potential of populations, but inferences are actually based on the structure and history of the specific gene or DNA region used, resulting in phylogenies which are gene - rather than species - trees (Zhang and Hewitt 2003, Ruedi and McCracken 2005).

Limitations associated with mtDNA analysis can be circumvented by combining it with analysis of ncDNA data, which is biparentally-inherited, and so allows for exploration of male gene flow and mating systems and presents a potential source of multiple independent gene lineages (Bradley and Baker 2001, Ruedi and McCracken 2005). The nuclear genome, however, shows lower variability because mutation rates are often an order of magnitude less than is usual for mtDNA and ncDNA lineages are also four times larger than mtDNA lineages, thus phylogeographic structure is expected to evolve more slowly in ncDNA markers (Ruedi and McCracken 2005).

Nuclear DNA polymorphisms existing in eukaryotic organisms provide practically limitless opportunities for the study of evolutionary mechanisms, but ncDNA markers present challenges at almost every stage of study, such as recombination, selection (non-neutrality), heterozygosity (heterozygous individuals have two different alleles and separation of these when using ncDNA is a big technical challenge), insertion/deletion polymorphisms, low divergence and polytomy. Further problems with ncDNA include recombination among markers, which may result in independent histories for sequences within nuclear haplotypes (Zhang and Hewitt 2003).

Thus both mtDNA and ncDNA have their associated limitations and advantages. It is expected that mtDNA will continue to be the first marker considered in genealogical, evolutionary and phylogeographic studies for animals, as its high intragenomic variability in evolutionary rates and its structural conservatism provide informative phylogenetic analyses and historical perspectives (Wang *at al.* 2003). It is also expected that ncDNA will be increasingly used to provide a broader understanding of past evolutionary processes (Ditchfield 2000, Zhang and Hewitt 2003, Ruedi and McCracken 2005, Bensch *et al*. 2006).

1.3.3 *Coding versus non-coding DNA*

Coding regions, which code for specific proteins and are therefore subject to selective and functional constraints, are relatively conservative in an evolutionary sense. Non-coding regions, on the other hand, often have no known function and tend to be selectively neutral and faster-evolving owing to their lack of functional constraints.

The cytochrome *b* coding region is perhaps the most extensively sequenced region to date within vertebrates (John and Avise 1998, Martin *et al.* 2000). As a result a large data set of cytochrome *b* sequences from a wide range of taxa, including many bat species, is easily available from the GenBank database. Access to this large reference database allows access to cytochrome *b* genes for comparative purposes, on which evolutionary biology depends, and as an aid in species identification (John and Avise 1998, Ditchfield 2000, Bradley and Baker 2001, Ruedi and McCracken 2005).

The levels of genetic divergence usually associated with sister species, congeners and confamilial genera are in a range in which the cytochrome *b* region is phylogenetically informative and unlikely to be severely compromised by saturation effects involving nucleotide substitutions at the same site (John and Avise 1998). Many phylogeographic studies of bats

have utilised the cytochrome *b* gene, since this coding gene presents a combination of fast-evolving nucleotide positions (synonymous third-positions) and more conserved first- and second-codon positions.

The displacement loop (D-loop) region of the mitochondrial DNA is a variable, rapidly evolving, non-coding region (part of the control region), which evolves at an estimated 6 - 25 % per million years (Petit *et al.* 1999) and is therefore useful for inferring processes at the intraspecific level (Ruedi and McCracken 2005). The D-loop region thus accumulates mutations more rapidly than the cytochrome *b* region and may potentially detect finer regional variation than that presented by cytochrome *b* (Ditchfield 2000). However, in comparative studies, the D-loop sometimes proves problematic, as it is difficult to align over highly divergent taxa (Kjer and Honeycutt 2007).

In any study involving DNA and genomes, the molecular markers chosen should preferably be accessible, reliable, easy to use and cost-effective, as is the case with the cytochrome *b* and D-loop regions (Schaal *et al*. 1998, Ditchfield 2000). The inferred relationships among populations due to sequence analysis are deemed stable when the cytochrome *b* and D-loop sequences correspond with each other (Martin *et al*. 2000). Therefore the two regions selected for this initial study of genetic variation in *M. leucostigma* are the mitochondrial cytochrome *b* and D-loop regions, as both regions provide standards for genetic comparisons in the construction of mammalian phylogenies (Lamb 2005).

1.4 Phylogeography

Phylogeography was a term introduced 20 years ago by Avise *et al.* (1987) and defined as "a field of study concerned with the principles and processes governing the geographical distribution of genealogical lineages, especially those within and among closely related species" (Avise 2000).

Avise (2000) assigned phylogeographic patterns into five main categories. Category I include species with deep gene trees, where major haplotypes are located in separate geographic areas. *Miniopterus schreibersii natalensis* was found to fit this category (Miller-Butterworth *et al.* 2003), which was unexpected in a bat capable of extensive migration. Category II is characterised by deep gene trees, in which major lineages co-exist in the same local population. Two kinds of population exhibiting this pattern are large ones with recently reduced numbers,

where intermediate lineages are lost through genetic drift, and those where hybridisation results in introgression of foreign lineages.

Shallow gene trees where closely related lineages are found in allopatry are characteristic of Category III, and involve populations that have recently expanded from a mutual area of origin or source area and have low ensuing gene flow. Bat populations, where female philopatric behaviour prevents haplotype emigration and where male-mediated gene flow links populations, display this pattern.

Category IV refers to shallow gene trees where closely related lineages are found in sympatry and is a general pattern in local populations connected by high gene flow levels or those that have lately colonised new areas. A haplotype network of populations that fit into this category would usually display a 'star-like' relationship of haplotypes with a common (ancestral) haplotype at the centre, and uncommon variants radiating from it. *M. myotis,* which is found across Europe, exhibits this type of phylogeographic pattern (Ruedi and Castella 2003), as does *C. pumilus* (Taylor *et al.* in press).

Category V is intermediate between categories III and IV, where there is a mixture of prevalent, often ancestral haplotypes coexisting with infrequent localised variants, or private alleles. This phylogeographic pattern is shown by the bat *Nyctalus noctula* (Petit *et al.* 1999).

Ruedi and McCracken (2005) added the dimension of phenotypic divergence to these five classical categories, and included characters such as morphological behaviour and echolocation variation.

1.5 Applications of mitochondrial DNA sequence data to population genetics and phylogeography of bats

Numerous studies on mitochondrial DNA variation on bat species have been undertaken in recent years. The last 10 years of the twentieth century has seen significant impact of population-genetic studies on our understanding of evolutionary processes and population and species history (Zhang and Hewitt 2003). This includes studies carried out on island bats to assess inter- and intraspecies diversity and relationships, as is the case in this study. Phylogenetic studies of bats have used the mitochondrial cytochrome *b* gene solely, or in conjunction with other mitochondrial regions such as the D-loop, NADH dehydrogenase gene 1

(ND1), ND2, cytochrome *c* oxidase subunit I, ribosomal 12S RNA, 16S RNA, $tRNA^{val}$ and/or nuclear DNA, such as the recombination activating gene 2 (*Rag2*). Mitochondrial data are frequently combined with morphological, echolocation, migratory, behavioural and geographic data to address specific questions, including, intra- and interspecies genetic diversity, lineages, species origins, population structure, diversification patterns, migration patterns, male and female patterns of dispersal and the existence of cryptic species.

Mitochondrial DNA sequences have been used to investigate molecular and phylogenetic relationships within and between species, to resolve taxonomies and uncover new species (*Glossophaga,* Hoffman and Baker 2001; *Plecotus*, Spitzenberger *et al.* 2001, Kiefer *et al.* 2002 and Juste *et al.* 2003; East Asian *Myotis,* Kawai *et al.* 2003; Hipposideridae and Rhinolophidae, Wang *et al*. 2003; African subgenus *Cistugo*, Bickham *et al.* 2004; *Artibeus*, Lim *et al.* 2004a; *Balantiopteryx*, Lim *et al.* 2004b; *Myotis vivesi*, Stadelmann *et al.* 2004a; *Myotis*, Stadelmann *et al.* 2004b; *Miniopterus*, Appleton *et al.* 2004,Goodman *et al.* 2007b, 2008; *Emballonura*, Goodman *et al.* 2006; *M. d. daubentonii* and *M. d. nathalinae*, Simões *et al.* 2007).

Mitochondrial DNA analysis can also be used to investigate dispersal patterns. A single colonisation event of an island by a mainland species was considered the conventional scenario for bats (Russell *et al.* 2008). Dávalos (2007) recommends revision of the usual model of one-way colonisation from continent to island, as well as of the ecological hypothesis intended to explain it, as more phylogenies discover multiple one-way dispersals and/or two-way invasions at variance with biogeographic convention (e.g. between Madagascar and Africa, Raxworthy *et al.* 2002, Russell *et al*. 2007 and 2008; between Polynesia and New Guinea, Filardi and Moyle 2005; two-way invasions among Caribbean bats, Dávalos 2007). Molecular mtDNA data have further been utilised to examine male and female migration patterns and their influence on population genetic structure (*M. myotis*, Castella *et al*. 2001; *Miniopterus schreibersii natalensis*, Miller-Butterworth *et al.* 2003; *Tadarida brasiliensis mexicana*, Russell *et al.* 2005). These studies are relevant to consideration of the patterns of dispersal between African *M. condylurus* and Madagascan and Comorian *M. leucostigma*.

Understanding of species distribution in archipelagos can be clarified by integration of geological history, evolutionary genetics and population genetics, as island size and geographical position are not necessarily constant over geological time (Pulvers and Colgan 2007). Conservation efforts and studies (Vespertilionidae from the Canary Islands, Pestano *et al.* 2003; endemic *Nyctalus azoreum* from the Azores, Salgueiro *et al.* 2004; endemic

Haplonycteris fischeri from the Philippines, Roberts 2006b; *Melonycteris* in the Bismarck Archipelago and Solomon Islands, Pulvers and Colgan 2007) have often focused on speciation processes on islands where endemic species are most vulnerable to extinction (Salgueiro *et al.* 2004).

Morphologically-based taxonomy is often not sufficient to recognize and delineate species, as it is unable to detect, for example, cryptic species. In recent years the number of cryptic species revealed by molecular DNA studies has steadily increased (Bickford *et al.* 2006, Beheregaray and Caccone 2007, Pfenninger and Schwenk 2007). Molecular techniques involving the use of mtDNA sequencing allow for detection and differentiation of morphologically similar species (Bickford *et al*. 2006). Jacobs *et al.* (2004) examined cytochrome *b* sequence variation in the widespread light and dark forms of southern African *Chaerephon pumilus* which also showed variation in diet and echolocation frequency, and found that the two forms were not distinct species as they were separated by only 0.9 % sequence divergence. Jacobs *et al.* (2006) investigated the possibility of a cryptic species of *Scotophilus dingani* by examining genetic, morphological and echolocation data on two yellow-bellied forms with different peak echolocation frequencies. The two phonic types were reciprocally-monophyletic, suggesting the possibility of their being sibling species. Two morphologically similar but phonically different types of the common pipistrelle were also shown through genetic analyses to belong to two distinct species (Hulva *et al*. 2004). Multidisciplinary approaches, incorporating both phenotypic and genetic aspects, are consequently key if biodiversity and species boundaries are to be characterized correctly.

Both morphological and genetic analysis of the cytochrome *b* region of *Miniopterus fraterculus*, which occupies divergent habitats in Madagascar and southern Africa, indicated that the Malagasy form was an unrecognised endemic species, referred to *M. sororculus* (Goodman *et al*. 2007b). A similar study of *M. sororculus* then led to the description of a new species of Madagascan *Miniopterus*, *M. petersoni* (Goodman *et al.* 2008a). Goodman *et al.* (2006) also described a new Malagasy species of *Emballonura*, *E. tiavato*, based on both morphological and genetic characters.

Studies incorporating both nuclear and mtDNA for estimates of population structure allow for comparison of both paternal and maternal lineages within species, as opposed to just the maternal lineage, as would be the case if only mtDNA were studied. Such studies may provide information on dispersal patterns, female philopatry (females faithful to their roosts), species

points of origin and colonisation patterns. Several studies utilised both of these markers in particular the cytochrome *b* and *Rag 2* genes (*Myotis myotis*, Castella *et al.* 2001; within Mormoopidae, Lewis-Oritt *et al.* 2001, Van den Bussche and Weyandt 2003; Natalidae, Dávalos 2005; *Corynorhinus*, Piaggio and Perkins 2005; Mormoopidae, Dávalos 2006; *Cynopterus brachyotis* and *Thoopterus nigrescens*, Campbell *et al*. 2007).

Mitochondrial DNA has been the marker of choice in most animal phylogeographic studies (Bermingham and Moritz 1998, Templeton 1998, Zhang and Hewitt 2003, Ballard and Whitlock 2004, Ruedi and McCracken 2005, Soltis *et al.* 2006). Several studies in recent years have incorporated a phylogeographic approach to phylogenetic analysis (*Myotis myotis*, Castella *et al.* 2000, 2001, Ruedi and Castella 2003; *Miniopterus schreibersii natalensis*, Miller-Butterworth *et al.* 2003; West Palaearctic *Plecotus*, Juste *et al.* 2004; the Azorean bat *Nyctalus azoreum*, Salgueiro *et al.* 2004; *Tadarida brasiliensis mexicana*, Russell *et al.* 2005; *Ptenochirus jagori, Cynopterus brachyotis* and *Macroglossus minimus*, Roberts 2006a). The rapid accumulation of phylogeographic data from mtDNA studies has allowed for a comparative approach to phylogeography facilitating the study of phylogeographic structure of multiple co-distributed species in a common geographic area. The comparative approach can help to assess historical geographic events and distribution which can assist in understanding the evolution of populations and species and also to detect common threats in their history which may be significant for conservation biology (Pestano *et al.* 2003, Stadelmann *et al*. 2004a, Freeland 2005a, Soltis *et al.* 2006).

As can be seen from the above studies, molecular analysis of the mitochondrial region of *Mops leucostigma* has the potential to provide information on this previously-unstudied (genetically) species from both Madagascar and the Comoros. A combination of morphological and genetic data is likely to prove more useful and accurate in species identification. This molecular analysis will complement the morphological analyses undertaken by Ratrimomanarivo *et al*. (in press, a).

1.6 Approaches to phylogenetic tree construction

Methods for constructing phylogenetic trees from molecular data may utilise either discrete character states, whilst phenetic trees may be calculated from a distance matrix of pairwise dissimilarities. Further an algorithm may be used to construct a single tree from the data, or a tree-searching approach that constructs several trees and then decides on the best tree or set of

trees based on some specified criterion (Vandamme 2003). The majority of distance matrix methods utilise stepwise-clustering approaches, while most character-state methods utilise the exhaustive search methods to compute the 'best' tree. Algorithmic and distance matrix (stepwise clustering) methods include neighbour joining (NJ), UPGMA and the Fitch-Margoliash method, character state methods include maximum parsimony (MP), maximum likelihood (ML) and Bayesian Analysis (Bollback 2002,Vandamme 2003, Van de Peer 2003).

1.6.1 *Phylogenetic /cladistic analysis*

Parsimony analysis works on the principle that taxa that share a common character do so because that character is inherited from a common ancestor. Inconsistencies are explained by reversal, parallelism, or convergence. These explanations, collectively termed homoplasy, are considered as "extra steps or hypotheses that are required to explain data". Maximum parsimony (MP) searches for the tree/trees with the minimum number of changes, and is based on the premise that the most likely tree is the one that requires the fewest number of mutations, to explain the data in the alignment. Often there are several equally parsimonious trees, that differ only slightly from one another, and are consistent with the same number of events, hence no single tree can be inferred (Steel and Penny 2000, Holder and Lewis 2003, Vandamme 2003).

Parsimony may not be efficient in reconstructing relationships between sequences that are evolving rapidly or have been separated for a long time, as it does not correct for multiple mutational events at the same site (Holder and Lewis 2003). In such cases it is appropriate to use methods that allow the implementation of models which correct for multiple substitutions at the same site. These include maximum likelihood and Bayesian likelihood methods. Maximum likelihood infers a phylogenetic tree by finding a single tree that exhibits the highest probability (likelihood) of producing the observed sequences and measures how well the observed data corresponds with the prediction reached by the model and tree hypothesis (Steel and Penny 2000, Hall 2001, Holder and Lewis 2003). A disadvantage of the maximum likelihood method is that it is computationally demanding and may require large amounts of computer time.

Bayesian inference of phylogeny generates an approximation of the posterior probability distribution of parameters such as branch lengths, tree topology and substitution model parameter estimates. It uses numerical integration methods such as the Markov Chain Monte Carlo (MCMC) and works on the premise that the optimal phylogenetic hypothesis is the one that maximizes the posterior probability (Holder and Lewis 2003). Confidence in the

phylogenetic tree structure is given in the form of posterior probabilities, as the frequency of a given clade is virtually identical to the probability of that clade, therefore bootstrapping is not required to assess the confidence of the tree. Bayesian likelihood analysis is less computer-intensive than maximum likelihood and can be performed in a lesser amount of time. For this reason it was used in this study in preference to maximum likelihood (Hall 2001, Huelsenbeck and Ronquist 2001, Huelsenbeck *et al.* 2002, Beaumont and Rannala 2004, Holder and Lewis 2003, Kelly 2005, Manel *et al.* 2005).

1.6.2 *Phenetic (genetic distance) analysis*

The neighbour-joining (NJ) method sequentially finds pairs of neighbours (OTUs), linked by an interior node, to construct a phylogenetic tree. It directly calculates the distance to internal nodes and minimises the length of all internal branches, and hence the tree length, generating the shortest possible tree instead of constructing clusters (as in UPGMA analysis) (Vandamme 2003).

1.6.2.1 Genetic distance models

In phenetic analyses of aligned nucleotide sequences, the initial step usually involves the computation of genetic (or evolutionary) distances between DNA sequences (Strimmer and von Haesler 2003). Nucleotide substitution models may be implemented to correct for multiple substitutions at the same site.

The first and simplest nucleotide substitution model, the Jukes–Cantor model (JC69) (1969), assumes that the equilibrium frequencies of the four nucleotides are equal and that transitions and transversions are equally likely. The Kimura 2-parameter model (K2P) (1989) differs from the JC69 model as it allows the rate of transitions per site (α) to differ from the rate of transversions (β) (Page 1996, Whelan *et al.* 2001, Strimmer and von Haesler 2003).

Base composition variation is a reason that certain nucleotide substitutions may be more frequent than others. Felsensteins 1981 model (F81) deals with this aspect and allows for the four nucleotide frequencies to be different, while assuming that the rates of transitions and transversions are equal. The Hasegawa, Kishino and Yano (HKY85) (1985) model is a combination of the K2P and F81 models. This model allows transitions and transversions to occur at different rates and also allows variation in nucleotide composition (Page 1996, Whelan

et al. 2001). The General Time Reversible (GTR) (Tavaré 1986) model allows for six different substitution rates as well as unequal base frequencies (Page 1996, Hall 2001).

Maximum parsimony analysis, a cladistic method, neighbour-joining analysis, a phenetic method, and Bayesian likelihood analysis will be used to analyse the mitochondrial cytochrome *b* and D-loop data generated in this study

1.6.3 *Choosing Nucleotide Substitution models*

MODELTEST is a computer program used to compare the likelihood of various models of nucleotide substitution and to select the best-fit model (of 56 possible models) given the dataset at hand (Posada 1998). MrModeltest version 2.2 (Nylander 2004) selects the most appropriate of a smaller set (24) of nucleotide substitution models (Swofford 1993). In both programmes, variations on the basic models are produced by consideration of invariable sites (+I), and/or a gamma distribution (+G) of rates across sites.

1.6.4 *Estimating the reliability of inferred trees*

Bootstrapping, jackknifing, and to a lesser extent, the Decay index/ Bremer support are techniques used in evaluating the reliability of specific clades or clusters within a tree. In the case of Bayesian phylogenetic trees, reliability is shown in the form of posterior probabilities (Holder and Lewis 2003, Vandamme 2003, Müller 2004, Morrison 2005).

Bootstrap analysis, selected for use in this study, is an extensively-used sampling technique in which the original data matrix is randomly re-sampled with replacements, to produce pseudo-replicate data sets which allow estimation of the reliability of a tree (Holder and Lewis 2003). The proportion or percentage of each clade among all the bootstrap replicates is computed and taken as the statistical confidence, or bootstrap value, of the group. Generally bootstrap support of more than 75 % indicates substantial confidence in a particular group or branch whilst support of less than 70 % should be viewed with caution (Hall 2001, Whelan *et al.* 2001, Holder and Lewis 2003, Vandamme 2003, Morrison 2005).

1.7 Aims and Objectives

The aim of this study was to analyse the genetic diversity of *Mops leucostigma* samples from their range in Madagascar and the Comoros. Analyses of mitochondrial cytochrome *b* and D-loop sequences (genetic distance, phenetic, cladistic, haplotype and population demographic) were designed to complement morphological (morphometric) analyses carried out on the same sample set by Ratrimomanarivo *et al.* (in press, a).

The objectives of this study were to:

a) Sequence the coding cytochrome *b* and non-coding D-loop regions of the mtDNA of *Mops leucostigma* populations from Madagascar and the Comoros.

b) To determine the phylogenetic status of the recently discovered *Mops leucostigma* population in the Comoros relative to Malagasy *Mops leucostigma*.

c) To examine, genetically, the proposed sister-species relationship between *Mops leucostigma* and the African mainland sister species *M. condylurus* (Koopman 1994).

d) To identify the number of *Mops leucostigma* haplotypes present within Malagasy and Comorian samples and to ascertain whether there is phylogeographic concordance in the distribution of haplotypes.

e) To carry out population demographic analysis of D-loop data in order to assess whether populations of *Mops leucostigma* on Madagascar are expanding.

f) To apply the genetic information to evaluation of the conservation status of *Mops leucostigma* and to make recommendations where appropriate.

2 MATERIALS AND METHODS

2.1 Sample collection and storage

Mops leucostigma samples were collected by Dr. Steven Goodman and colleagues from various locations within Madagascar and the Comoros between September 2004 and January 2006. These samples of heart muscle were stored in 80 % ethanol at - 20 ºC until used.

Twenty-four geographically representative Madagascan sites were selected for study; 1 - 5 samples from each location, depending on availability, were selected for DNA extraction. A further four samples from one location in the Comoros were also included in the sample set (Table 1, Fig.1). Fig. 1 and Fig. 2 represent sample distribution in relation to habitat and altitude respectively. Outgroups included: 3 specimens of *Mops condylurus*, regarded as the sister-species of *M. leucostigma*, obtained from St. Lucia, South Africa; a more distantly related *Mops* taxon, *Mops midas* (Sundevall 1843); and a Molossid from a different genus, *Otomops martiensseni* (Matschie 1897) (Table 2).

2.2 DNA isolation

Total genomic DNA was extracted from 25 mg heart muscle tissue preserved in 80 % ethanol using a standard extraction protocol outlined in the QIAGEN DNeasy® Tissue Handbook (2005). DNA was stored in Qiagen buffer AE at - 20 ºC. Working stocks were stored at 4 ºC for up to 2 months.

2.3 DNA quantification

2.3.1 *Determination of DNA concentration*

The concentration of each DNA sample (ng ml^{-1}) was determined using a Nanodrop spectrophotometer. The spectrophotometer was zeroed against Qiagen buffer AE prior to reading the sample DNA concentration.

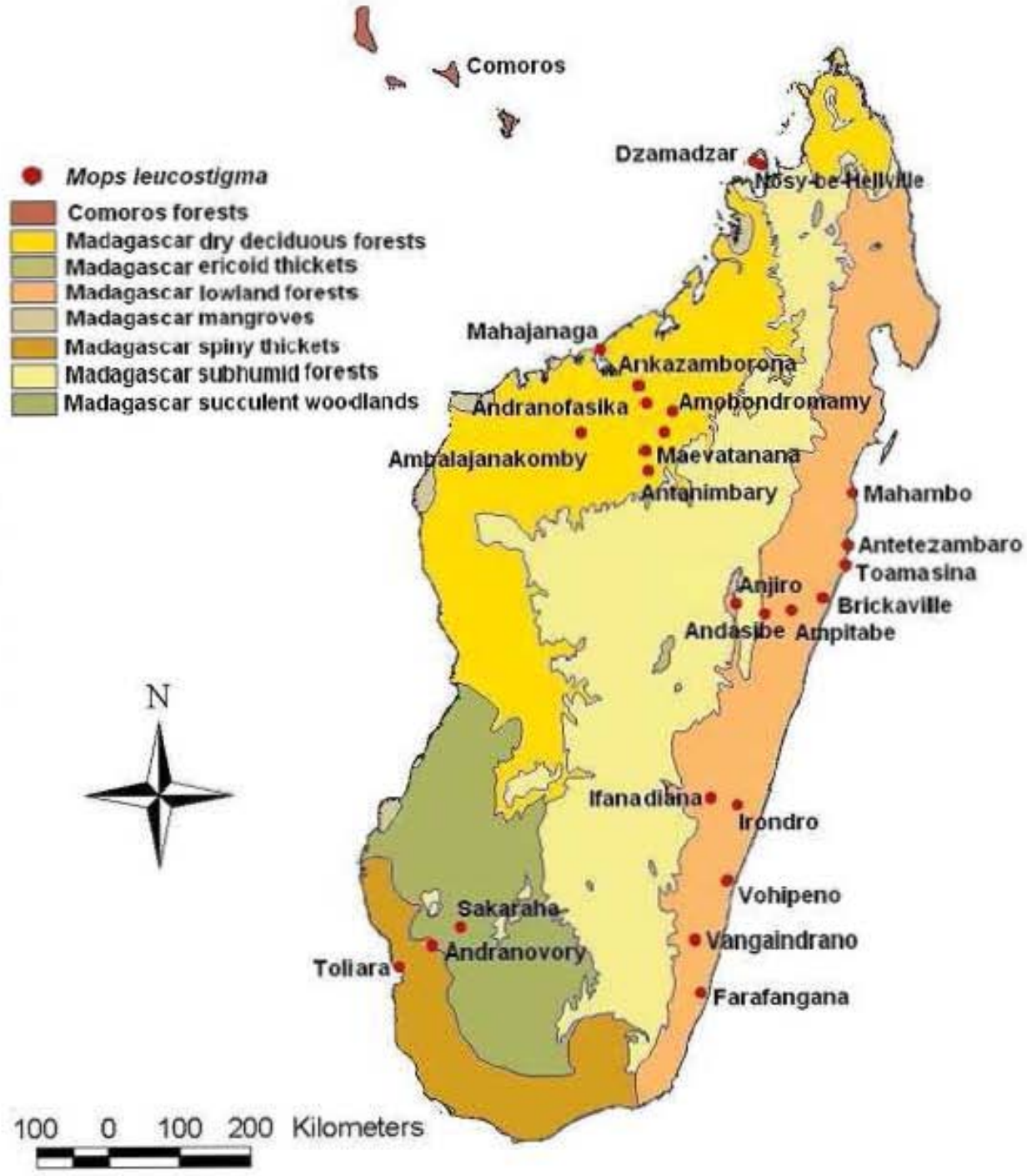

Figure 1: Map showing *Mops leucostigma* sampling sites in Madagascar and the Comoros in relation to habitat (Olsen *et al*. 2002).

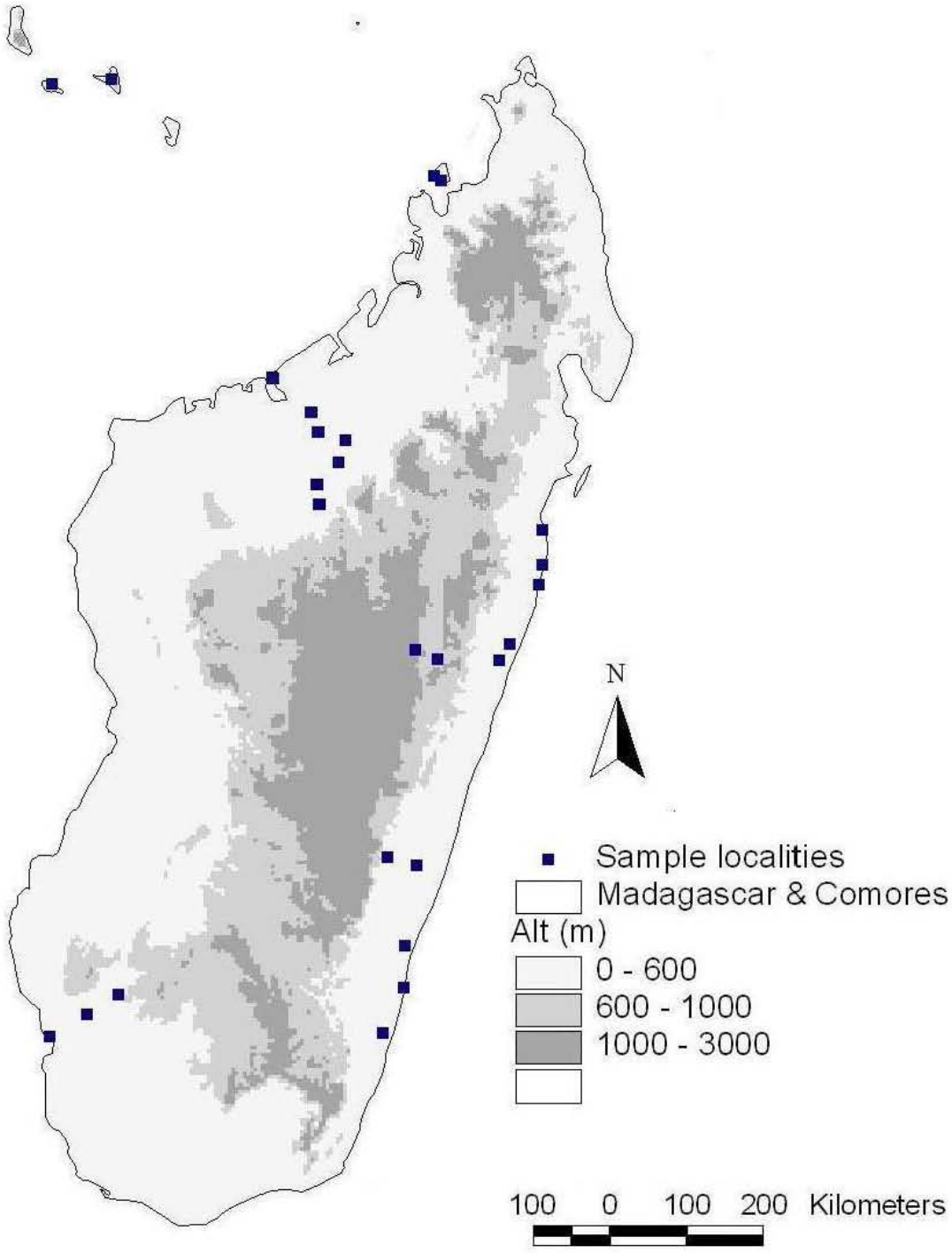

Figure 2: Map showing *Mops leucostigma* sampling sites in Madagascar and the Comoros in relation to altitude.

Table 1: Sample table indicating sample location, catalogue number, FMNH (Field Museum of Natural History) number, gender and collector. Sample sequences used in the cytochrome *b* and D-loop analyses respectively are also indicated.

Location	Geographic Co-ordinates		Cat. No.	FMNH	Sex	Cyt *b*	D-loop	Collector(s)
	Latitude	Longitude						
Maevatanana	16º57.275'	46º49.608'	RHF-733	185051	F	Y	-	F.H. Ratrimomanarivo, Andriarileva
			RHF-732	185050	M	Y	-	
			RHF-731	185049	F	Y	-	
Ambalanjanakomby	16º41.738'	47º04.241'	RHF-797	185069	M	Y	-	F.H. Ratrimomanarivo, Andriarileva
Ambondramamy	16º26.173'	47º09.329'	RHF-851	185079	F	Y	-	F.H. Ratrimomanarivo, Andriarileva
			RHF-852	185080	F	Y	-	
			RHF-853	185081	M	Y	-	
			RHF-854	185082	F	Y	-	
Anjiro	18º52.945'	47º58.245'	RHF-532	184701	M	Y	Y	F.H. Ratrimomanarivo Rakotomavo
			RHF-533	184702	F	Y	Y	
			RHF-534	184703	M	-	Y	
			RHF-535	184704	F	Y	Y	
			RHF-536	184705	M	-	Y	
Antanimbary	17º11.104'	46º51.306'	RHF-674	185039	F	Y	Y	F.H. Ratrimomanarivo, Randrimasinarivo
			RHF-675	185040	M	Y	Y	
			RHF-676	185041	F	Y	Y	
			RHF-677	185042	F	Y	Y	
			RHF-678	185043	F	Y	Y	
Toamasina	18º07.734'	49º24.281'	SMG-14492	184092	M	Y	Y	S.M. Goodman
			SMG-14493	184093	F	-	Y	
			SMG-14494	184094	M	Y	Y	
			SMG-14495	184095	M	-	Y	
			SMG-14496	184096	M	Y	Y	
Mahajanga	15º43.069'	46º19.489'	RHF-423	184687	F	Y	-	F.H. Ratrimomanarivo S.M. Goodman
			RHF-424	184688	F	-	Y	
			RHF-425	184689	F	Y	-	
	15º42.500'	46º18.688'	RHF-427	184691	F	-	Y	
			RHF-433	184697	M	Y	Y	

Sakahara	22º 54.567'	44º31.338'	RHF-198	184478	F	-	Y	F.H. Ratrimomanarivo Rakotomavo
			RHF-199	184479	M	-	Y	
			RHF-200	184285	F	-	Y	
			RHF-202	184287	M	Y	Y	
	22º54.542'	44º31.616'	RHF-204	184289	M	-	Y	
Andranofasika	16º20.166'	46º50.503'	RHF-871	185088	F	-	Y	F.H. Ratrimomanarivo Andriarileva
			RHF-872	185089	F	-	Y	
Ankazomborona	16º06.961'	46º45.400'	RHF-901	185095	M	-	Y	F.H. Ratrimomanarivo, Andriarileva
			RHF-902	185096	F	-	Y	
			RHF-903	185097	F	-	Y	
			RHF-904	185098	F	-	Y	
Dzamadzar	13°21'09.5"	48°11'30.7"	SMG-15079	188545	F	Y	Y	S.M. Goodman
			SMG-15080	188546	F	Y	Y	
			SMG-15082	188548	F	-	Y	
Nosy-be Hellville	13°24'25.4"	48°16'42.5"	SMG-15031	188522	M	Y	Y	S.M. Goodman
Mahambo	17°29.254'	49°27.108'	RHF-1370	187852	M	Y	Y	F.H. Ratrimomanarivo, J. Ranaivo
			RHF-1372	187854	M	-	Y	
Antetezambaro	17°53.377'	49°27.038'	RHF-1395	187870	M	Y	Y	F.H.Ratrimomanarivo, J. Ranaivo
			RHF-1396	187871	M	Y	Y	
			RHF-1399	187874	M	-	Y	
Brickaville	18°49.087'	49°04.332'	RHF-1512	187960	M	-	Y	F.H.Ratrimomanarivo, J. Ranaivo
			RHF-1514	187962	F	-	Y	
Moramanga	18°59.363'	48°14.004'	RHF-1772	188151	M	Y	-	F.H. Ratrimomanarivo, J. Ranaivo
Ampitabe	19°00.130'	48°56.580'	RHF-1561	188008	F	-	Y	F.H. Ratrimomanarivo, J. Ranaivo
			RHF-1562	188009	M	Y	-	
			RHF-1563	188010	M	-	Y	
			RHF-1565	188012	M	-	Y	
Ifanadiana	21°18.491'	47°38.253'	RHF-1306	185526	F	Y	Y	F.H. Ratrimomanarivo, E. Rakotonandrasana
			RHF-1307	185527	F	Y	Y	
			RHF-1310	185530	F	Y	Y	
Irondro	21°24.129'	47°58.928'	RHF-1256	185503	F	Y	Y	F.H.Ratrimomanarivo, E. Rakotonandrasana
			RHF-1259	185506	M	Y	Y	
			RHF-1260	185507	F	-	Y	
Vohipeno	22°20.707'	47°50.773'	RHF-1143	185433	M	-	Y	F.H.Ratrimomanarivo, E. Rakotonandrasana
			RHF-1144	185434	M	Y	Y	

			RHF-1145	185435	F	-	Y	
Vangaindrano	23°21.380'	47°35.750'	RHF-995	185339	F	Y	Y	F.H. Ratrimomanarivo
			RHF-996	185340	F	Y	Y	
			RHF-998	185342	M	-	Y	
Farafangana	22°49.444'	47°49.741'	RHF-1070	185374	F	-	Y	F.H.Ratrimomanarivo
			RHF-1073	185377	M	Y	Y	
			RHF-1074	185378	M	Y	Y	
Andranovory	23°08.618'	44°08.676'	RHF-174	184275	F	Y	-	F.H.Ratrimomanarivo
			RHF-175	184276	M	Y	Y	
Toliara	23°23.704'	43°43.219'	RHF-138	184268	F	Y	-	F.H.Ratrimomanarivo, Rakotomavo
			RHF-139	184269	F	Y	-	
RFI Comore	12°13.497'	44°25.884'	SMG-15626	194387	M	Y	Y	S.M. Goodman, M. Ruedi, N. Weyenthe
	12°16.882'	43°44.272'	SMG-15668	194502	M	Y	-	
			SMG-15669	194503	M	Y	-	
			SMG-15671	194505	F	Y	Y	

Table 2: Outgroups used in the genetic analyses: Sample locations, FMNH accession numbers and GenBank accession numbers for cytochrome *b*and D-loop data. DM2 and DM3 have not been submitted to GenBank and have no accession numbers.

Outgroup	Location	FMNH/ Sample Code	Cyt *b*	D-loop	Accession Number
Mops condylurus	St. Lucia (South Africa)	DM1	Y	Y	EF474030
Mops condylurus	St. Lucia (South Africa)	DM2	Y	Y	-
Mops condylurus	St. Lucia (South Africa)	DM3	Y	Y	-
Mops midas M263	Sakaraha (Madagascar)	FMNH184306	Y	-	EF474034
Mops midas M266	Satrokala (Madagascar)	FMNH184309	Y	-	EF474035
Mops midas SA2	Hoedspruit (South Africa)	-	Y	-	EF474031
Otomops martiensseni	Durban (South Africa)	-	Y	-	AY591535.1

2.3.2 *Assessment of the integrity of DNA samples*

The integrity of each sample was assessed using agarose gel electrophoresis. A 1 % agarose gel (in 0.5 x TBE) was prepared, to which 100 µl ethidium bromide (EtBr) (0.05 mg ml^{-1}) was added. 6 µl of each sample was mixed with 4 µl of loading dye, either 6x Orange Loading Dye solution (Fermentas Life Sciences) or bromophenol blue (Appendix A), prior to loading into the wells. One lane of each gel was loaded with a marker of known molecular weight, either 6 µl O'Gene Ruler (Fermentas Life Sciences) in which the dye was premixed or 2 µl Molecular Weight Marker III (Roche Molecular Biochemicals). The gel was electrophoresed in 0.5 x TBE buffer containing 100 µl EtBr (0.05 mg ml^{-1}) at 100 Volts for approximately two hours. DNA bands in the gels were stained with EtBr ad nthen visualised with a Uvitec UV transilluminator; images were photographed with the Uvitec camera and saved on disk using the Uvisave feature. The presence of bright bands close to the wells, and the absence of significant low molecular-weight smear was taken as an indication that the DNA was of high integrity and suitable as a substrate for PCR.

2.4 DNA sequencing using the Polymerase Chain Reaction

2.4.1 *PCR-Amplification of target fragments*

The mitochondrial cytochrome *b* gene was successfully PCR-amplified for 49 samples, and the D-loop region for 61 samples. The cytochrome *b* gene was amplified as two overlapping fragments using the primers of Irwin *et al*. (1991). The 5' hypervariable region of the D-loop was amplified as a single fragment using primers P and F/E (Wilkinson and Chapman 1991) (Table 3). PCR-amplification of 76 samples of the D-loop was initially performed using primers P and F. Samples that failed to amplify with this primer pair were then PCR-amplified using primers P and E.

Amplifications were performed in 25 µl reaction volumes each consisting of 9 µl genomic DNA solution (containing 30 ng DNA) and 16 µl of mastermix (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)). PCRs were performed using the thermal cycling parameters displayed in Table 4a and 4b on a Perkin Elmer GeneAmp PCR System 2400 thermal cycler.

Table 3: Oligonucleotide primer sequences for PCR-amplification of the cytochrome *b* gene and D-loop region of the mtDNA of *Mops leucostigma* and *M. condylurus*.

Amplified Region		Primer	Direction	Primer Sequence 5' to 3'
Cytochrome *b*	5' fragment	L14723 (L23)	Forward	ACCAATGCAATGAAAAATCATGGTT
		H15553 (H53)	Reverse	TAGGCAAATAGGAAATATCATTCTGGT
	3' fragment	L15146 (L46)	Forward	CATGAGGACAAATATCATTCTGAG
		H15915 (H15)	Reverse	TCTCCATTTCTGGTTTACAAGAC
D-loop 5' hypervariable region		P	Forward	TCCTACCATCAGCACCCAAAGC
		F	Reverse	GTTGCTGGTTTCACGGAGGTAG
		E	Reverse	CCTGAAGTAGGAACCAGATG

Table 4: Thernal cycling parameters for PCR amplification of a) the cytochrome *b* gene and b) the D-loop region.

a) Cytochrome *b*

	Purpose	Cycle/s	Temperature	Time (minutes)
1.	Denaturation	1	95 ºC	2
2.	i) Denaturation	40	95 ºC	1
	ii) Primer Annealing		55 ºC	1.30
	iii) Primer Extension		72 ºC	2
3.	Primer Extension	1	72 ºC	7
4. PCR product holding temperature		Hold	15 ºC	∞

b) D-loop

	Purpose	Cycle/s	Temperature	Time (minutes)
1.	Denaturation	1	94 ºC	4
2.	i) Denaturation	36	94 ºC	1
	ii) Primer Annealing		50 ºC	1.30
	iii) Primer Extension		72 ºC	2
3.	Primer Extension	1	72 ºC	10
4. PCR product holding temperature		Hold	15 ºC	∞

2.4.2 *Purification of PCR products*

Cytochrome *b* and D-loop PCR products were purified by electrophoresis in 1.5 % agarose gels (in 0.5 X TBE) at 15 Volts for ± 24 hours. Gels were viewed with a Uvitec UV transilluminator. Amplified bands, identified by molecular weight, were excised and stored in Eppendorf tubes at - 20 ºC. Target fragments were purified from excised bands following the protocol provided with the QIAquick® Gel Extraction Kit (Qiagen Inc.) (for use with a microcentrifuge). A final volume of 30 µl DNA was eluted in Buffer AB for each sample. The concentration of DNA in each sample was measured by spectrophotometry (see section 2.3.1) to ensure that each sample contained at least 200 ng of DNA (concentration 10 ng μl^{-1}) as required for sequencing.

2.4.3 *DNA sequencing*

Single stranded dideoxy sequencing of DNA amplicons was performed in both directions using the primers used in the initial amplifications. Sequencing reactions were carried out by Inqaba Biotechnical Industries Pty. Ltd., Hatfield, Pretoria, South Africa. GenBank Accession numbers for cytochrome *b* and D-loop *Mops leucostigma* sequences are given in Table 9, Appendix A.

2.5 Data Analyses

2.5.1 *Sequence alignment*

Consensus sequence alignments were generated with the BioEdit Sequence Alignment Editor (Version 5.0.9 for Windows 95/98/NT) (Hall 1999) and its accessory, Clustal W (Thompson *et al*. 1994) alignment application. For all samples the forward and reverse sequences and electropherograms were compared against each other by visual inspection to confirm sequence homology and to locate inconsistencies. Observed discrepancies were corrected manually to obtain consensus sequences for each sample. Samples were aligned using Clustal W, and further corrected manually. All sequences were trimmed to a common length of 1008 nucleotides for the cytochrome *b* gene, and 380 nucleotides for D-loop. Alignments were opened in Clustal X version 1.81 (Thompson *et al.* 1997) in order to convert and save the files into .nxs and .aln formats for use in other programs.

2.5.2 *Choosing the appropriate nucleotide substitution model for each dataset*

Aligned sequences for both cytochrome *b* and D-loop were analysed in MrModeltest version 2.2 (Nylander 2004), to determine the nucleotide substitution model that best fit the sequence dataset. The HKY + I model was selected for both the cytochrome *b* and D-loop datasets, and all further analyses were carried out using this model where appropriate.

2.5.3 *Analysis of data saturation*

The program DAMBE (Data Analysis in Molecular Biology and Evolution) version 4.5.34 (Xia 2000) was used to assess the degree of saturation present in both the cytochrome *b* and D-loop data sets. For both saturation curves the F84 model of evolution was used, as the HKY + I model was not available. The Xia *et al.* (2003) test was used to measure substitution saturation. This test calculates Iss, an index of substitution saturation for the dataset, as well as the Iss

critical value, Iss.c.Sym, to which it is compared. If the probability that Iss.c ≥ Iss.c.sym < 0.05, this can be interpreted to mean that there is little saturation in the dataset.

2.5.4 *Phenetic and cladistic analysis*

2.5.4.1 Maximum parsimony

Maximum Parsimony analysis was carried out in PAUP version 4.0b10 (Swofford 2002). The random addition sequence option (n = 100) for discrete, unordered characters was utilised and 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 (1000 pseudoreplicates) (Felsenstein 1985, Felsenstein and Kishino 1993, Hillis and Bull 1993).

2.5.4.2 Bayesian analysis

The HKY + I model was implemented in MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001) for both data sets. Four Markov chains were run for 15 million generations each, and the first 5000 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 = branch lengths are unconstrained: exponential (10.0).

2.5.4.3 Neighbour-joining and genetic distances analysis

Neighbour-joining trees were produced in PAUP 4.0b10 (Swofford 2002) using the HKY + I model. Nodal support was estimated using bootstrap resampling analysis (1000 pseudoreplicates) in PAUP. Genetic distances (HKY + I) for each dataset were also calculated in PAUP.

2.5.5 *Phylogeographic analysis*

2.5.5.1 Population genetic analysis

Haplotype analysis of both the cytochrome *b* and D-loop datasets was performed using the program DnaSP (DNA Sequence Polymorphism) version 4.10.9 (Rozas *et al.* 2003). This was

used to ascertain the number of haplotypes present in each dataset. Additionally for the D-loop dataset population genetic demographic analysis was performed according to Russell *et al.* (2005). Neutrality tests, Fu and Li's (1993) *D** test statistic and Fu and Li's *F** test statistic were calculated to determine whether the populations were undergoing expansion. These tests were not performed for the cytochrome *b* dataset since this region is not selectively neutral. Expansion coefficients (*S/d*) were calculated for D-loop data (larger expansion coefficients are an indication of population growth and smaller expansion coefficients are indicative of population stationarity). Population expansion is also indicated by unimodal pairwise difference distributions, high haplotype diversity (*h*), low nucleotide diversity (π), non-significant *F** and *D** and significant F_S (Russell *et al.* 2005). Following Rogers and Harpending (1992), the time since expansion began was calculated using the formula $\tau = 2ut$ t. Tau (τ) was calculated and obtained from DnaSp, *u* is the mutation rate per site (μ) per million years, *k* is the sequence length, and t is the time (in generations) since expansion. From Rogers and Harpending (1992) two rates for D-loop mutations per site per generation (1.73×10^{-7} and 3.3×10^{-7}) were used.

2.5.5.2 Haplotype analysis

Statistical parsimony haplotype networks, which consider population level phenomena by analysing moelcularl characters of individuals from several populations (Gemeinholzer 2008), were generated for both cytochrome *b* and the D-loop using TCS version 1.21 (Clement *et al*. 2000).

3 RESULTS

3.1 DNA isolation and quantification

DNA isolation yielded 200 µl genomic DNA per sample with concentrations which ranged from 4.2 to 46.6 ng μl^{-1}. After agarose gel electrophoresis samples yielded a distinct single bright band of high molecular weight, and little low molecular weight smear, indicating that the genomic DNA was of high integrity and hence suitable for PCR amplification of the cytochrome *b* and D-loop target fragments.

3.2 PCR Amplification and purification of target fragments

PCR amplification of the cytochrome *b* gene and D-loop region was performed successfully although during PCR non-target fragments co-amplified with target fragments of interest in some samples. In these cases target bands were excised from the gel in order to purify the target DNA and separate it from co-amplified non-target fragments and streaks.

3.3 Recovery of PCR-amplified genes

After DNA was extracted from excised gel bands, samples formed single clear, bright bands with DNA concentrations ranging from 7.6 to 119 ng μl^{-1} in a volume of 25 µl, which was adequate for sequencing.

3.4 Sequence analysis

3.4.1 *Data saturation*

The program DAMBE version 4.5.35 (Xia 2000) was used to establish the level of data saturation in both the sequence data sets, by plotting transitions and transversions against divergence.

3.4.1.1 Cytochrome *b*

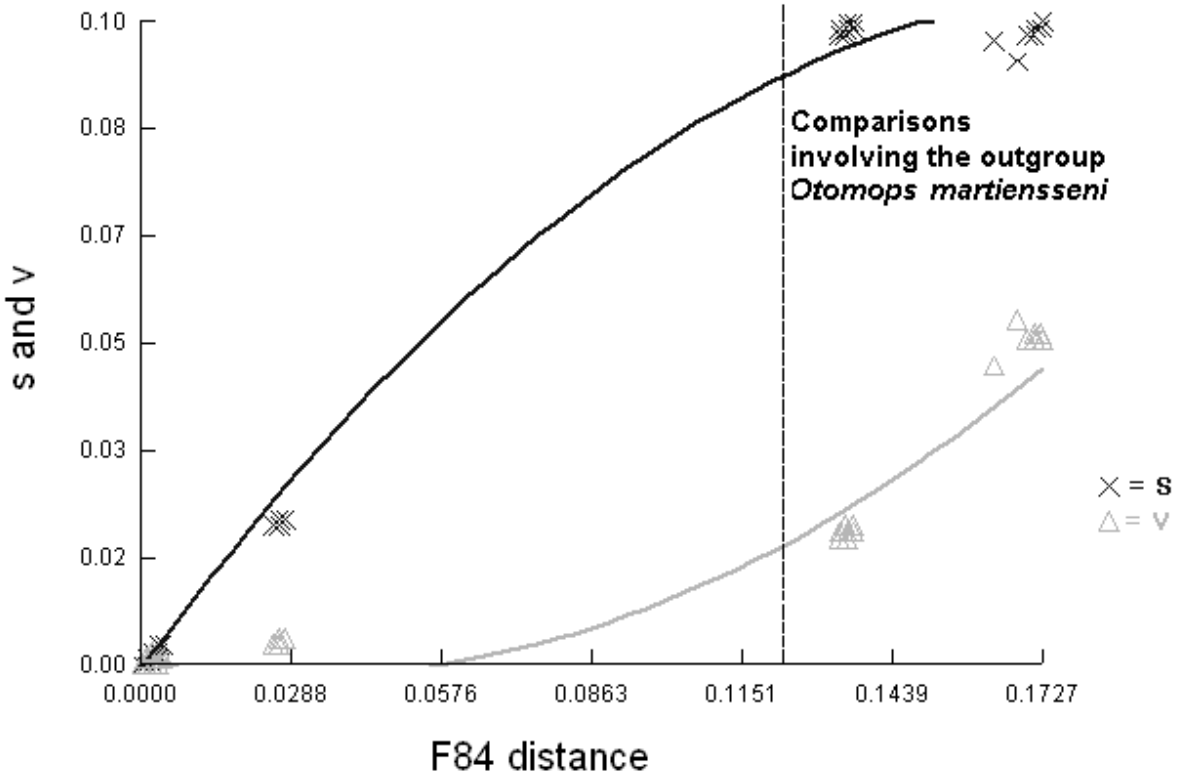

Figure 3: Saturation curve for the cytochrome *b* data set (s = transitions, v = transversions). Solid lines represent the least squares best fit.

There was little saturation in the cytochrome *b* data set (Fig. 3), with the exception of comparisons involving the outgroup, *Otomops martiensseni* where showed transitions plateau slightly. The Xia *et al.* (2003) test of substitution saturation yielded an Iss (index of substitution saturation) value of 0.0750, which was significantly lower than the Iss.cSym value (critical value assuming a symmetrical topology) of 0.7590, $P < 0.001$.This indicated that there is little saturation in the cytochrome *b* data set.

3.4.1.2 D-loop

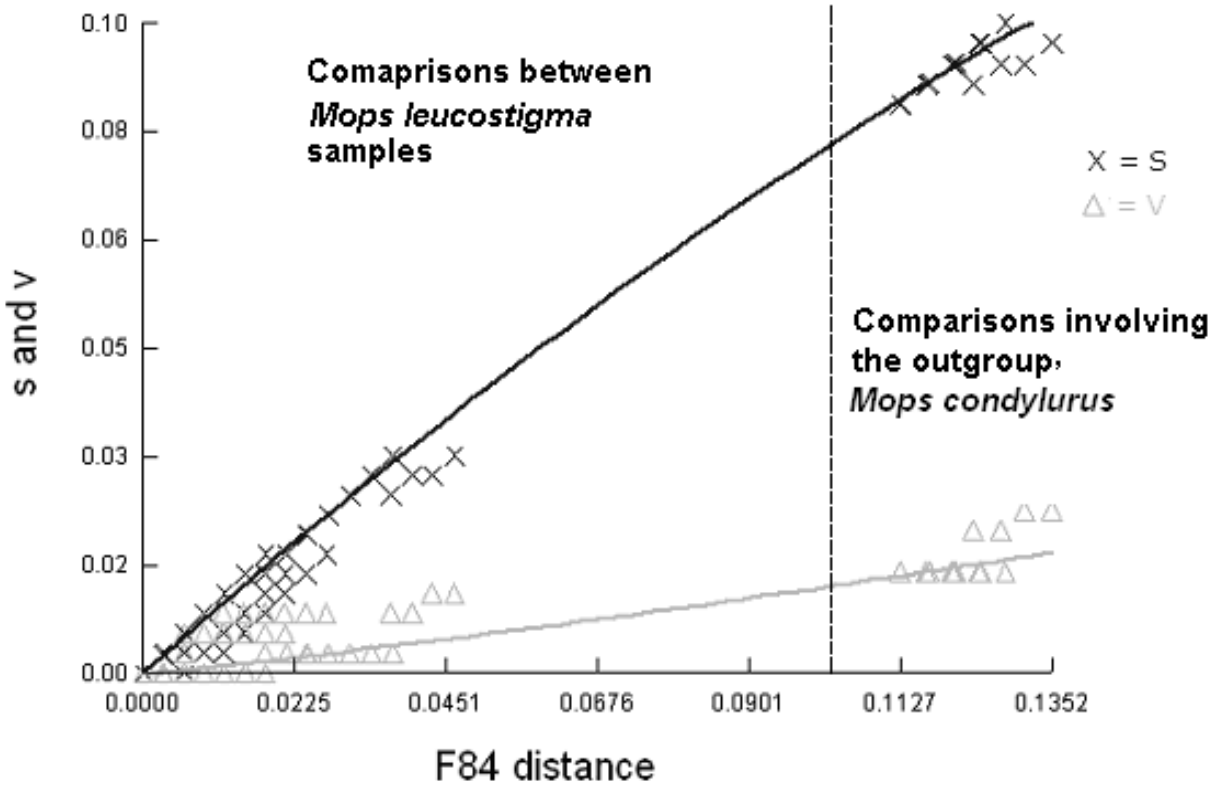

Figure 4: Data saturation curve for the D-loop data set (s = transitions, v = transversions). Solid lines represent the least squares best fit.

The D-loop data set appeared to follow a straight-line model (Fig. 4), signifying little data saturation. The Xia *et al.* (2003) test of substitution saturation showed an Iss (index of substitution saturation) value of 0.4072, which was significantly lower than the Iss.sSym value (critical value assuming a symmetrical topology) of 0.7005, $P < 0.002$. This indicated that there is little saturation in the D-loop data set (excluding outgroups). Fig. 4 provides evidence that for ingroup versus outgroups comparisons the D-loop region is saturated for substitutions.

3.4.2 *Data statistics*

The *Mops leucostigma* cytochrome *b* dataset of 1008 nucleotides contained 999 conserved sites and 9 variable sites, of which 1 was parsimony-informative and 8 were singletons. The *Mops leucostigma* D-loop dataset of 338 nucleotides contained 318 conserved sites and 20 variable sites, of which 18 were parsimony-informative and 2 were singletons.

3.5 Phylogeographic analysis

3.5.1 *Haplotype analysis*

3.5.1.1 Cytochrome *b* haplotype analysis

Haplotype analysis was carried out on 1008 nucleotides of the cytochrome *b* sequence of 49 *M. leucostigma* samples using DnaSp version 4.10.9 (Rozas *et al.* 2003). Analysis indicated 9 variable sites, yielding 9 haplotypes (Table 5). The haplotype (gene) diversity (h) was 0.367 (standard deviation 0.088) and the nucleotide diversity per site (Pi) was 0.00048 (standard deviation 0.00014). The average number of nucleotide differences (k) was 0.48.

Two of the nine cytochrome *b* haplotypes were shared by more than one sample. Haplotype 1 consisted of 39 Malagasy *M. leucostigma* from varied locations across the island, whilst haplotype 9 comprised three samples from the Comoros (Table 5, Fig. 6). The remaining seven *M. leucostigma* haplotypes each comprised one sample. The four *M. leucostigma* samples from the Comoros formed two haplotypes, 8 and 9.

3.5.1.2 D-loop haplotype analysis

Haplotype analysis was carried out on 380 nucleotides of the D-loop of 61 *M. leucostigma* samples using the program DnaSp version 4.10.9 (Rozas *et al.* 2003). There were 12 haplotypes based on 20 variable sites (Table 6). The haplotype (gene) diversity was 0.758 (standard deviation 0.050) and the nucleotide diversity (Pi) was 0.00912 (standard deviation 0.00146). The average number of nucleotide differences (k) was 3.07. Haplotype 1 was the most common haplotype and contained 28 samples. Haplotypes 7, 8 and 9 comprised 9, 5 and 5 samples respectively, whilst the remaining haplotypes comprised between 1 and 3 samples.

Fu and Li's D^* test statistic and Fu and Li's F^* test statistic, calculated using DnaSp were 0.92024 and 0.33863 respectively, and were non-significant ($P > 0.10$), consistent with the assumption of an expanding population. The expansion coefficient, S/d, was high at 6.51, and also indicative of an expanding population. Distribution of pairwise differences followed an essentially unimodal distribution (Fig. 5) with a non-significant raggedness statistic r: 0.0492 ($p > 0.05$), indicative of an expanding population (Rogers and Harpending 1992). However the non-significant value of Fu's (1997) F_s statistic (-1.110; $P > 0.05$) did not indicate an expanding population.

Table 5: Haplotypes present in the cytochrome *b* dataset (1008 nucleotides) of 49 *Mops leucostigma* samples and outgroups *Mops condylurus*, *Mops midas* and *Otomops martiensseni*. H - haplotype, N - Number of samples.

Species	H	N	Variable Sites (out of 1008)	Location	Sample Code
M. leucostigma	1	39	AATGCTCCTACCTCTCTCCTTCCAGCTATGCC	Farafangana	FMNH 185378, 185377;
				Vohipeno	FMNH 185434;
				Irondro	FMNH 185503,185506;
				Ifanadiana	FMNH 185526, 185527, 185530;
				Toliara	FMNH 184268,184269;
				Antetezambaro	FMNH 187870, 187871;
				Ampitabe	FMNH 188009;
				Andranovory	FMNH 184275;
				Moramanga	FMNH 188151;
				Sakaraha	FMNH 184287;
				Mahajanga	FMNH 184687, 184689, 184697;
				Anjiro	FMNH 184701, 184704;
				Antanimbary	FMNH 185039,185041, 185042, 185043;
				Maevatanana	FMNH 185050;
				Ambalajanakomby	FMNH 185069;
				Ambondromamy	FMNH 185079,185080,185081,185082;
				Vangaindrano	FMNH 185339,185340;
				Toamasina	FMNH 184092, 184094, 184096;
				Nosy be Hellville	FMNH 188522;
				Dzamadzar	FMNH 188545, 188546;
	2	1	.....A	Mahambo	FMNH 187852;
	3	1	.....C....	Andranovory	FMNH 184276;
	4	1	.....C.....C.....	Anjiro	FMNH 184702;
	5	1	.....T.....	Antanimbary	FMNH 185040;
	6	1	.....A.	Maevatanana	FMNH 185049;
	7	1	.....A.....	Maevatanana	FMNH 185051;
	8	1	..A.....C.....	Comoros	FMNH 194387;
	9	3	..A.....	Comoros	FMNH 194502,194503,194505;
M. condylurus	10	3	CG.CTCTTCGTT..CTCTT.CTTG.TG.CA..	St. Lucia, South Africa	DM1, DM2, DM3;
M. midas	11	2			FMNH 184306, 184309
M. midas	12	1	Not shown owing to large number of variable sites.		GenBank EF474031
O. martiensseni	13	1			-

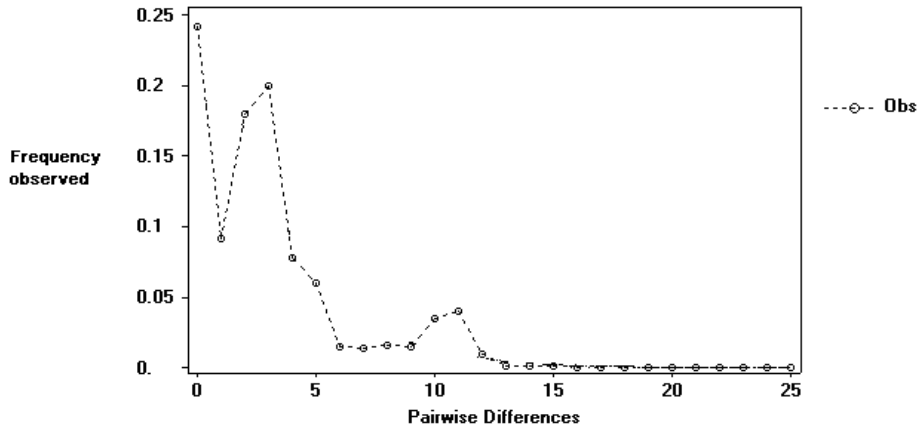

Figure 5: Observed distribution of pairwise distances for the D-loop data set.

Considering the majority of evidence to indicate the existence of an expanding population, an estimated time since expansion was obtained using the formula $\tau = 2ut$. The value of tau (τ), the mutation rate in generational units, was 0.474 (obtained from DnaSp). t the estimated time since expansion, was calculated at between 3779 years (using 33% divergence) and 7210 years (using 17% divergence) before present according to (Rogers and Harpending 1992).

3.5.2 *Haplotype networks*

3.5.2.1 Cytochrome *b* haplotype networks

Forty-five *Mops leucostigma* samples formed 9 haplotypes. Haplotypes 1 – 7 represented Malagasy samples only, whilst haplotypes 8 and 9, separated from each other by one mutational step, were exclusively from the Comoros (Fig. 6a and Table 5). The network, which had a star-like topology, comprised one central haplotype (H1) of 35 samples, from which Malagasy haplotypes 2, 3, 5, 6 and 7 were separated by a single mutational step. Haplotype 1 was distributed across all Malagasy locations, whilst 7 of the haplotypes on the lateral branches of the network comprised one sample and hence were specific to one area. Haplotype 9, from the Comoros, was the only location-specific haplotype that comprised more than one sample (3 samples), and was also separated from H1 by one mutational step. All *Mops leucostigma* samples were connected by a 95 % parsimony connection.

The outgroup *M. condylurus* was separated from the main Malagasy and Comorian *M. leucostigma* network by 23 mutational steps, through an unsampled haplotype between H4 and H1 (a less-than 95 % parsimony connection).

Table 6: Haplotypes present in the D-loop dataset (380 nucleotides) of 61 *Mops leucostigma* samples and outgroup *Mops condylurus*. H - haplotype, N - number of samples.

Species	H	N	Variable sites (out of 380)	Location	Sample Code
M. leucostigma	1	28	AGACACGACTGCAATGGGCTAAATGTAACCGAATTTATAGGCAGATTCT	Farafangana	FMNH 185374, 185377, 185378;
				Vohipeno	FMNH 185433, 185434;
				Irondro	FMNH 185503, 185506;
				Ifanadiana	FMNH185526, 185527, 185530;
				Mahambo	FMNH 187852;
				Antetezambaro	FMNH 187874;
				Ampitabe	FMNH 188008, 188012;
				Andranovory	FMNH 184276;
				Sakaraha	FMNH 184478, 184285, 184289;
				Anjiro	FMNH 184702, 184703;
				Vangaindrano	FMNH 185342;
				Toamasina	FMNH 184093, 184094, 184095, 184096;
				Nosy be Hellville	FMNH 188522;
				Dzamadzar	FMNH 188545, 188546;
	2	1	..CACT.....	Vohipeno	FMNH 185435;
	3	1	..CA.....	Irondro	FMNH 185507;
	4	3	.....T.....T...G.C.....T.....	Mahambo	FMNH 187854;
				Brickaville	FMNH 187962;
				Vangaindrano	FMNH 185339;
	5	2	G.....A...C..A.....GT..GG.....G....	Antetezambaro/Dzamadzar	FMNH 187870/ FMNH 188548;
	6	2	G.....G..A...C..A.....GT..GG.....G....	Antetezambaro/Brickaville	FMNH 187871/ FMNH 187960;
				Ampitabe	FMNH 188010;
				Sakaraha	FMNH 184479, 184287;
	7	9	.....T...G.....	Anjiro	FMNH 184701;
				Antanimbary	FMNH 185040, 185041, 185042;
				Vangaindrano	FMNH 185340;
				Toamasina	FMNH 184092;
				Comoros	FMNH 194505, 194387;
	8	5	.....GT...G.....	Andranofasika	FMNH 185088, 185089;
				Ankazamborona	FMNH 185095;
	9	5	.....T.A.....C	Mahajanga/Ankazamborona	FMNH 184688, 184691, 184697/ 185097, 185098;
	10	1	.....T...G.C.....T.....	Anjiro	FMNH 184704;
	11	3	.....T.....	Anjiro	FMNH 184705;
				Antanimbary	FMNH 185040, 185043;
	12	1	.....GT..GG.....	Ankazamborona	FMNH 185096;
M. condylurus	13	2	.A....A.ACA.GG.ATAACGGGCACGGTT...C.CGC.AATGA.GGT.	St. Lucia, South Africa	DM1, DM3;
	14	1	.A....A.ACA.GG.ATAACGGGCACGGTT...C.CGCGAATGA.GGT.		DM2

Most samples occurred at altitudes below 500 m (Fig 6b); there was no apparent association between haplotype distribution and altitude. Similarly, there was no apparent correlation between gender (Fig. 6d) and haplotype distribution, or aspect (east vs. and west facing slopes) and haplotype distribution (Fig. 6c).

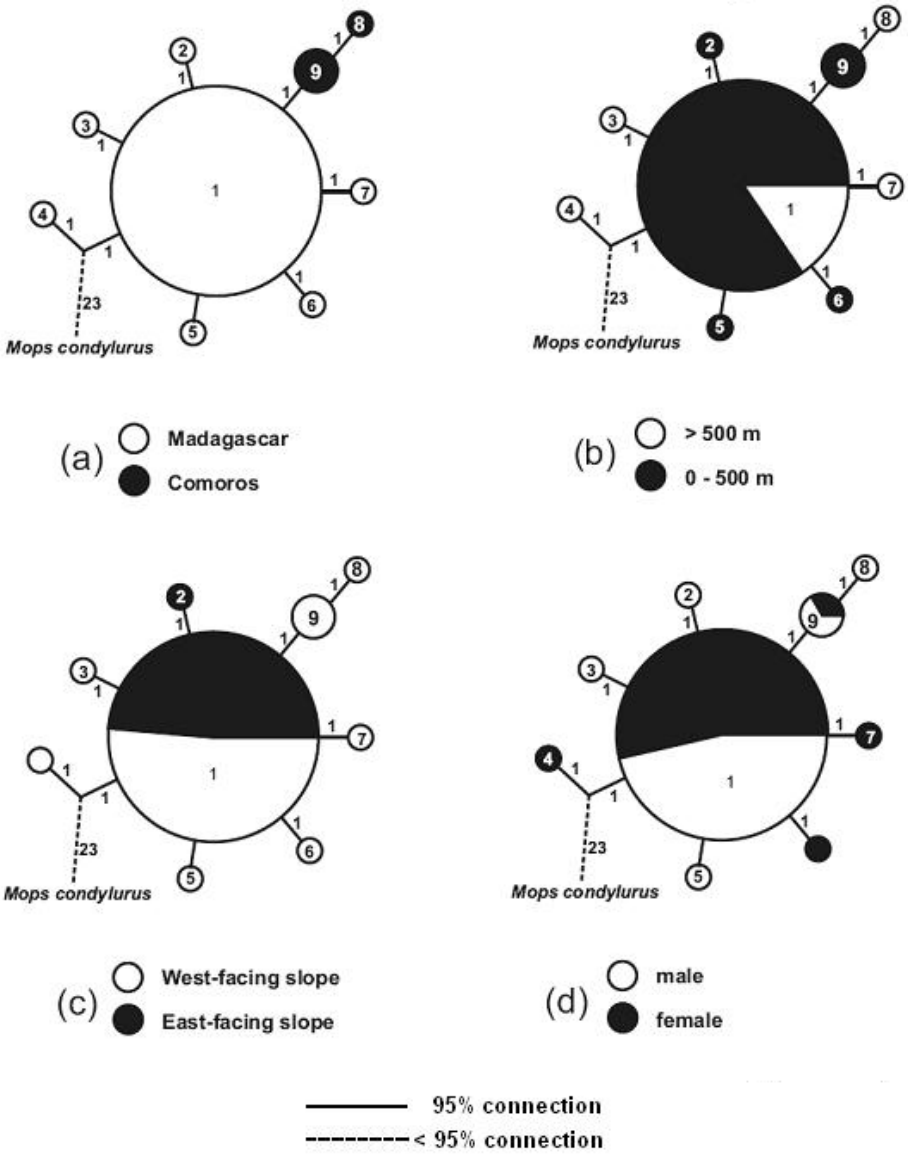

Figure 6: Haplotype networks showing mutational relationships between 9 mitochondrial cytochrome *b* (1008 nucleotides) haplotypes of *Mops leucostigma* obtained from 49 samples with reference to the outgroup, *M. condylurus*: (a) distribution of samples from Madagascar and the Comoros, (b) distribution of samples between low and high altitudes on Madagascar, (c) distribution of samples between east- and west-facing slopes on Madagascar, (d) distribution of samples among males and females on Madagascar. Numbers within circles are haplotype numbers; numbers adjacent to connecting lines representing mutational steps.

3.5.2.2 D-loop haplotype networks

The *M. leucostigma* D-loop dataset was composed of four major haplotypes, H1 comprising 28 samples, was the most prevalent haplotype, followed by H7 (9 samples) and H8 and H9 (5 samples each). A further 8 haplotypes were present at a lower frequency (Table 6).

Either 1 or 2 mutations separated all *M. leucostigma* haplotypes, with the exception of H12 and H5, which were separated by 5 mutational steps. The reticulating D-loop haplotype network showed no overall phylogeographic concordance, as the Comorian samples were part of a mixed haplotype (H8), together with Madagascan *M. leucostigma* samples from Ankazamborona and Andranofasika, which was internal to the network (Fig 7a). There were also no readily apparent associations between altitude (Fig 7b), aspect (Fig 7c) or (gender) and haplotype distribution. All connections between Malagasy and Comorian *Mops leucostigma* occurred with the 95 % connection.

The outgroup, *M. condylurus,* was separated from the *M. leucostigma* network by 33 mutational steps, and connected to an unsampled haplotype between H8 and H11 by a less than 95 % parsimony connection limit.

3.6 Phenetic and cladistic analysis

3.6.1 *Maximum parsimony, neighbour joining and Bayesian analysis*

NJ, MP, and Bayesian trees were constructed for the cytochrome *b* and D-loop datasets. Given that all phylogenetic and phenetic methods produced trees with congruent topologies and to avoid redundancy, only the Bayesian trees for cytochrome *b* and the D-loop are presented for cytochrome *b* and D-loop (Figs. 8 and Fig. 9 respectively), although neighbour-joining and maximum parsimony bootstrap support is indicated at key nodes.

3.6.1.1 Cytochrome *b*

Bayesian analysis was performed on the cytochrome *b* dataset using the HKY + I substitution model, as indicated by MrModeltest version 2.2. It generated a 99 percent credible set of 143551 trees, from which a 50 percent majority rule consensus tree was created (Fig. 8).

The phylogenetic tree representing relationships within and between *M. midas*, *M. condylurus* and *M. leucostigma* was rooted on the outgroup *Otomops martiensseni*. *Mops midas* samples formed a strongly-supported monophyletic clade (1.00 pp., NJ and MP bootstrap 99 %) and appeared ancestral to a strongly-supported reciprocally-monophyletic clade (1.00 pp., NJ and MP bootstrap 99 %) comprising *M. condylurus* and *M. leucostigma* samples. This clade formed two subclades, one consisting of a well-supported *M. condylurus* (1.00 pp., NJ and MP 99 % bootstrap) clade, which was sister to a monophyletic *M. leucostigma* subclade, which was less well supported (0.51 pp., NJ and MP 98 % bootstrap).

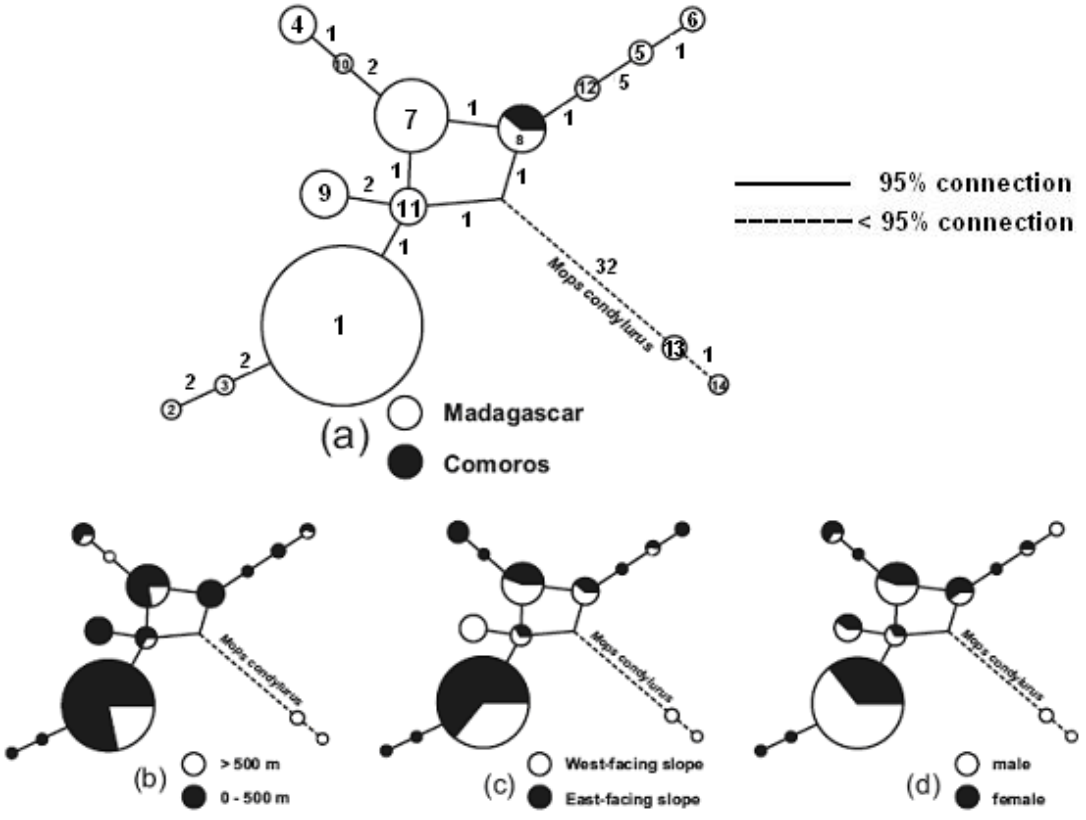

Figure 7: Haplotype networks showing mutational relationships between 12 mitochondrial D-loop (380 nucleotides) haplotypes of *Mops leucostigma* obtained from 61 samples with reference to the outgroup, *M. condylurus*: (a) distribution of samples from Madagascar and Comoros, (b) distribution of samples between low and high altitudes on Madagascar, (c) distribution of samples between east- and west-facing slopes on Madagascar, (d) distribution of samples among males and females on Madagascar. Numbers within circles are haplotypes; numbers adjacent to connecting lines represent mutational steps.

The *M. leucostigma* clade was essentially an undifferentiated polytomy and demonstrated little resolution beyond a weakly-supported monophyletic subclade (0.70 pp., NJ 63 % and MP 56 % bootstrap) consisting of the four Comorian samples, which was based on one shared substitution which differentiated Malagasy and Comorian samples (Table 5). There was no resolution of samples from any of the 24 Malagasy locations, irrespective of position on the island. Likewise, there was differentiation into clades based on gender, altitude or aspect (east and west facing slopes).

3.6.1.2 D-loop

Bayesian analysis was performed on the D-loop dataset using the HKY + I substitution model and generated a 99 percent credible set of 54 451 trees, from which a 50 percent majority rule consensus tree, depicting the relationships within *M. leucostigma* rooted on *M. condylurus*, was created (Fig. 9).

The outgroups *Mops midas* and *Otomops martiensseni* were omitted from the analysis owing to saturation in comparisons involving these samples and because, as mentioned by Kjer and Honeycutt (2007), there may have been alignment problems in these relatively highly-divergent taxa.

There was strong support for the grouping of both Malagasy and Comorian *leucostigma* into a monophyletic *Mops leucostigma* clade (1.00 pp., NJ 99 % and MP 100 % bootstrap). As with the cytochrome *b* tree there was little definite geographically significant resolution within this clade, although the tree contains several subclades. The subclades reflect tentative groupings of locations (Fig. 9), but not strong geographic concordance, as no well-supported subclades consist exclusively of samples from any area.

One sample from Vohipeno and one from Irondro formed a subclade with strong support (1.00 pp., NJ 89 %, MP bootstrap 87 %) but not all samples from these locations were exclusively contained in this clade. Samples from two locations in north-western Madagascar, Mahajanga and Ankazamborona, formed a separate subclade with strong support (0.96 pp., NJ 77 % and MP bootstrap 89 %) consisting exclusively of 3 Mahajanga and 2 Ankazamborona samples. The Ankazamborona samples do not occur exclusively within this clade, but also form part of another subclade. However it was observed that this clade contains the female samples while the males are included in the other subclade.

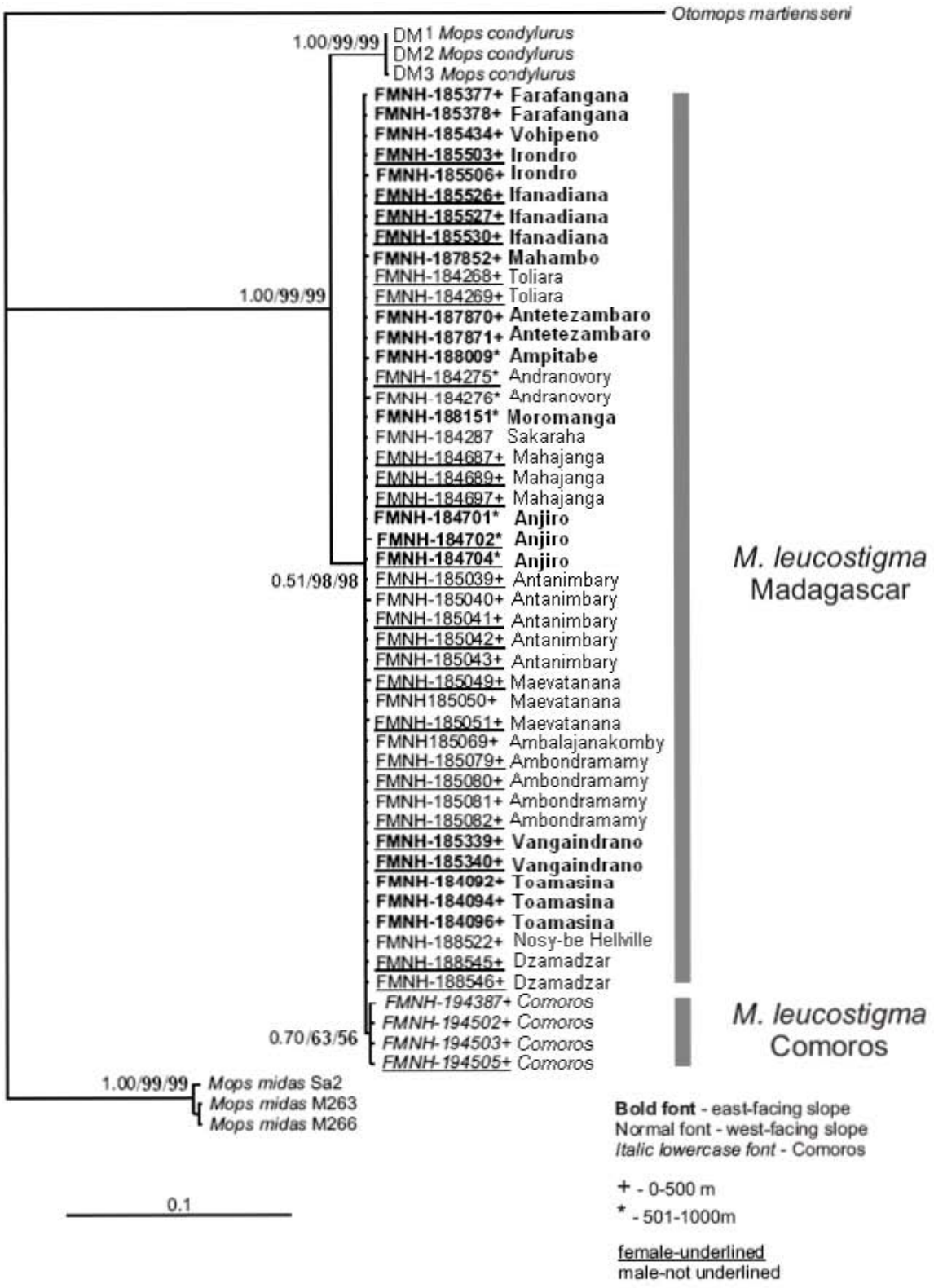

Figure 8: Bayesian phylogram, based on 1008 nucleotides of the cytochrome *b* gene, showing relationships between 49 samples of *Mops leucostigma* with reference to the outgroups, *M. condylurus*, *M. midas* and *Otomops martiensseni*. Support indicated is, in order: Bayesian posterior probability, neighbour joining and maximum parsimony bootstrap support.

Samples from Anjiro, Mahambo, Brickaville and Vangaindrano formed an essentially unsupported subclade (0.92 pp., NJ and MP bootstrap 83 %), and the latter 3 locations formed a further essentially-unsupported clade (0.83 pp., NJ 70 % and MP 68 % bootstrap). Two Antetezambaro samples, and one each from Brickaville and Dzamadzar formed a group with strong support (0.97 pp., NJ and MP 99 % bootstrap).

The distinctiveness of the Comorian samples, as displayed by the cytochrome *b* phylogenetic and haplotype analysis, is not supported by the D-loop data. Comorian *M. leucostigma* was not monophyletic and formed part of an unsupported subclade (0.51 pp., NJ 73 % and MP 63 % bootstrap) together with samples from Ankazamborona and Andranofasika in Madagascar.

Groupings also did not exhibit any apparent relationships with gender, altitude or aspect (east and west-facing slopes).

3.6.2 *Genetic distances*

3.6.2.1 Cytochrome *b*

The overall mean genetic distance between *M. leucostigma* samples based on 1008 nucleotides of the cytochrome *b* dataset was 0.22 % (Table 7).

Table 7: Genetic distances ($x10^2$) based on 1008 nucleotides of the cytochrome *b* gene, between 9 *Mops leucostigma* haplotypes and the outgroups, *M. condylurus*, *M. midas* and *Otomops martiensseni.* H – haplotype.

Taxon	H	1	2	3	4	5	6	7	8	9	10	11	12
	1	-											
	2	0.01	-										
	3	0.01	0.20	-									
	4	0.20	0.30	0.30	-								
M. leucostigma	5	0.01	0.20	0.20	0.30	-							
	6	0.01	0.20	0.20	0.30	0.20	-						
	7	0.01	0.20	0.20	0.30	0.20	0.20	-					
	8	0.20	0.30	0.30	0.40	0.30	0.30	0.30	-				
	9	0.01	0.20	0.20	0.30	0.20	0.20	0.20	0.01	-			
M. condylurus	10	2.44	2.54	2.54	2.44	2.54	2.54	2.54	2.64	2.54	-		
M .midas	11	13.80	13.78	13.78	13.80	13.92	13.91	13.67	13.81	13.68	13.78	-	
O. martiensseni	12	17.24	17.37	17.37	17.24	17.38	17.37	17.10	17.37	17.22	16.90	16.46	-

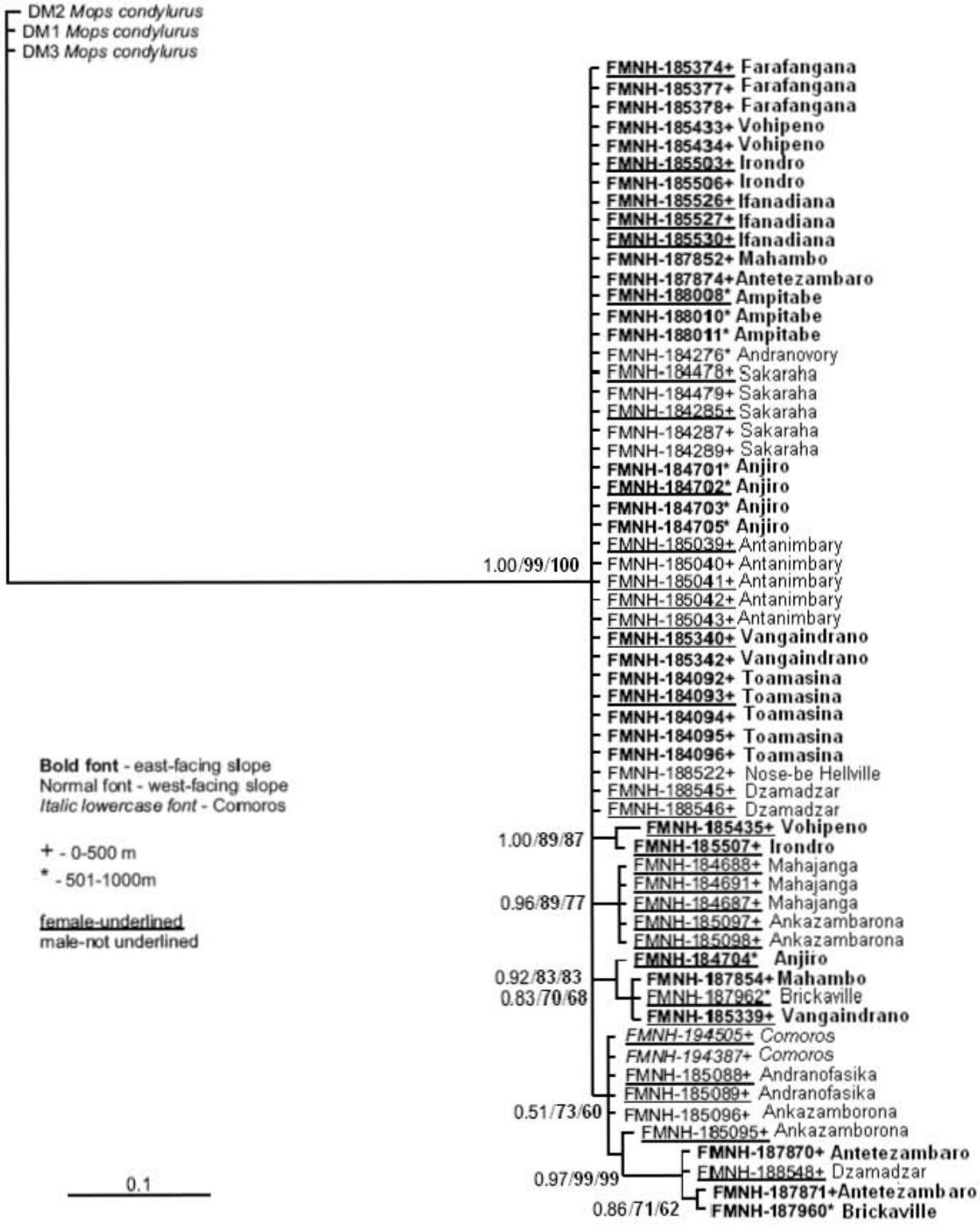

Figure 9: Bayesian phylogram based on 380 nucleotides of the mitochondrial D-loop, showing relationships between 61 samples of *M. leucostigma* with reference to the outgroup, *M. condylurus*. Support indicated, is in, order Bayesian posterior probability and neighbour-joining and maximum parsimony bootstrap support.

Genetic distances between Malagasy *M. leucostigma* haplotypes ranged from 0.01 to 0.30 % (Table 7). Comorian *M. leucostigma* samples were separated from Malagasy *M. leucostigma* by genetic distances of 0.01 to 0.40 % (mean 0.20 %) and from each other by 0.01 %.

Genetic distances of 2.44 to 2.64 % were observed between *M. leucostigma* and *M. condylurus*, with a mean distance of 2.54 % separating the two taxa. *M. midas* is separated from *M. condylurus* and *M. leucostigma* by mean genetic distance of 13.80 and 13.78 % respectively. *Otomops martiensseni* is separated from *M. midas* by a distance of 16.46 %, from *M. condylurus* by 16.90 % and from *M. leucostigma* by a mean distance of 17.29 % (Table 7). The entire *Mops* group is separated from *O. martiensseni* by a mean genetic distance of 16.90 %.

3.6.2.2 D-loop

Genetic distances within 380 nucleotides of the D-loop dataset of *M. leucostigma* are given in Table 8.

Table 8: Genetic distances ($x10^2$) based on 380 nucleotides of the mitochondrial D-loop between 12 *M. leucostigma* haplotypes and the outgroup, *M. condylurus*. H – haplotype.

Taxon	H	1	2	3	4	5	6	7	8	9	10	11	12	13
	1	-												
	2	2.27	-											
	3	0.61	0.61	-										
	4	1.55	2.80	2.17	-									
	5	2.84	4.11	3.46	3.16	-								
M. leucostigma	6	3.16	4.46	3.80	3.50	0.31	-							
	7	0.61	1.85	1.23	0.92	2.19	2.51	-						
	8	0.92	2.17	1.54	1.24	1.87	2.19	0.31	-					
	9	0.92	2.17	1.54	1.87	3.16	3.49	0.92	1.24	-				
	10	1.24	2.48	1.86	0.31	2.84	3.16	0.61	0.92	1.55	-			
	11	0.31	1.54	0.92	1.24	2.51	2.84	0.31	0.61	0.61	0.92	-		
	12	1.24	2.48	1.85	1.55	1.55	1.87	0.61	0.31	1.55	1.24	0.92	-	
M. condylurus	13	11.71	13.16	12.40	12.11	12.09	12.48	11.70	11.30	12.12	11.70	11.31	11.69	-

The mean genetic distance between 61 *M. leucostigma* samples was 1.91 %. Genetic distances between 12 *Mops leucostigma* haplotypes ranged from 0.31 to 4.46 % (Table 8). *M. leucostigma* samples from the Comoros (H8) were separated by genetic distances of between 0.00 % and 2.17 % from Malagasy *M. leucostigma* samples, with a mean genetic distance of 1.46 %. *M. leucostigma* displayed a mean genetic divergence of 11.98 % from *M. condylurus* (range 11.30 to 13.16 %).

4 DISCUSSION

4.1 Dataset Analysis

The level of saturation in both the cytochrome *b* and D-loop datasets was assessed using saturation plots (Xia 2000) and the test of substitution saturation by Xia *et al*. (2003). Analyses indicated little saturation in either dataset, signifying that they were suitable for further phylogenetic and phenetic analyses.

The D-loop dataset contains a lower percentage of conserved sites than the cytochrome *b* dataset (83.7 % versus 99.1 %). This difference is reflective of the more rapidly evolving, less conservative nature of the non-coding D-loop region and its potential to detect finer regional variation than the coding cytochrome *b* region, which is less variable as it is subject to selective constraints, particularly at codon positions 1 and 2 (section 1.3.3).

4.2 Genetic Distances and Phylogeny of *Mops leucostigma*

Most analyses provided strong support for a monophyletic Malagasy and Comorian *M. leucostigma* group (cytochrome *b* - MP and NJ bootstrap 98 %; D-loop - NJ bootstrap 99 %, MP bootstrap 100 %, 1.00 pp), with the exception of the Bayesian analysis of the cytochrome *b* data (0.51 pp.) This outcome, which does not correspond with the other support estimates, may be explained by one or more of the Markov chains being stuck on a sub-optimal likelihood plateau, few unambiguous characters along that specific branch or the prescence of homoplasy (Figs. 8 and Fig. 9). Malagasy and Comorian samples were separated by cytochrome *b* genetic distances of between 0.01 and 0.40 % (Table 7), which is less than the range reported for inter-population distances of other bat species (1.4 to 1.9 %) (Baker and Bradley 2006). Other species have also shown genetic distances much lower than the range reported by Baker and Bradley (2006), such as a maximum cytochrome *b* genetic distance of 0.35 % between 39 *Chaerephon leucogaster* samples (Ratrimomanarivo *et al.* in press, b) and a maximum cytochrome *b* distance of 0.1 % between 22 *Mops midas* samples from Madagascar and South Africa (Ratrimomanarivo *et al.* 2007). These low distances suggest that Malagasy and Comorian *M. leucostigma* form a genetically rather uniform group and may be sub-populations of one large population. Additionally they suggest that the body of water between Madagascar and the Comoros does not provide a barrier to inter-island dispersal and gene flow.

Mops condylurus forms a well-supported (MP and NJ bootstrap 99 %, 1.00 pp) monophyletic group, which is sister to *M. leucostigma*. The strongly-supported reciprocal monophyly of the two taxa may be taken as an indication that they are good phylogenetic species according to the PSC (Fig. 8). *Mops condylurus* and *M. leucostigma* were separated by a mean cytochrome *b* genetic distance of 2.54 % (Table 7), which is less than the sister species divergence values of 3.3 to 14.7 % reported Baker and Bradley (2006), but greater than their intraspecific values of 0.6 to 2.3 %. However the inter- and intraspecific data reported by Baker and Bradley (2006) were based on 10 studies each, and did not include any Molossidae. It appears that at least some of the distance values obtained for molossid bats may be low relative to non-molossids. For instance, the cytochrome *b* genetic distances separating *Chaerephon leucogaster* from its sister species, *C. Pumilus,* range from 1.8 to 2.7 %, (Ratrimomanarivo *et al.* in press, b). A 4.1 % cytochrome *b* distance was observed between the sister species *Otomops madagascariensis* (Dorst, 1953) and *O. martiensseni* (Matschie, 1897) (Lamb *et al*. submitted). Hence the genetic distance of 2.5 % separating *Mops leucostigma* and *M. condylurus* is in keeping with interspecific values observed for some other molossid species and supports the status of *M. condylurus* and *M. leucostigma* as sister species.

Chiroptera in general have been considered to have mutation rates slower than the mammalian average (Nabholz *et al.* 2008). Simões *et al.* (2007) examined cytochrome *b* genetic variation in populations of *Myotis daubentonii daubentonii* and *Myotis daubentonii nathalinae* and found the two sub-species to be separated by a divergence of 2.5 – 3.0 %. The authors mentioned that although Baker and Bradley (2006) reported a range of 3.3 – 14.7 % cytochrome *b* divergence among sister bat taxa, the figure for *Myotis* may be as low as 2.3 – 3.8 % (Rodriguez and Ammerman 2004). Sister taxa in several other bat species have been found to display shallow genetic divergence, for example two distinct species, *Eptesicus serotinus* and *E. nilssonii*, which are clearly distinct morphologically and occur in sympatry with no indication of hybridisation, exhibit a cytochrome *b* divergence of 1.7 % (Ruedi and Mayer 2001). Cytochrome *b* divergence between *Myotis myotis* and *M. blythii* was less than 2.6 % (Mayer and von Helversen 2001), while the sibling species *M. myotis* and *M. oxygnathus* showed a divergence of 2.5 % (Ruedi and Mayer 2001). Salgueiro *et al.* (2004) also found extremely shallow genetic divergence between two species of *Nyctalus*, *Nyctalus leisleri* and *N. azoreum* (1.2 %). This was unexpected as these species are phenotypically quite distinct on the basis of size, pelage colouration, echolocation frequency and patterns of daytime activity, as are *M. condylurus* and *M. leucostigma*, which are 2.5 % divergent. Salgueiro *et al.* (2007) considered that the lower divergences may be due to recent speciation or introgression of mitochondrial

haplotypes. Furthermore divergent morphological characters may be adaptive features that have evolved rapidly in response to selection (Salgueiro *et al.* 2007). Incomplete lineage sorting may also tend to reduce divergence between sister-species, although this does not appear to be the case for *M. leucostigma* and *M. condylurus* which appear to be reciprocally monophyletic, although the *M. condylurus* sample was small.

Mops midas is separated from the monophyletic *M. leucostigma*/ *M. condylurus* group by a mean genetic distance of 13.79 %, which is at the high end of the sister species range (3.3 – 14.7 %) and above the middle of the inter-generic range (8.4 – 15.7 %) reported by Baker and Bradley (2006). In terms of genetic distances, *M. midas* appears somewhat equidistant between *M. leucostigma* (13.8 % separation) and *Otomops martiensseni* (16.5 % separation). The *midas*/ *leucostigma /condylurus* group may have diverged basally within *Mops*, or it is possible that *M. midas* belongs to a different genus, although such a conclusion would need to be based on a comprehensive study of genetic variation in Molossidae.

The *Mops* group is separated from the outgroup, *Otomops martiensseni,* by a mean cytochrome *b* genetic distance of 16.9 %, which is slightly greater than but consistent with distances expected for an inter-genus comparison (8.4 % to 15.7 %) under the genetic species concept (Baker and Bradley 2006).

D-loop data (Fig. 9) supported the principal findings based on phylogenetic analysis of cytochrome *b* data as: (1) *Mops leucostigma* and *M. condylurus* appear as reciprocally-monophyletic sister taxa and (2) *Mops leucostigma* samples from Madagascar and the Comoros form a monophyletic group in which the Comoros samples are separated from the Malagasy samples by low genetic distances (0.00 % - 2.19 %).

The lack of distinctiveness of the Comoros samples and the fact that they share a D-loop haplotype with Malagasy samples from Andranofasika and Ankazomborona is striking, given their separation from Madagascar by 300 km of ocean. D-loop distances followed a similar trend to those shown by cytochrome *b*, although they were greater than corresponding cytochrome *b* distance values, which is to be expected given that the D-loop is non-coding and that fixation of substitutions is less subject to functional constraints.

4.3 Phylogeographic analysis of genetic variation in *Mops leucostigma*

4.3.1 *Haplotype and nucleotide diversity*

Haplotype diversity values for *Mops leucostigma* (cytochrome *b*, 0.367 and D-loop, 0.758) appear low when compared with those of other molossid species, such as *Otomops martiensseni* (0.876, 0.952), *Otomops madagascariensis* (0.945, 0.968) (Lamb *et al.* 2008 and unpublished data), *Chaerephon leucogaster* (0.718, 0.870) (Ratrimomanarivo *et al.* in press, b) and *Tadarida brasiliensis* (0.987, 0.998) (Russell *et al.* 2005). *Mops leucostigma* cytochrome *b* haplotype diversity is also lower (0.70 to 0.97) than that reported for *Triaenops* populations (Russell *et al*. 2007).

A similar trend was displayed by the nucleotide diversity per site of *Mops leucostigma* cytochrome *b* and D-loop sequences (0.0005 and 0.0090 respectively), which appear low relative to values for comparable populations of *O. madagascariensis* (0.0070 and 0.0200), *O. martiensseni* (0.0040 and 0.0300) (Lamb *et al.* 2005, and unpublished), *M. midas* (0.0008 and 0.0040) (Ratrimomanarivo *et al*., 2007) and *Chaerephon leucogaster* (0.0010 and 0.0070) (Ratrimomanarivo *et al.* in press, b).

Thus it appears that the genetic variation displayed by *Mops leucostigma* falls at the lower end of the range for Molossidae, revealing values comparable to those of *Mops midas,* which has been described as extremely conservative (Ratrimomanarivo *et al*. 2007). These low haplotype and nucleotide diversity values are consistent with the low genetic diversity, as indicated by genetic distance analysis of *Mops leucostigma* populations on both Madagascar and the Comoros.

4.3.2 *Haplotype networks*

Haplotype distributions within *M. leucostigma* show no geographic concordance and little diversity, as no cytochrome *b* haplotype differs from any other (Malagasy or Comorian) by more than 4 mutational steps. This is indicative of widespread genotype mixing across these islands, and frequent gene flow between populations, possibly combined with a relatively recent origin and/or a low mutation rate. The exclusivity of Comorian haplotypes H8 and H9, seen in the cytochrome *b* data, was not supported by D-loop data, further emphasizing the lack of geographic exclusivity and high genetic similarity of the Comorian samples. This could be due to relatively frequent gene flow between Madagascar and Mohéli and Anjouan in the Comoros.

Another explanation might be relatively recent colonization, most probably of the Comoros by animals from Madagascar, as the Comorian cytochrome b haplotypes are separated by 1 or 2 mutations from the most common *Mops leucostigma* cytochrome *b* haplotype (H1) from Madagascar.

Haplotype distribution was not found be correlated with gender or altitude, further indicating a lack of phylogeographic structure in *M. leucostigma*. The morphological distinction between eastern and western populations (Ratrimomanarivo *et al.* in press, a) was not reflected in the haplotype distribution, possibly indicating, insofar as cytochrome *b* and D-loop diversity are representative of general genomic diversity, that these morphological differences are not genetically-based.

Mops leucostigma appears to conform to Avise's phylogeographic Category IV, which is characterised by shallow gene trees of closely related lineages found in sympatry, and to Category IV* in which animals are phenotypically different but show weak or no genetic divergences (Avise 2000). This lack of differentiation may be explained by local hybridisation or the existence of morphotypes maintained by selection (Ruedi and McCracken 2005). These patterns are commonly observed in local populations connected by high levels of gene flow or that recently colonised new areas. A typical indication of such populations is a "star-like" relationship of haplotypes, consisting of a common haplotype at the centre of the network and rare variants radiating from this ancestral sequence (Ruedi and McCracken 2005), which was observed in the cytochrome *b* haplotype network (Fig. 6).

Other species have been seen to conform to category IV and show similar patterns to that displayed by *M. leucostigma*. *Nyctalus noctula* exhibited low geographic structure among its D-loop haplotypes, with a widespread lineage occurring over its entire range and several private haplotypes in single colonies. Closely related sequences differed by only one or two mutations from the most common haplotype (resembling *M. leucostigma* haplotype networks) (Figs. 6 and 7) (Petit *et al.* 1999). *Nyctalus azoreum*, an endemic bat from the Azores archipelago displayed a star-like haplotype network with a few widespread D-loop haplotypes and others that were closely related and restricted to specific islands. Ruedi and McCracken (2005) state that the occurrence of shared haplotypes between populations on remote islands may suggest rare over-water migrations or that the haplotypes represent a source population that reached the archipelago and persisted on the different islands as shared ancestral polymorphisms. This may also apply to the sharing of haplotypes between the Comoros and Madagascar.

Certain *Rhinolophus* species have been shown to exhibit similar phylogeographic patterns to *Mops leucostigma*. *Rhinolophus ferrumequinum* and *R. cornutus* from the Japanese Archipelago (Sakai *et al.* 2003), despite occupying a fragmented insular landscape that could have increased genetic differences among island populations, showed no significant genetic structure within or among insular populations, with haplotypes being widespread and closely related (Ruedi and McCracken 2005).

Findings from a study on *Rhinolophus philippinensis* in Sulawesi also somewhat mirror those found for *M. leucostigma*, as the species consisted of three different morphotypes but were found to be only marginally divergent at the D-loop level (0.0 - 2.2 %) and did not form monophyletic groups (Kingston and Rossiter 2004), hence conforming with category IV*.

4.4 Expansion of *M. leucostigma* populations

High haplotype and low nucleotide diversity, a high expansion coefficient and a unimodal mismatch distribution (Hull and Girman 2005) (section 3.5.1.2) are consistent with an exponentially-expanding population. Furthermore a star-like haplotype tree, as observed particularly for the *Mops leucostigma* cytochrome *b* data in this study, is representative of a population undergoing expansion. The time since expansion of *M. leucostigma* populations was estimated at between 3379 and 7210 years BP, which predates the arrival of humans on Madagascar, approximately 2300 BP (Burney *et al.* 2004). *Mops leucostigma* is presently predominantly synanthropic and occupies human dwellings (Goodman *et al*. 2005, Ratrimomanarivo *et al.* in press, a). Prior to the existence of human dwellings on the island the species must have occupied natural roost sites, which gives rise to the question of whether this species expanded its geographical range as a consequence of human colonisation, and if so what the reason may have been. However as the date for expansion predates the human colonisation of Madagascar, this also begs the question of what the natural roost sites were.

According to dating analysis conducted by Ratrimomanarivo *et al.* (in press, a), who based their inference on cytochrome *b* data, Comorian and Malagasy *M. leucostigma* populations diverged sometime between 34 000 and 373 000 years ago in the late Pleistocene and Holocene. These dates indicate that *M. leucostigma* may have reached the Comoros long before human colonisation of Madagascar.

The minimal genetic structuring and high frequency of a few main haplotypes seen in both cytochrome *b* and D-loop *M. leucostigma* networks could also be attributed to possible contraction of ranges during retreat to Pleistocene glacial refugia and subsequent range expansion following the glacial period. The earliest divergence date of *M. leucostigma* (34 000 years) predates the last glacial maximum (~ 18 000 years ago), but the star-like structure of the cytochrome *b* haplotype network suggests that the most common haplotype may have been one which survived repeated glaciations and has persisted since the last glacial maximum, thus explaining its frequency among *Mops leucostigma* populations. Petit *et al.* (1999) examined the genetic consequences of refugia on the distribution of *Nyctalus noctula*. They found that this species underwent expansion and range shifts following restriction and isolation in three refugia during the last glaciations, and that this, combined with male-mediated gene flow, may have resulted in low population genetic structuring.

It is surprising that no *Mops* species were recorded from the Comoros prior to the report of Racey *et al.* (in press), given that the divergence of Comorian and Malagasy *M. leucostigma* was estimated at between 34 000 and 373 000 years ago (Ratrimomanarivo *et al.* in press, a). Goodman (in 2006 and 2007) (Ratrimomanarivo *et al.* in press, a) also found no evidence of *Mops* on the island of Mayotte, which is the closest of the Comoros islands to Madagascar, though synanthropic populations of *M. leucostigma* were found on the more distant Mohéli and Anjouan islands. A possible explanation for the absence of *M. leucostigma* on Mayotte is the extensive construction on the island in past decades, which has left very few of the old-style architectural buildings, which tend to serve as synanthropic sites (Ratrimomanarivo *et al.* in press, a). Populations of *M. leucostigma* on the Comoros (cytochrome *b* H8 and H9) are no more distant (1 or 2 mutational steps) from the predominant Malagasy haplotype (1) than are other Malagasy haplotypes. It is therefore possible that, although the haplotypes (H8 and H9) arose > 34 000 years ago, colonization of the Comoros by these haplotypes was more recent and due to over-water dispersal.

4.5 Conservation

Since *Mops leucostigma* is widespread and has been shown to be an expanding population, it is likely that the species is not threatened. Further, the IUCN (Andriafidison *et al.* 2008) has most recently designated the species as Least Concern where previously it was regarded as Data Deficient (Hilton-Taylor 2000, IUCN 2007). The morphological, bioclimatic, dating and genetic research undertaken in this study and in Ratrimomanarivo *et al*. (in press, a) support the

latest classification. The species is not under threat; however human-associated threats related to its synanthropic occurrence, hunting for meat, slash and burn agriculture, habitat disturbance and destruction (such as human habitat encroachment and conversion into cattle pasture) (Goodman 2006), justify general conservation measures, such as continued monitoring, for synanthropic bats.

The cytochrome *b* dataset shows two exclusive Comoros haplotypes, which due to recent widespread construction in the archipelago, may possibly be under threat, as the newer types of buildings may not necessarily incorporate the architectural features, such as roof spaces, which make suitable roosts. Thus Comoros populations of *M. leucostigma* may warrant conservation attention. However the exclusivity of the Comoros haplotypes is based on four samples only, and was not supported by the D-loop data. Thus a larger sample size should be examined to confirm the distinctiveness of Comorian populations before designation as a possible management unit.

4.6 General Discussion

Morphologically, Ratrimomanarivo *et al.* (in press, a) demonstrated phenotypic variability between *Mops leucostigma* populations from the eastern and western slopes of the island, and similarity between Comorian and western Malagasy *M. leucostigma* populations (section 1.1.1). The results were supported by AMOVA tests using the cytochrome *b* dataset (Ratrimomanarivo *et al.* in press, a).

In contrast, the genetic examination of *Mops leucostigma* undertaken here displays very low levels of genetic structure and variability in both the cytochrome *b* gene and D-loop regions, and no apparent correlation between haplotype distribution and gender, altitude or aspect (east- and west-facing slopes).

The lack of genetic structure and variability in *M. leucostigma* may be explained by extensive current or historical dispersal and gene flow. As Brown *et al*. (2007) mention, persistent gene flow can swamp genetic differentiation and speciation in highly mobile species that are distributed across a continuous habitat. *Mops leucostigma* in Madagascar is widely distributed with no potential barriers to gene flow. Furthermore gene flow between Mohéli and Anjouan in the Comoros and Madagascar is probable as is indicated by the high genetic similarity between samples from both areas.

In migratory species, gene flow typically results in minimal population substructure, whilst considerable genetic structure is expected in non-migratory species (Miller-Butterworth *et al.* 2003, Russell *et al.* 2005). The dispersal ability of bats through powered flight allows high gene flow between populations and consequently low genetic structuring. This is frequently seen in many species with wide geographic ranges, such as *Tadarida brasiliensis, Nyctalus noctula, Pteropus* species and *Leptonycteris curasoae* (Webb & Tidemann 1996, Wilkinson & Fleming 1996, Petit *et al.* 1999, Castella *et al.* 2001, Russell *et al.* 2005). However exceptions do occur due to factors such as philopatry, vicariant barriers in the form of mountains and large bodies of water, and isolation of gene pools due to migration patterns in migratory species (Petit *et al.* 1999, Castella *et al*. 2000, Miller-Butterworth 2003, Ruedi and McCracken 2005). Therefore despite the potential of flight dispersal, varying degrees of genetic structure may be exhibited by bat species (Castella *et al*. 2001), as in the case for *Miniopterus schreibersii natalensis* (Miller-Butterworth *et al.* 2003), where strong population genetic structuring is found although the species is migratory. The extensive genotype mixing and low genetic structure observed in *M. leucostigma* could be as a result of widespread dispersal across the island, and possibly across to the Comoros as all locations are well within the flight range of the species, as molossids are capable of high and fast flight and have the potential for covering relatively large distances (probably tens to hundreds of kilometres) (Taylor 2008).

There are many possibilities concerning the origin of dispersal of *Mops leucostigma*. Since the origin of Chiroptera has been dated by Teeling *et al.* (2005) at 71 - 58 MYA and the African mainland and Madagascar have been separated from each other for about 160 to 140 million years (Russell *et al.* 2008), it appears that the presence of (African) bats on Madagascar can be attributed to over-water colonisation. One possibility is an origin in mainland Africa, which would involve dispersal of individuals (of *Mops condylurus*) eastwards over the Mozambique channel to Madagascar and the subsequent divergence and adaptation of the Malagasy form, isolated due to restricted gene flow, into modern-day *Mops leucostigma*. The converse hypothesis may also apply, involving an origin in Madagascar and a westward dispersal to mainland Africa, with subsequent diversification to form modern-day *Mops condylurus*. A similar overwater dispersal scenario for bats appears to be the best explanation for their existence on the Comoros Islands, an archipelago located between northernmost Madagascar and the African coastline, composed of *in situ* volcanic islands which range in age from 0.5 to 10 - 15 million years (Nougier *et al*. 1986) and which have lacked any land connections since formation (Ratrimomanarivo *et al.* in press, b). Several of the Malagasy and western Indian Ocean molossid bats are proposed to have had African origins preceding their dispersal over the

Mozambique Channel (Ratrimomanarivo *et al*. 2007, Ratrimomanarivo *et al*. in press, b, Lamb *et al*. submitted).

Ratrimomanarivo *et al.* (2007) reported that *Mops midas* (Sundevall 1843) populations in both Africa and Madagascar exhibited no significant morphological or genetic variation, probably due to regular and recent exchanges between them. Ratrimomanarivo *et al.* (in press, b) examined morphological and genetic diversity within populations of *Chaerephon leucogaster* and found, as has been reported for *Mops leucostigma*, morphological size variations between individuals from differing geographic areas. *Chaerephon leucogaster* animals from Mayotte were morphologically largely identical to those from western Madagascar, as is the case for *Mops leucostigma*, where Comorian and western Malagasy populations were similar.

Ratrimomanarivo *et al.* (in press a) showed that individuals of *C. leucogaster* from the extreme south and south west and extreme north of Madagascar were larger in certain measurements than those from the central west. However, unlike for *M. leucostigma*, there was some phylogeographic concordance as unique D-loop haplotypes were found in the north and extreme south west of the island, although they were only separated by one mutational step from the most common haplotype.

4.7 Conclusions

In terms of a genetic species concept, *M. leucostigma* and *M. condylurus* appear to have evolved into separate species, or to be on this path. They also appear to be good species under a phylogenetic species concept. Consequently, the genetic findings of this study together with the morphological findings of Ratrimomanarivo *et al.* (in press, a) support the conclusions of Peterson *et al*. (1995), that *M. leucostigma* and *M. condylurus* should be considered specifically distinct.

Comorian *Mops leucostigma* was shown to be genetically referable to Malagasy *M. leucostigma*, specifically to populations from western Madagascar, again supporting the morphological data. The morphological variation demonstrated by Ratrimomanarivo *et al.* (in press, a) between *Mops leucostigma* populations from the eastern and western-facing slopes of Madagascar is not supported by cytochrome *b* and D-loop sequence analyses and is thought to be largely adaptive in nature, associated with different climatic regimes. The species is genetically homogeneous, with no discernable genetic correlates with geography, gender,

elevation and /or slope aspect. There is seemingly widespread genotype mixing over the entire geographic range on Madagascar, with no area containing an exclusive genotype. The Comoros contained two exclusive cytochrome *b* haplotypes, the closest of which was separated from the most common Malagasy haplotype by one mutational step, but was not distinct at the D-loop level.

Due to the lack of genetic diversity of the species across Madagascar and the similarity between Comorian and Malagasy populations, it appears likely that across island and inter island gene flow is common.

The most recent designation of *Mops leucostigma* by the IUCN as Least Concern (Andriafidison *et al.* 2008) is supported by morphological, bioclimatic, dating and genetic research undertaken in this study and in Ratrimomanarivo *et al*. (in press, a). The species is not under immediate threat, however general conservation measures may be needed, as this synanthropic species may be susceptible to anthropogenic threat.

The conclusions of this study are based on DNA sequences of two mitochondrial regions and hence have a matrilineal bias. Further studies should focus on nuclear DNA (sequencing and microsatellites) to provide a broader perspective of the genetic history and population structure of *Mops leucostigma*.

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6 APPENDICES

6.1 Appendix A

GenBank Accession Numbers for *Mops leucostigma* samples

FMNH	Accession Numbers	
	Cytochrome *b*	D-loop
185374	-	FJ546258
185378	FJ546209	FJ546260
185433	-	FJ546261
185434	FJ546210	FJ546262
185435	-	FJ546263
185377	FJ546211	FJ546259
185503	FJ546212	FJ546264
185506	FJ546213	FJ546265
185507	--	FJ546266
185526	FJ546214	FJ546267
185527	FJ546215	FJ546268
185530	FJ546216	FJ546269
187852	FJ546217	FJ546270
187854	-	FJ546271
184268	FJ546218	-
184269	FJ546219	-
187870	FJ546220	FJ546272
187871	FJ546221	FJ546273
187874	-	FJ546274
187960	-	FJ546275
187962	-	FJ546276
188008	-	FJ546277
188009	FJ546222	-
188010	-	FJ546278
188012	-	FJ546279
184275	FJ546223	-
184276	FJ546224	FJ546280
188151	FJ546225	-
184478	-	FJ546281
184479	-	FJ546282
184285	-	FJ546283
184287	FJ546226	FJ546284
184289	-	FJ546285
184687	FJ546227	-
184688	-	FJ546288
184689	FJ546228	-
184691	-	FJ546289
184697	FJ546229	FJ546290
184701	FJ546230	FJ546291
184702	FJ546231	FJ546292

184703	-	FJ546293
184704	FJ546232	FJ546294
184705	-	FJ546295
185039	FJ546233	FJ546296
185040	FJ546234	FJ546297
184041	FJ546235	FJ546298
185042	FJ546236	FJ546299
185043	FJ546237	FJ546300
185049	FJ546238	-
185050	FJ546239	-
185051	FJ546240	-
185069	FJ546241	-
185079	FJ546242	-
185080	FJ546243	-
185081	FJ546244	-
185082	FJ546245	-
185088	-	FJ546301
185089	-	FJ546302
185095	-	FJ546303
185096	-	FJ546304
185097	-	FJ546305
185098	-	FJ546306
185339	FJ546246	FJ546307
185340	FJ546247	FJ546308
185342	-	FJ546309
184092	FJ546248	FJ546310
184093	-	FJ546311
184094	FJ546249	FJ546312
184095	-	FJ546313
184096	FJ546250	FJ546314
188522	FJ546251	FJ546315
188545	FJ546252	FJ546316
188546	FJ546253	FJ546317
188548	-	FJ546318
194387	FJ546254	FJ546287
194502	FJ546255	-
194503	FJ546256	-
194505	FJ546257	FJ546286