Genetic variability of *Chaerephon atsinanana* (Chiroptera) within the context of the Afro-Malagasy Molossidae; a mitochondrial and nuclear perspective.

by

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As the candidate's supervisor I have/have not approved this dissertation for submission.

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OVERALL ABSTRACT

This study has focused on genetic variability and structure in *Chaerephon atsinanana*, a newly-described molossid bat found in the mid to southern region of the eastern watershed of Madagascar. As these bats are strong fliers, and are able to traverse the riverine and mountain barriers within the landscape, it was hypothesized that they would show relatively low levels of intraspecific genetic structure, consistent with patterns shown for other Molossidae on Madagascar (*Mormopterus jugularis*, *Mops midas*, *Mops leucostigma*, and *C. lecuogaster*.

Phylogenetic (neighbor-joining, parsimony and Bayesian inference) and population genetic analyses of maternally-inherited mitochondrial control region sequences revealed the presence of 6 distinct haplotype groups separated by genetic distances of up to 8.14% (mean 4.95%). There were high levels of genetic structure among the haplotype groups (overall F_{ST}= 0.994). Thus the hypothesis of low levels of genetic structure was rejected. Bayesian skyline analyses and significantly ragged mismatch distributions were consistent with ancient stable *C. atsinanana* populations which were of constant size during the last two major Pleistocene glacial periods. This made retreat into and expansion from glacial refugia an unlikely explanation for such high levels of structure. An alternative hypothesis is that *C. atsinanana* haplotype groups are spatially structured as a result of philopatry. As mitochondria are maternally-inherited, this data is consistent with the existence of female philopatry in *C. atsinanana.*

The second aim of this study was to examine the genetic structure of *C. atsinanana* with nuclear sequence markers, which are biparentally-inherited, in order to provide information on the male contribution to gene flow and the possible presence of male philopatry in this molossid bat species. The initial objective was to amplify and sequence candidate nuclear markers in order to identify those which were variable among *C. atsinanana* samples. I attempted to amplify and sequence a set of 12 nuclear markers, identified from the literature, which had been reported to show high levels of variability, or which were untested and showed the potential for high levels of variability. Of these, the intron markers PNPO-3, SLC38A7-8, CARHSP1-1, GAD2-1, OSTA-5 had not previously been used in phylogenetic analyses while FES, GHR, RHO1 CHRNA1, STAT5, PRKC1 and THY had been. I was not able to amplify and/or sequence SLC38A7-8, CARHSP1-1, GAD2-1, OSTA-5, CHRNA1, STAT5 and THY across the range of the *C. atsinanana* samples. PNPO-3, FES, GHR, RHO1 and PRKC1were successfully amplified and sequenced, but showed no variability and very little polymorphism, and were therefore unsuitable for testing hypotheses related to genetic variability of *C. atsinanana* populations.

These five nuclear sequence markers were further used to investigate phylogenetic relationships among 5 genera (*Chaerephon*, *Mops*, *Mormopterus*, *Otomops* and *Sauromys*) and 13 species of Afro-Malagasy molossid bats, and to provide a nuclear phylogenetic perspective on the newly-described *C. atsinanana*. PNPO-3 is a novel nuclear intron marker, previously unused in phylogenetic studies. This intron provides resolution primarily at the genus level, and is less informative at interspecific level. These five nuclear markers were combined with already existing mitochondrial cytochrome *b* (Cyt *b*) and nuclear Rag2 data retrieved from GenBank.

This study provides strong support for the monophyly of the *Chaerephon* and *Mops* taxa included, with the exception of *C. jobimena*, which was weakly associated with this group. There was no support for the generic affiliation of *C. jobimena* or for the monophyly of either of the genera *Chaerephon* or *Mops* individually. This leads to the suggestion that *Mops* and *Chaerephon* be combined into a single genus, with crown age of 14.82 (6.44-25.54) MYA, or 21.97 (12.16-33.44) MYA if *C. jobimena* is included. *Otomops* forms a strongly supported clade consistent with its generic status, comprising two subclades corresponding to the recognised sister species *O. martiensseni* and *O. madagascariensis*, which last shared a common ancestor 8.35 (2.87-17.47) MYA. This study provides good nuclear support for the mitochondrially-defined subclades of *O. martiensseni*, which last shared a common ancestor 4.18 (1.08-9.96) MYA. It would appear appropriate to name the clade from north east Africa and Arabia as a new species of *Otomops*, as the clade from southern and western Africa includes the type locality. This study provides weak support based on individual gene regions for associations of *Sauromys* with *Otomops* and *Mormopterus*, although these do not stand up in the concatenated datasets which offer better resolving power, indicating that *Sauromys* is not phylogenetically associated with *Chaerephon*/*Mops*, *Otomops* and *Mormopterus*. These results provide some support for the membership of *Mormopterus* in the proposed Old World Molossid tribe, Tadarini, but also support *Mormopterus* as a basal genus within the Molossidae, consistent with its designation as a separate tribe, Mormopterini.

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban. This work was carried out from January 2011 to January 2013, under the supervision of Prof. Jennifer M. 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.

DECLARATION 1 – PLAGIARISM

I, Melanie Napier, declare that

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

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

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

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OVERALL INTRODUCTION

This molecular study, based on analyses of nuclear and mitochondrial DNA regions, has focused on the molossid bats of Madagascar, the islands of the western Indian Ocean (WIO) and the south eastern portion of Africa. There were two aspects to the study. The first part comprised a population genetic study, based on mitochondrial control region sequences, of *Chaerephon atsinanana*, a newly-described molossid bat from eastern Madagascar (Goodman *et al*. 2010). The second part of the study was initially aimed at studying nuclear DNA sequence variation within *C. atsinanana*. As none of the five nuclear markers which I was able to amplify and sequence were significantly variable in *C. atsinanana*, I decided to use these to analyse phylogenetic relationships among a suite of molossid bats from the Afro/ Malagasy/ WIO region, to address issues raised in the studies of Lamb *et al.* (2011) and Ammerman *et al*. (2012) on molossid molecular phylogenetics.

The family Molossidae Gervais, 1856, consists of 16 genera and approximately 100 species with a cosmopolitan distribution (Simmons 2005). They are divided into two subfamilies, Molossinae and Tomopeatinae. The latter comprises the genus *Tompeas* Miller, 1900 (Sudman *et al*. 1994) while the former consists of 15 genera: *Molossus* Geoffroy, 1805; *Tadarida* Rafinesque, 1814; *Myopterus* Geoffroy, 1818; *Cheiromeles* Horsfield, 1824; *Mops* Lesson, 1842; *Promops* Gervais, 1856; *Molossops* Peters, 1865; *Mormopterus* Peters, 1865; *Chaerephon* Dobson, 1874; *Nyctinomops* Miller, 1902; *Platymops* Thomas, 1906; *Eumops* Miller, 1906; *Otomops* Thomas, 1913; *Sauromys* Roberts, 1917 and *Cynomops* Thomas, 1920 (Simmons 2005).

Molossid bats are strong flyers, with long narrow wings and are able to fly for long periods (6-7 hours) of time (Altringham, 2011). They feed on insects while in flight and usually search for food in groups (Altringham, 2011). They roost in groups from one to thousands and even millions (Feldhamer *et al*. 2007). Molossid bats do not hibernate as they occupy warm regions (Feldhamer *et al*. 2007). Some male molossid species have polygamous mating behaviours, in that during mating season they mate with more than one female (Feldhamer *et al*.2007).

Molossidae are distinguishable from other bat families by their tail, which is not enclosed in a membrane (Ammerman *et al*. 2012), hence the common name, free-tailed bats. They also have unique features such as short, smooth fur; tough skin and narrow wings (Vaughan, 1966). Numerous molossid species have similar morphological features and are distinguishable by certain elusive outer structural characteristics (Freeman 1981). There appears to be convergence in certain morphological characters classically used for taxonomic classification of Molossids. These characteristics, which include palatal emargination, separation of the ears, basisphenoid pits, and lip

wrinkles, might be functional adaptations to eating beetles, and therefore might not be optimal for the recovery of phylogeny (Freeman 1981). This points to the need for molecular studies to complement existing classifications based on morphology, such as those of Lamb *et al*. (2011) and Ammerman *et al*. (2012), based on nuclear and mitochondrial DNA sequences.

Of the 16 molossid genera, eight (*Tadarida*, *Platymops*, *Myopterus*, *Otomops*, *Mormopterus*, *Mops*, *Sauromys*, and *Chaerephon*) are found in mainland Africa, Arabia and the islands of the western Indian Ocean, including Madagascar (Lamb *et al*. 2011). *Otomops*, *Mormopterus*, *Mops*, *Sauromys* and *Chaerephon* formed part of this study and occupy areas across mainland Africa, Madagascar, the Seychelles (Aldabra) and the Comoros Archipelago, including four main islands, Anjouan, Mohéli, Mayotte and Grande Comore (Weyeneth *et al*. 2008). It was unfortunately not possible to obtain samples of *Tadarida*, *Platymops* and *Myopterus* for inclusion in this work.

Chaerephon is found on mainland Africa and its offshore islands, as well as Arabia, Madagascar, the Comoros Archipelago and Aldabra. *Chaerephon*, which was previously included in *Tadarida*, is today regarded as a valid genus (Freeman 1981; Simmons 2005). Agnarsson *et al*. (2011), Lamb *et al*. (2011) and Ammerman *et al*. (2012) recovered a monophyletic *Chaerephon*/*Mops* clade in their molecular analyses, but did not recover support for either *Charephon* or *Mops* as separate distinct genera. The latter two studies found no support for an association of *Chaerephon jobimena* Goodman & Cardiff, 2004 with other species of *Charephon*, based on both nuclear and mitochondrial sequence data.

There are currently 21 recognized species of *Chaerephon* (Goodman & Cardiff 2004; Simmons 2005; Goodman *et al*. 2010), of which five are included in this study, namely: *C. atsinanana* Goodman *et al*. 2010, *C. jobimena* Goodman & Cardiff, 2004, *C. leucuogaster* Grandidier, 1869, *C. pumilus* Cretzschmar, 1826 and *C. pusillus* Miller, 1902. Based primarily on mitochondrial Cyt *b* sequence studies, *C. pumilus* from Madagascar was found to be distinct from the nominate *C. pumilus* and was described as a new species, *C. atsinanana* (Goodman *et al*. 2010). *Chaerephon atsinanana* is found in the south eastern region of Madagascar over an altitude range of near sea level to 1100 m (Goodman *et al*. 2010). *Chaerephon atsinanana* and three other *Chaerephon* species, *C. pusillus*, *C. leucogaster* and *C. jobimena*, are known to occupy synanthropic day roosts in places such as roof and attic spaces in public and private buildings (Ratrimomanarivo *et al*. 2009a; Goodman *et al*. 2010). While *C. leucogaster* and *C. jobimena* have also been observed to roost in natural forest areas, *C. atsinanana* and *C. pusillus* have never been documented roosting in natural settings (Goodman *et al*. 2010).

Chaerephon jobimena, described on the basis of morphological characters (Goodman & Cardiff 2004), appears genetically more similar to *Tadarida aegyptiaca* Geoffroy, 1818 (Lamb *et al*. 2011). *Chaerephon lecuogaster* occurs primarily in the arid western portion of Madagascar, as well as on Pemba (offshore of Tanzania) and Mayotte (Comoros). *Chaerephon leucogaster* was included in the genus *Tadarida* (Peterson *et al*. 1995), and is recognised as a member of *Chaerephon* by Hutson *et al*. (2001), Goodman & Cardiff (2004) and Simmons (2005). Koopman (1993) regarded *C. leucogaster* as conspecific with *C. pumilus*, however Russ *et al*. (2003) differentiate it from *C. pumilus* based on its distinct echolocation characteristics and smaller size. Goodman *et al*. (2010), based on mitochondrial sequence data, found *C. lecuogaster* to be distinct from the nominate *C. pumilus* from Massawa, Eritrea, and from *C. pusillus* (from the Comoros and Aldabra), *C. pumilus* sensu lato (s. l.) from south eastern Africa and *C. atsinanana* from eastern Madagascar.

The genus *Mops* consists of 15 species (Wilson & Reeder 2005), three of which were investigated in this study, namely *M. condylurus* Smith, 1833, *M. leucostigma* Allen, 1918 and *M. midas* Sundevall, 1843. *M. condylurus* has been found in mainland Africa, while its sister species, *M. leucostigma* is found in the Comoros archipelago and Madagascar (Eger & Mitchell 2003). Some morphological studies place *M. leucostigma* in the genus *Mops* (Goodman & Cardiff 2004; Simmons 2005) whereas others place it in *Tadarida* (Russ *et al*. 2003). The phylogenetic studies of Ratrimomanarivo *et al*. (2007) and Lamb *et al*. (2011) recover *M. leucostigma* and *M. condylurus* as sister species, although Lamb *et al*. (2011) do not find support for *Mops* as a distinct genus. The distribution of *M. midas*, which roosts in groups of hundreds (Smithers 1983), includes areas of both Madagascar and Africa (Ratrimomanarivo *et al*. 2007). Two subspecies were recognised, namely *M. m. miarensis* from mainand Africa and *M. m. midas* from Madagascar (Koopman 1994). However, Simmons(2005) and Ratrimomanarivo *et al*. (2007), based on morphological data and mitochondrial sequence data respectively, established that there were no conclusive subspecies-level differences amongst Madagascan and African forms, and that there was only one species, *M. midas* on both Madagascar and mainland Africa.

Ten species of *Mormopterus* have been reported worldwide (Wilson & Reeder 2005), of which two were investigated in this study, namely *M. acetabulosus* Hermann, 1804 and *Moropterus jugularis* Peters, 1865. *M. acetabulosus* is endemic to Mauritius and Rèunion (Goodman *et al*. 2008) and its occurrence in Madagascar and Africa is not confirmed (Van Cakenberghe and Seamark 2011). *M. jugularis* is endemic to Madagascar (Simmons 2005), where it has been found roosting in groups of less than 100 (Ratrimomanarivo *et al*. 2009b).

Sauromys and *Platymops* are regarded as subgenera of *Mormopterus* by some authors (Freeman 1981; Koopman 1993) and as valid genera by others (Simmons 2005; Van Cakenberghe and Seamark 2011). *Sauromys petrophilus* Roberts, 1917, is the only member of a monotypic genus which is distributed in small colonies across southern Africa (Jacobs and Fenton, 2002). *Sauromys* has previously been included as a subgenus of *Tadarida* (Koopman 1975; Hayman and Hill 1971) and of *Mormopterus* (Freeman 1981; Koopman 1994), while Peterson (1965) and Simmons (2005) regard it as a valid genus. Van Cakenberghe and Seamark (2011) support this based on the distinctive morphological characters and ecology of *S. petrophilus*. Ammerman *et al*. (2012) recovered an association between *S. petrophilus* and *Tadarida.*

Otomops consists of seven species worldwide (Wilson & Reeder 2005), of which two sister species, *O. madagascariensis* Dorst, 1953 and *O. martiensseni* Matschie, 1897 were investigated in this study. *O. madagascariensis* is endemic to Madagascar (Simmons 2005) while *O. martiensseni* has been found in mainland Africa and Yemen (Van Cakenberghe and Seamark 2011). Two reciprocally monophyletic mitochondrial clades of *O. martiensseni* have been described one from north east Africa and Yemen, and the other from southern and western Africa (Lamb *et al*. 2008).

A range of molecular markers may be used to carry out molecular genetic studies (Parker *et al*. 1998). These include sequences of both mitochondrial and nuclear markers, and fingerprinting techniques, including microsatellite (simple sequence repeat) analysis. Nuclear DNA is diploid and biparentally-inherited, whereas mitochondrial DNA is haploid and maternally inherited (Freeland 2011). Mitochondrial markers tend to have a higher rate of evolution than nuclear markers (Freeland 2011). In the recent past, phylogenetic inference based on mitochondrial DNA has been commonly used in the taxonomic evaluation of mammal species, including bats (Baker & Bradley 2006, Goodman *et al*. 2010). The Cyt *b* gene and control region of the mitochondrial DNA have been used most frequently for analysis in phylogenetic studies. Organellar genes are useful in detecting early stages of speciation, and variation among populations of a species because the organelle genome is usually not affected by recombination and achieves reciprocal monophyly in about 1/4 the time of the average nuclear gene in diploid sexual organisms (Birky 2013). Many of the nuclear markers initially used were relatively slowly evolving, and were useful in establishing deep phylogenetic divergence among major lineages. However faster-evolving nuclear markers, including nuclear introns, are able to provide phylogenetic resolution at lower taxonomic levels (eg. Matthee *et al*. 2001; Zhu & Ge 2005; Stock *et al*. 2008). A combination of both nuclear and mitochondrial markers is useful for resolution of phylogenetic relationships at different levels, and provides robust results which take account of both the male and female contribution to gene flow.

The taxonomy of the Molossidae was initially based on morphological attributes, which may not be optimal for the recovery of phylogeny owing to convergence due to functional adaptation (Freeman 1981). Lamb *et al*. (2011) and Ammerman *et al.* (2012) reported on the phylogeny of subgroups of molossid bats based on mitochondrial and nuclear markers. These studies proposed the first molecular phylogenetic hypotheses for the Molossidae, raising many further questions. Phylogenetic and phylogeographic studies have been carried out for the following molossid taxa from the western Indian Ocean region: *M*. *midas* (Ratrimomanarivo *et al*. 2007); *M. leucostigma* (Ratrimomanarivo *et al*. 2007); *C*. *lecuogaster* (Ratrimomanarivo *et al*. 2009a); *C. pumilus* from south eastern Africa (Taylor *et al*. 2009); *M*. *jugularis* (Ratrimomanarivo *et al*. 2009b); *O*. *madagascariensis* and *O. martiensseni* (Lamb *et al*. 2008). Goodman *et al*. (2010) described *C. atsinanana* from eastern Madagascar, formerly *C. pumilus*, as a new species, closely allied to *C. leucogaster* from western Madagascar and *C. pusillus* from the Comoros and Aldabra.

The initial part of this study focused on genetic variability and structure among populations of the newly-described *C*. *atsinanana* based on the maternally-inherited mitochondrial control region. This work, which forms the first data chapter of this dissertation, was published as a multi-author comparative study with data from *C. leucogaster* (Lamb *et al*. 2012). I was a coauthor of this paper, as I contributed most of the *C*. *atsinanana* sequences, as well as analyses, and contributed to the writing of sections of this paper relating to *C. atsinanana*. For the purposes of this dissertation, I have written my contribution to this paper as a separate chapter, including some new analyses.

The second data chapter of this thesis is an analysis of phylogenetic relationships among five genera and 13 species of Afro-Malagasy molossid bats. The initial objective was to amplify and sequence candidate nuclear markers so as to carry out a study of nuclear variability among *C*. *atsinanana* populations, which I show in Chapter 1 of this dissertation to comprise a number of deeply divided, highly structured lineages, hypothesized to be the result of female philopatry revealed by the maternally-inherited mitochondrial control region marker. The aim was to study nuclear variability among these populations in order to establish whether similar levels of structure could be recovered, consistent with the possible existence of male philopatry. Of the 12 nuclear regions initially investigated, the markers PNPO-3 (Igea *et al*. 2010), FES, GHR, RHO1 (Venta *et al*. 1996) and PRKC1 (Matthee *et al*. 2001) were successfully amplified and sequenced. However they showed no variability and very little polymorphism, and were thus unsuitable for testing hypotheses related to genetic variability of *C. atsinanana* populations. As a result, these five nuclear markers, combined with already existing mitochondrial Cyt *b* and nuclear Rag2 data were used to

further investigate phylogenetic relationships among the Molossid species of the WIO region and south eastern Africa. This work is reported on in Chapter 2 of this dissertation, and will be submitted for publication with Prof. J. Lamb and Prof. S. Goodman as co-authors.

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CHAPTER 1

POPULATION STRUCTURE AND HISTORICAL DEMOGRAPHY OF MADAGASCAN *Chaerephon atsinanana* (CHIROPTERA) BASED ON THE MITOCHONDRIAL CONTROL REGION

1.1 ABSTRACT

This study has focused on genetic variability and structure in *Chaerephon atsinanana*, a newly-described molossid bat found in the mid to southern region of the eastern watershed of Madagascar. As these bats are strong fliers, and can traverse the riverine and mountain barriers within the landscape, it was hypothesized that they would show relatively low levels of intraspecific genetic structure. Phylogenetic analyses of the mitochondrial control region of 107 samples revealed several well supported clades among the eight *C. atsinanana* haplotypes, indicative of population structure. Population genetic analyses of control region sequences revealed the presence of 6 major haplotype groups (overall 8 haplotypes) separated by genetic distances of up to 8.14% (average 4.95%). Analysis of molecular variance revealed very high overall levels of genetic structure among the haplotype groups (F_{ST} =0.994). There was also significant structure (P<0.05) among altitude-based groups (44.85 % of variance) and latitude-based groups (27.76% of variance). Thus the hypothesis of low levels of genetic structure was rejected. A second hypothesis was that there would be signatures of Pleistocene-era population expansions in *C. atsinanana*, as found in the Malagasy bats *C. leucogaster* and *Myotis goudoti*. Diversity and neutrality statistics, Bayesian skyline analyses and significantly ragged mismatch distributions were consistent with ancient stable *C. atsinanana* populations of constant size throughout the last two major Pleistocene glacial periods. It therefore appeared that expansion from glacial refugia is an unlikely explanation for such high levels of structure. An alternative hypothesis is that *C. atsinanana* haplotype groups are spatially structured due to behavioral barriers to gene flow, for example female philopatry, as revealed by the maternally inherited mitochondrial control region marker.

Keywords: Chiroptera, gene flow, genetic structure, historical demography, Malagasy, *Chaerephon*

1.2 INTRODUCTION

The Family Molossidae (Order Chiroptera), also known as free-tailed or mastiff bats, consists of 100 species divided into17 genera (Simmons 2005). These bats have a world-wide pan-tropical distribution, and occur on many islands and on every continent except Antarctica. They have long narrow wings and are strong fliers, with the ability to consume their insect prey in flight.

In this study based on mitochondrial control region sequence data, I report on the historical demography and population genetic structure of *C. atsinanana*, a newly-described species of molossid bat endemic to the mesic southern portion of the eastern watershed of Madagascar (Goodman *et al.* 2010). *Chaerephon atsinanana* has been shown to be sister to a clade consisting of *C. pumilus* Cretzschmar, 1830-1831 from mainland Africa and Arabia, *C. lecuogaster* A. Grandidier, 1869 from western Madagascar and *C. pusillus* Miller, 1902 from the Comoros and Aldabra (Lamb *et al.* 2011). Three species of *Chaerephon* are known to occur on Madagascar. Eastern *C. atsinanana* is allopatrically distributed with respect to *C. leucogaster*, which is endemic to the drier western portion of Madagascar. The third species, *C. jobimena* Goodman & Cardiff, 2004 is not monophyletic with other species of *Chaerephon* based on nuclear Rag2 and mitochondrial cytochrome *b* (Cyt *b*) and control region data (Lamb *et al*. 2011), bringing into question the validity of its generic designation.

A variety of factors are known to affect levels of genetic structure in bats. Dispersal ability has been reported to play an important role in determining the demography of natural populations (Proctor *et al.* 2004). Roosting ecology is an important aspect of social structure in bats; it is known to affect dispersal strategy (Chen *et al.* 2010), and may therefore play a role in determining genetic structure. The Malagasy *Chaerephon* species have adopted synanthropic day roosts in roof and attic spaces in buildings (Ratrimomanarivo *et al*. 2009; Goodman *et al*. 2010). *Chaerephon atsinanana* has never been found to roost in natural settings, including caves, in Madagascar (Goodman *et al*. 2010). Philopatry, in which females and/or males return consistently to, or remain in a roost or area, is a social isolation mechanism which can lead to genetic structuring in bats. For example, Worthington-Wilmer *et al.* (1994) observed extremely high levels of genetic structure in the Australian ghost bat, *Macroderma gigas*, and attributed this to the presence of philopatry in these bats.

Glacial periods have been known to influence the distribution of organisms on Madagascar as a result of the cooler and drier climate associated with the glacial periods (Wilmé *et al*. 2006). During Pleistocene glacial periods, organisms have been known to retreat into refugia, from

which they expand in interglacial periods (Vences *et al*. 2009). Analysis of fossil pollen cores in Madagascar appears to show that greater levels of Pleistocene era climatic change may have occurred in the western portion of Madagascar than in the east, which was relatively stable (Virah-Sawmy *et al*. 2009a, 2009b). Habitat shifts associated with Pleistocene climatic cycles resulted in areas of refugia in the more humid montane areas and dryer conditions in low-lying areas of Madagascar (Burney *et al.* 2004; Wilmé *et al*. 2006)

This has been shown to influence genetic structure; for example, studies on Malagasy bats have revealed the expansion of populations of *C. leucogaster* (Ratrimomanarivo *et al.* 2009) and *Myotis goudoti* Smith, 1834, (Weyeneth *et al*. 2011) from Pleistocene refugia. As *C. atsinanana* would have occupied the same landscape during this period, it is possible that this bat underwent a similar Pleistocene-era expansion. Simulations by Knowles and Alvarado-Serrano (2010) have shown that genetic differentiation does not only depend on the long term isolation of organisms in refugial populations, and that it can also occur as a result of range expansion across a heterogeneous environment. Some of the population genetic signatures of range expansions include unimodal mismatch distributions, star-shaped haplotype networks and a high number of singletons (Excoffier *et al*. 2009).

Strong flying bats such as Molossidae would be expected to be panmictic over large expanses (Russell *et al*. 2005) and therefore to show low levels of genetic structure. As *C. lecuogaster*, endemic to western Madagascar, has been reported to display relatively low levels of genetic structure, one might expect a similar pattern in *C. atsinanana*.

High levels of population substructure in mammals are often associated with separation due to vicariance created by barriers such as rivers and mountain ranges (Avise *et al*. 1987). However, Malagasy forms of *Chaerephon* are strong flying bats which are likely to be capable of traversing such barriers in the landscape, as reflected in their presence at altitudes from 0 to 1100 meters. Thus vicariance is not likely to be responsible for the creation of significant population genetic structuring in *C. atsinanana*.

The aim of this study was to examine the historical demography and genetic structure of *C. atsinanana* populations, based on the mitochondrial control region, in order to evaluate two hypotheses. The first hypothesis was that there would be low levels of genetic structuring in *C. atsinanana* populations. This was based on the presence of relatively low levels of phylogeographic structure in another species of *Chaerephon* on Madagascar, *C. leucogaster*, as well as the expectation of panmixia in strong-flying bats such as Molossidae (Russell *et al*. 2005). The second hypothesis was that there would be signatures of Pleistocene era population expansions in *C.*

atsinanana, as there is evidence for such expansion of populations of the Malagasy bats *C. leucogaster* (Ratrimomanarivo *et al.* 2009) and *Myotis goudoti* Smith, 1834 (Weyeneth *et al*. 2011).

1.3 MATERIALS AND METHODS

SAMPLING

I sequenced the control region region of 69 *C. atsinanana* samples. In the analyses, my sequences were combined with sequences generated in our laboratory as part of other projects by Waheeda Buccas and Theshnie Naidoo, in order to form a comprehensive dataset, based on all available samples. Overall, the mitochondrial control region was sequenced for a total of 107 samples of *C. atsinanana* (Fig. 1). One to 13 samples (average 6) were sequenced from each of 17 localities across this species' distribution range (Appendix 1). The outgroups included in the mitochondrial control region tree (Fig. 2) included, *C. leucogaster* (western Madagascar) and *C. pusillus* (Comoros). The bats were treated according to the guidelines of American Society of Mammalogists (Sikes, Gannon & The Animal Care and Use Committee of the American Society of Mammalogists, 2011). Tissue samples were provided in the form of wing punches and heart, liver, kidney or muscle tissue preserved in lysis buffer or 80% ethanol.

GENERATION OF SEQUENCE DATA

DNA was isolated from tissue samples using a DNeasy® DNA isolation kit (QIAGEN) according to manufacturer's instructions. The primers used to amplify a single fragment of the mitochondrial control region were P (5' TCCTACCATCAGCACCCAAAG C 3') and E (5' CCTGAAGTAGGAACCAGATG 3') (Wilkinson & Chapman 1991). PCR-amplifications were performed in 25 µl volumes. Each reaction contained 9 µl DNA (3 ng μl^{-1}), 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Roche), 0.2 µl *Taq* polymerase (5 u/µl) (Super-Therm, Southern Cross Biotech, SA) and 4 µl of each primer (6 µM) (forward and reverse). The following thermal cycle was used for amplification: 94 °C for 4 min, followed by 40 cycles of (94 °C for 60 s, 55 °C for 90 s and 72 °C for 120 s) and followed by 72 °C for 7 min.

A QIAquick® Gel Extraction Kit (QIAGEN) was used to purify excised gel bands following the manufacturer's instructions.

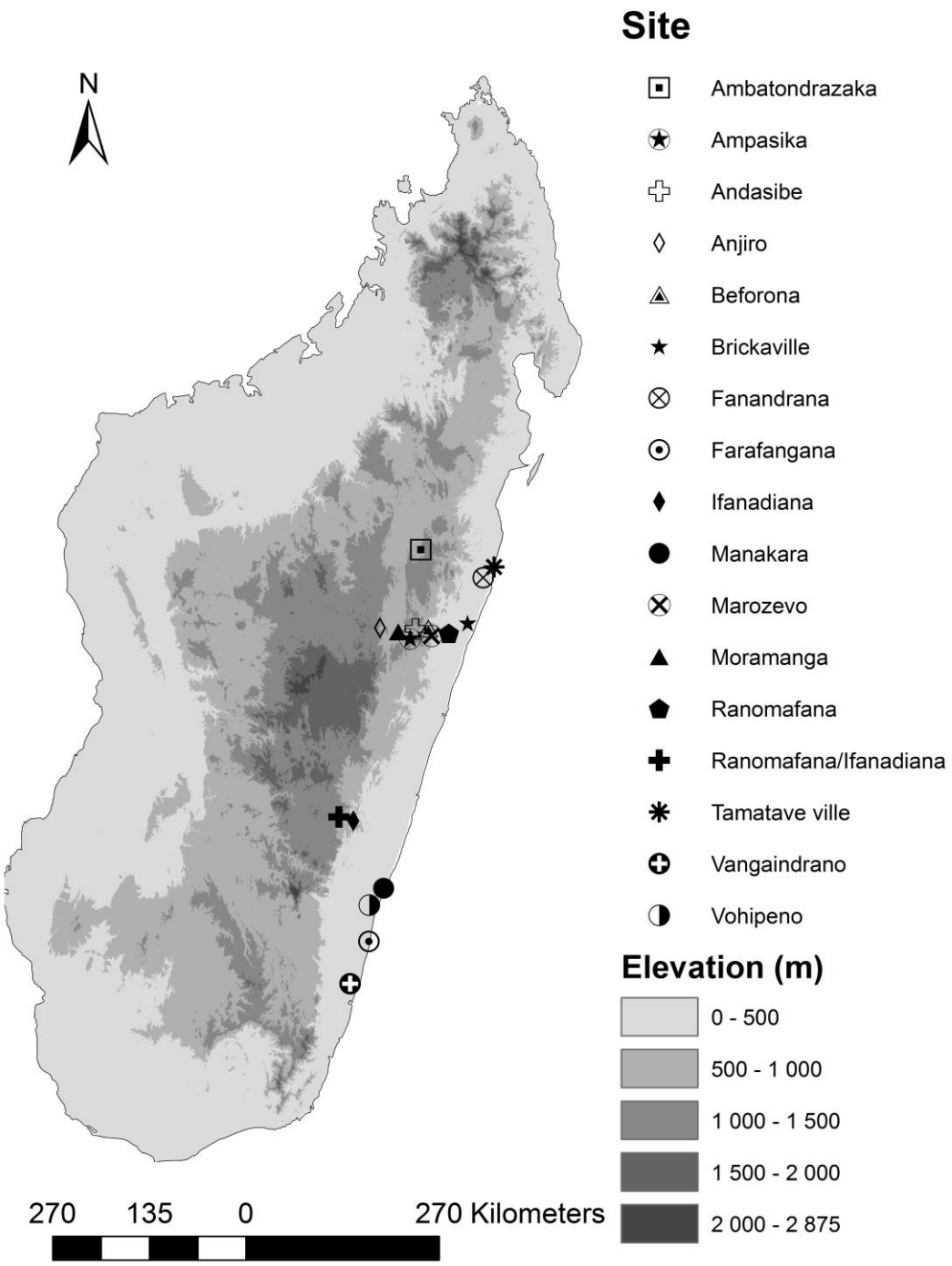

Figure 1. Map of Madagascar showing the collection localities of *Chaerephon atsinanana* samples used in this study.

The purified DNA was sequenced at Inqaba Biotec (Hatfield, Pretoria, South Africa) or at the University of Stellenbosch sequencing facility (Stellenbosch, South Africa). All sequences obtained were deposited in GenBank (Appendix 1). Sequences were edited in BioEdit 7.0.9.0 for Windows 95/98/NT (Hall 1999) and aligned using the CLUSTAL W option (Thompson, Higgins & Gibson 1994) within BioEdit. Alignments were further refined by eye.

PHYLOGENETIC ANALYSES

The most appropriate evolutionary model, HKY+G, was determined in jModelTest 0.1.1 (Posada 2008). Parsimony, neighbor-joining and maximum likelihood analyses were carried out in PAUP 4.0b10 (Swofford 2002). Node support was calculated through bootstrap (1000 replicates) resampling analysis (Felsenstein 1985). For parsimony analyses, starting trees were obtained by stepwise addition. The addition sequence was random, with 1 tree held at each step and with 10 replicates. The tree-bisection- reconnection (TBR) branch-swapping algorithm was used. In maximum likelihood analyses, starting trees were obtained by neighbor-joining followed by TBR branch swapping.

HAPLOTYPE ANALYSES

DnaSP 5.10 (Librado & Rozas 2009) was used to generate haplotype data files from the control region dataset. These data files were used in analysis of molecular variance (AMOVA) and to create statistical parsimony haplotype networks in TCS v.1.2.1 (Clement e*t al*. 2000). Genetic distances between the control region haplotypes were calculated in PAUP 4.0b10 using the HKY+G model (Swofford 2002).

ANALYSIS OF MOLECULAR VARIANCE

The distribution of control region sequences among individuals, populations and groups of populations was analysed using AMOVA carried out using Arlequin 3.0 (Excoffier *et al*. 2005). Fixation indices were calculated using standard methods and a non-parametric permutation approach was used to test their significance (Excoffier *et al*.1992). AMOVA was run using three different population grouping structures: (1) samples formed one group only, with no subgroups (2) samples were divided into groups north and south of 20ºS and (3) samples were divided into three altitude groups (0-100 m, 101-600 m, and 601-1000 m). The northern group consisted of 61

samples while the southern group consisted of 46 samples. Altitudes of0-100 m, 101- 600 m, 601-1000 were represented by 38, 30 and 39 samples respectively (Table 3).

HISTORICAL DEMOGRAPHY AND POPULATION STRUCTURE

The historical demography of *C. atsinanana* was inferred through a variety of methods in DnaSP version 4.0 (Rozas *et al.* 2003). Nucleotide diversity (π) and halotype diversity (*h*) (Nei 1987) were calculated, and Fu's (1997) Fs and Fu & Li's (1993) F* and D* were calculated to test for deviations from neutrality (as would be anticipated in the case of population expansion). DnaSP version 4.0 (Rozas *et al.* 2003) was used to generate a mismatch distribution under a population growth decline model and it was clear that the shape of the mismatch distribution did not correspond to that of an expanding population therefore a goodness of fit test was not necessary. DnaSP shows a graphic representation of the observed and expected values for expanding and stable populations (Rozas *et al*. 2003). Demographic stability is illustrated by multimodal distributions while a unimodal pattern is consistent with sudden expansion (Slatkin & Hudson 1991). Mismatch distribution analysis was used estimate whether each population group was stationary or had undergone a historical population expansion (Rogers & Harpending 1992). Low π with high *h*, significant Fs but non-significant D* and F*, a unimodal mismatch distribution and a high ratio of number of segregating sites (S) to average number of pairwise differences (*d*) (S/*d*) are signals of a historical population expansion event (see Russell *et al.* 2005).

A Bayesian skyline plot was created to predict past population dynamics of *C. atsinanana*. The analysis was carried out in BEAST v. 1.2 (Drummond *et al.* 2005) in conjunction with BEAUti v.1.5.1 (Drummond and Rambaut, 2009) and TRACER 1.2.1 (Rambaut & Drummond 2009). Stationarity was assed in TRACER 1.2.1 (Rambaut & Drummond 2009) as follows: the plot of state (x-axis) vs. likelihood (y-axis) formed a horizontal line and a plot of likelihood (x-axis) vs. frequency (y-axis) resembles a bell curve (http://beast.bio.ed.ac.uk). The HKY substitution model was selected because the programme BEAST v.1.2 (Drummond *et al.* 2005) offers limited models, it does not offer the HKY+G model. The parameter *m* (the number of grouped intervals) was set at five. The MCMC analysis was run for 10 000 000 generations (sampled every 1000 generations) and the first 10% were discarded as burn-in.

1.4 RESULTS

Analysis of the *C. atsinanana* control region alignment, trimmed to 301 nucleotides, yielded 66 variable characters, 41 of which were parsimony-informative, and two singletons. Eight haplotypes were derived from 107 *C. atsinanana* control region sequences. The nucleotide diversity was 0.03448 ±0,002, and the haplotype diversity was 0.793±0.026. Parsimony, maximum likelihood and neighbor joining analyses yielded congruent trees, which are presented as one neighbor joining tree with node support given as bootstrap values derived from all three analyses (Fig. 2). *Chaerephon atsinanana* formed a monophyletic clade with respect to the outgroups, *C. pusillus* and *C. lecuogaster*. Good support is considered when two of the three bootstrap support values are greater than 90%. Moderate support is considered when two of the three bootstrap support values are greater than 70% and weak support is considered when two of the three bootstrap values are greater than 60%. Haplotypes 6 and 7, and Haplotypes 4 and 5 formed well-supported subgroups. There were other supported subgroups within the *C. atsinanana* clade, including: H6, H7, H8 (moderate support); H6, H7, H8 and H1 (good support) and H6, H7, H8, H1, H2 and H3 (moderate support). The mean diversity among *C. atsinanana* haplotypes was 4.8% (range 0.01 to 8.14%). Genetic distances between *C. atsinanana* and *C. leucogaster* ranged from 10.83% to 15.15%, and those between *C. atsinanana* and *C. pusillus* were 11.86% to 16.27% (Table 1).

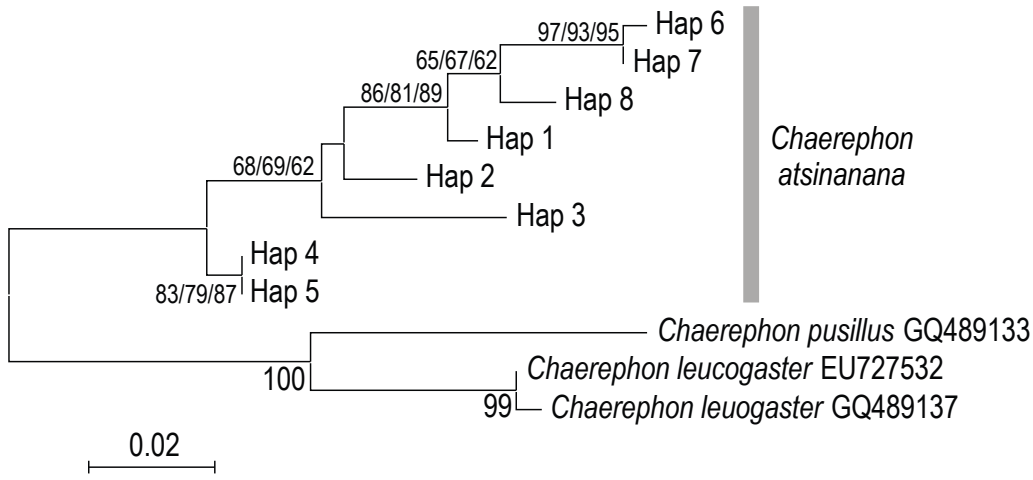

Figure 2. Phylogenetic relationships based on analysis of genetic relationships among haplotypes of *Chaerephon atsinanana* based on 301 nt of the mitochondrial control region of *Chaerephon atsinanana* (eastern Madagascar) DNA with respect to the outgroups, *Chaerephon leucogaster* (western Madagascar) and *Chaerephon pusillus* (Comoros). Bootstrap values are given as [parsimony % / maximum likelihood % / neighbor joining %]. Hap = haplotype

Table 1. HKY+G genetic distances (below diagonal) and p-distances (above diagonal) between haplotypes of *Chaerephon atsinanana* and outgroups, *Chaerephon leucogaster* and *Chaerephon pusillus*, based on analysis of 301 nucleotides of the mitochondrial control region. Hap.= haplotype.

	Hap.	1	2	3	4	5	6	7	8	9	10
C. atsinanana	1		3.63	5.93	5.12	5.13	3.65	2.92	2.57	10.56	11.40
C. atsinanana	2	3.82		5.13	2.91	2.91	5.93	5.14	4.77	10.56	11.40
C. atsinanana	3	6.35	5.44		5.78	5.79	6.57	7.40	6.98	12.91	3.40
C. atsinanana	4	5.47	3.00	6.31		0.01	5.80	5.06	5.51	9.66	10.13
C. atsinanana	5	5.48	3.00	6.32	0.01		5.92	5.16	5.51	9.66	10.13
C. atsinanana	6	3.84	6.35	7.23	6.33	6.34		0.73	4.04	12.56	12.94
C. atsinanana	7	3.04	5.49	8.14	5.47	5.48	0.74		3.27	12.08	12.56
C. atsinanana	8	2.65	5.06	7.68	5.90	5.90	4.25	3.44		10.56	11.41
C. leucogaster	9	11.83	11.83	15.11	10.83	10.83	14.58	14.02	12.36		0.40
C. leucogaster	10	12.36	12.36	15.68	11.35	11.35	15.15	14.58	12.90	0.41	
C. pusillus	11	12.89	12.89	16.27	11.86	11.86	13.42	12.87	13.44	9.12	9.63

HAPLOTYPE NETWORKS

The haplotype network (Fig. 3a) based on 301 nucleotides of the mitochondrial control region of *C. atsinanana* consisted of eight haplotypes, with adjacent haplotypes separated by between one and 17 steps. Individual haplotypes were represented at between one and six localities. All samples represented by haplotype 2 shared a 26-nucleotide insertion, and samples with haplotype 3 all shared a 1-nucleotide deletion. Four localities contained only one haplotype (H), namely Toamasina (Tamatave Ville) – H3, Ranomafana Ifanadiana – H5, Fanandrana – H6 and Ambatondrazaka - H8. Three localities contained two haplotypes, namely Beforona (haplotypes 2 and 7, separated by 14 mutations), Ranomafana Ifanadiana (haplotypes 4 and 5, separated by one mutation) and Fanandrana (haplotypes 6 and 7, separated by 1 mutation).

AMOVA

In the analyses of molecular variance, all three group designs (Tables 2 and 3) revealed high levels of structure. The overall F_{ST} was 0.994. High and significant levels of structure were observed when populations were divided into latitude groups north and south of 20^{o}S: in this case 27.76% of the variance was among groups, 67.36% among populations within groups, and 4.88% within populations (Table 2 and Fig. 3b). AMOVAs also revealed significant levels of structure when

populations were divided into 3 altitude groups, with 44.85% of the variance occurring among population groups, 54.92% among populations within groups, and 0.22% within populations (Table 2 and Fig. 3b).

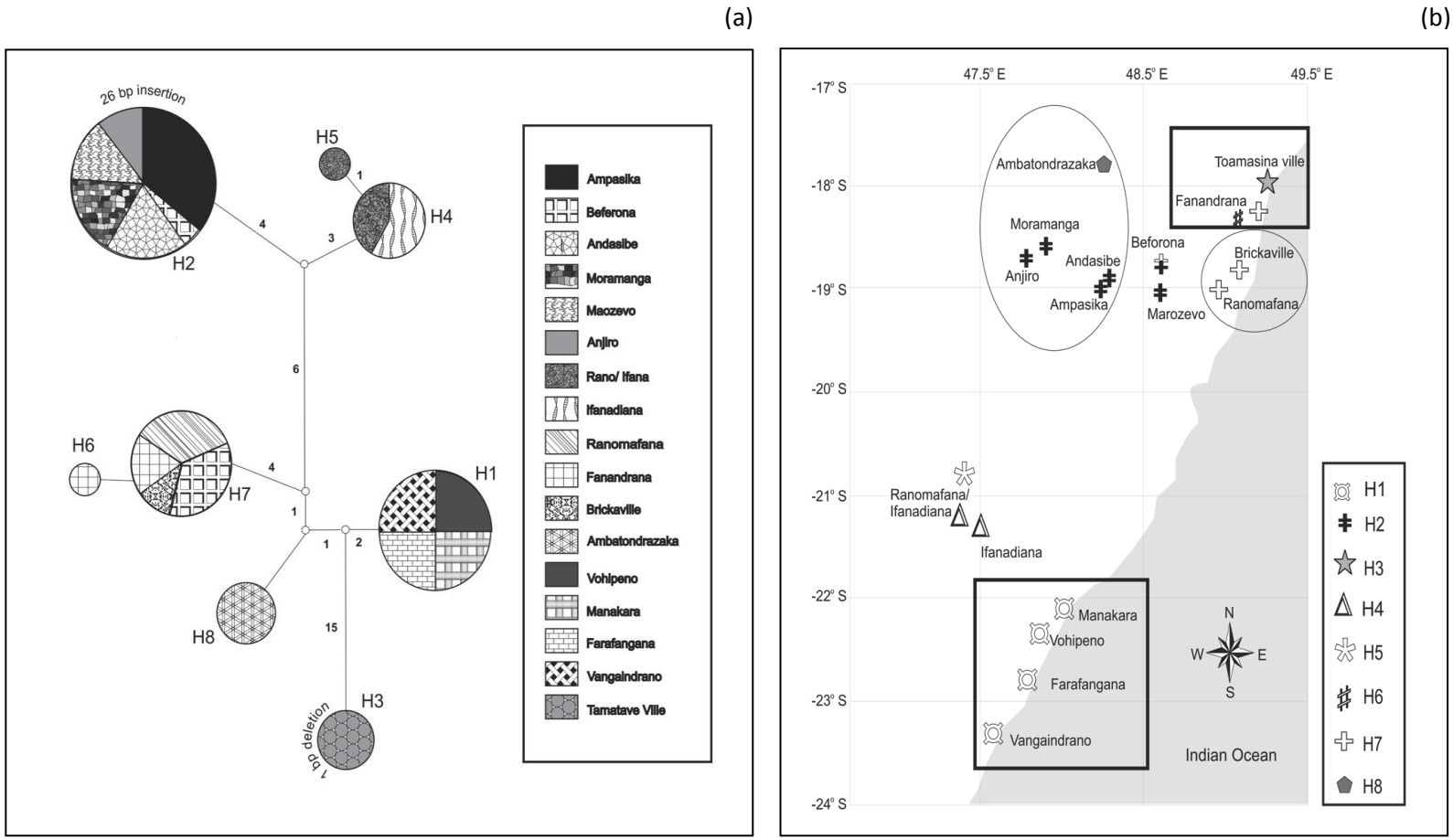

Figure 3. (a) Statistical parsimony haplotype network showing mutational relationships between 107 mitochondrial control region haplotypes (sequence length 301 nt) of *Chaerephon atsinanana* from eastern Madagascar. (b) Distribution of eight haplotypes of *C. atsinanana* in eastern Madagascar. Also represented in (b) are the groupings analysed in the AMOVA. Altitude groups: heavy outlines = 0-100 m; no outlines = 101-600 m; light outlines = 601- 1000 m. The latitudinal groups were north and south of 20°S. H = haplotype. H1=28 samples, H2=32 samples, H3= 6 samples, H4= 17 samples, H5=1 sample, H6=1 sample, H7=15 samples and H8= 7 samples of *Chaerephon atsinanana*.

Table 2. Results of analysis of molecular variance (AMOVA) for three grouping structures of *Chaerephon atsinanana*. (1) Groups were north & south of 20°S, (2) groups were 0-100 m, 101-600 m, 601-1000 m (Fig.3b).

Grouping criterion	Source of variation	Fixation Indices	% of variation	*P* value
No groups		F_{ST}: 0.994		
Latitude[1]	Among groups	FCT: 0.277	27.76	0.03
	Among populations within groups	FSC: 0.932	67.36	0.00
	Within populations	F_{ST}: 0.951	4.88	0.00
Altitude[2]	Among groups	FCT: 0.449	44.85	0.04
	Among populations within groups	FSC: 0.997	54.92	0.00
	Within populations	F_{ST}: 0.951	0.22	0.00

HISTORICAL DEMOGRAPHY AND POPULATION STRUCTURE

One hundred and seven *C. atsinanana* control region sequences were divided into eight haplotypes. The haplotype diversity was 0.793 ± 0.026 and the nucleotide diversity 0.03448 ± 0.002 (Table 4). Fu's F* was 2.029 and Fu and Li's D* was 1.917, both significant (P<0.02), indicating no deviation from neutrality, contrary to the expectation for an expanding population. Fu's Fs was 13.015 (P<0.02). The *C. atsinanana* mismatch distribution was multimodal (Fig. 4a) and the raggedness statistic was highly significant (P<0.00001), contrary to the expectation of non-significance for an expanding population. The mismatch distribution did not fit the expectation for a single population of constant size, or for a single expanding population, but was consistent with that expected for an ancient stationary population with structured subdivisions (Harpending 1994). Overall, the diversity and neutrality statistics were not consistent with those expected for an expanding population (Russell *et al.* 2005).

Based on the Bayesian Skyline plot, *C. atsinanana* populations appear to have displayed a constant population size from ~30 000 to ~232 560 years ago (Fig. 4b). There also appears to be a relatively recent drop in the population size, from about 30 000 years ago until the present, although the confidence limits in this part of the plot are very wide.

Table 3. Distribution of *Chaerephon atsinanana* control region haplotypes at study localities in eastern Madagascar. Data are presented as numbers of specimens; numbers in brackets are the percentage of samples from the locality present with that haplotype. Latitude and altitude variables refer to the grouping criteria used in the AMOVA (see Table 2 and Fig. 3b). CR = control region; Hap = haplotype; S = south of 20°S; N = north of 20°S.

Locality	Hap 1	Hap 2	Hap 3	Hap 4	Hap 5	Hap 6	Hap 7	Hap 8	No. CR	Latitude *C. atsinanana*	Altitude(m) *C. atsinanana*
Ambatondrazaka								7 (100)	7	N	601-1000
Ampasika		13 (100)							13	N	601-1000
Anjiro		3 (100)							3	N	601-1000
Andasibe		8 (100)							8	N	601-1000
Beforona		1 (11)					8 (89)		9	N	101-600
Brickaville							1 (100)		1	N	601-1000
Fanandrana						1 (25)	3 (75)		4	N	0-100
Farafangana	6 (100)								6	S	0-100
Ifanadiana				9 (100)					9	S	101-600
Manakara	6 (100)								6	S	0-100
Marozevo		3 (100)							3	N	101-600
Moramanga		4 (100)							4	N	601-1000
Ranomafana Atsinanana							3 (100)		3	N	601-1000
Ranomafana Ifanadiana				8 (89)	1 (11)				9	S	101-600
Toamasina ville			6 (100)						6	N	0-100
Vangaindrano	5 (100)								5	S	0-100
Vohipeno	11 (100)								11	S	0-100
No. (control region)	28 (26)	32 (29)	6 (6)	17 (16)	1 (1)	1 (1)	15 (14)	7 (7)	107 (100)	-	-

Table 4. Diversity and neutrality statistics based on the analysis of 301 nucleotides of the mitochondrial control region of *Chaerephon atsinanana***.**

Parameter	**Quantitative analyses**	**Quantitative analyses**	**Expectation #**
Nucleotide diversity per site (π)	0.03448	Moderate	low
Haplotype diversity *(h)*	0.793	Moderatly high	high
Expansion coefficient (S/d)	3.399	Moderate- Moderatly low	high
Fu & Li (1993) F*	2.02897 *	Significant	not significant
Fu & Li (1993) D*	1.91681*	Significant	not significant
Fu (1997) Fs	13.015 *	Significant	significant
Raggedness statistic	0.2246**	Significant	not significant
Mismatch distribution	Multimodal		unimodal
F_{ST} (variance between localities)	0.994		

—Trends expected for a model of demographic population expansion (Hull & Girman 2005);
* — $P < 0.02$; ** — $P < 0.00001$.

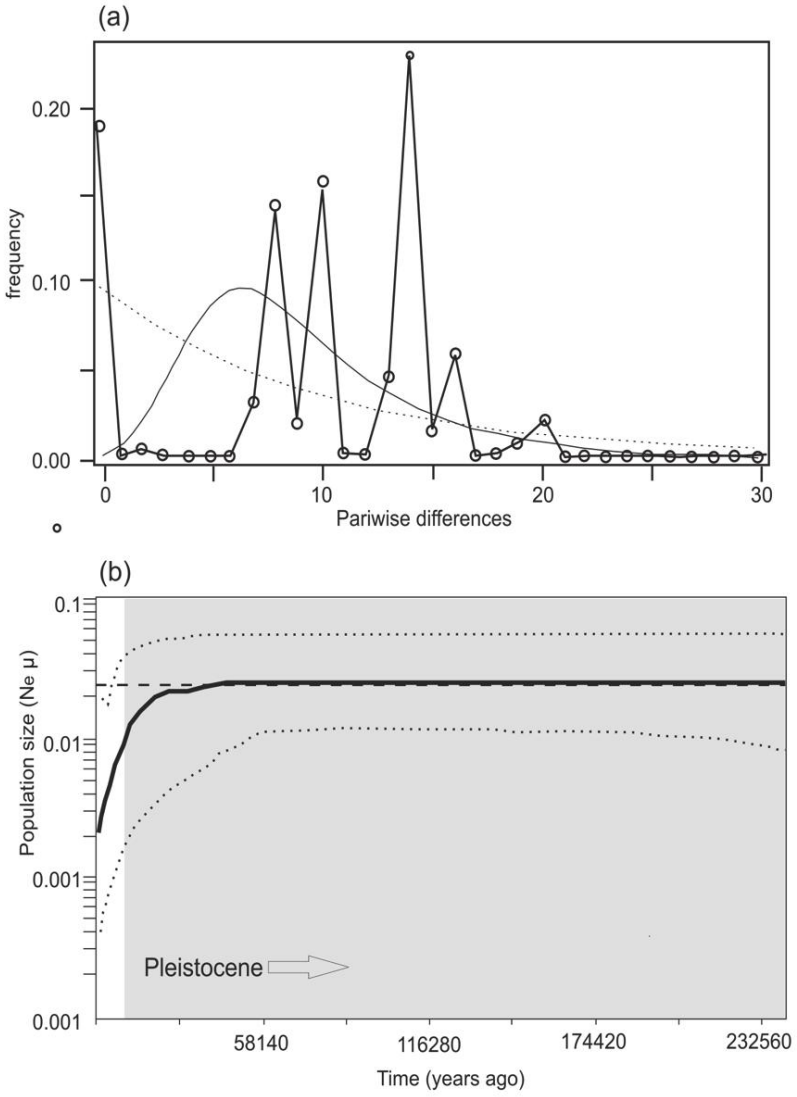

Figure 4. (a) Mismatch distribution for *Chaerephon atsinanana* based on 301 nucleotides of the mitochondrial control region. The observed distribution is represented by a heavy solid line. The distribution expected under the growth–decline model is represented by a feint solid line whereas the distribution expected under a model of constant population size is represented by a feint dotted line. (b) Bayesian skyline plot of past population sizes of *C. atsinanana*. The heavy line represents the median, and the feint lines bound the 95% credibility interval. The shaded area represents the Pleistocene era. The dashed line is horizontal, giving an indication that the population size was constant over much of the late Pleistocene.

1.5 DISCUSSION

The aim of this study was to examine the historical demography and population genetic structure of *C. atsinanana* populations, based on the mitochondrial control region, in order to evaluate two hypotheses. The first hypothesis was that there would be low levels of genetic structuring in *C. atsinanana* populations. This was based on the presence of relatively low levels of phylogeographic structure in another form of *Chaerephon* on Madagascar, *C. leucogaster*, as well as the expectation of panmixia in strong-flying bats such as Molossidae (Russell *et al*. 2005).

The second hypothesis was that there would be signatures of Pleistocene era population expansions in *C. atsinanana*, as there is evidence for such expansion of populations of *C. leucogaster* (Ratrimomanarivo *et al.* 2009) and of the Malagasy bat, *Myotis goudoti* Smith, 1834 (Weyeneth *et al*. 2011). This study shows that, contrary to expectations, *C. atsinanana* displays extremely high levels of genetic and phylogeographic structure, and does not show signatures of population expansion during the Pleistocene or at other times.

HISTORICAL DEMOGRAPHY

The population genetic analyses of *C. atsinanana* are consistent with a population structure that has been stable for a long period of time. This is indicated by the diversity and neutrality statistics, which are not consistent with population expansion in *C. atsinanana* (see Russell *et al*. 2005). Additionally, the mismatch distribution plot was multimodal and significantly ragged (Table 4, Fig. 4a), and thus not compatible with a model of demographic population expansion (Hull & Girman 2005) but rather with an ancient stationary population with genetic subdivisions (Slatkin & Hudson 1991; Rogers & Harpending 1992; Schneider & Excoffier 1999). Consistent with the above, Bayesian skyline plots show no Pleistocene era population expansion in *C. atsinanana*, as was observed for *C. leucogaster* (Ratrimimanarivo *et al*. 2009).

The Bayesian skyline plot shows evidence of a population contraction at the end of the Pleistocene, but, as the confidence limits on this part of the plot are very wide, it is not clear whether this is an accurate reflection of population sizes in *C. atsinanana* during this period. Further, *C. atsinanana* does not show a star-shaped haplotype network, as expected for expanding populations, and only two singletons are present in the alignment, in contrast with the expectation of a high number of singletons in an expanding population (Excoffier *et al*. 2009).

Thus it appears that *C. leucogaster* from arid western Madagascar showed Pleistocene era population expansions, whereas the allopatrically distributed *C. atsinanana* from mesic eastern Madagscar did not. Analysis of fossil pollen cores appears to show that greater levels of Pleistocene era climatic change may have occurred in the western portion of Madagascar than in the east, which was relatively stable (Virah-Sawmy *et al*. 2009a, 2009b). Thus habitat shifts associated with Pleistocene climatic cycles (Burney *et al.* 2004; Wilmé *et al*. 2006) are likely to have resulted in post refugial expansion of *C. lecuogaster*, but not *C. atsinanana*. Consistent with this, the Bayesian skyline plot reveals that the population size of *C. atsinanana* appears to have been stable over the last ~230 000 years of the Quaternary, and shows no evidence of a population expansion. In modern times, Madagascar has experienced a high human population growth rate, and population pressures have resulted in large scale deforestation to create land for use in agriculture and to generate wood for use in construction (Harper *et al.* 2007). The increase in man-made structures has resulted in the creation of more roosting sites for *C. atsinanana*, creating likelihood that this bat would undergo a population expansion to fill newly-available roosting sites. In contrast, there appears to be a population contraction in recent times, although the confidence limits in this part of the Bayesian skyline plot are very wide.

GENETIC STRUCTURE

The six major haplotypes are separated by high numbers of mutational steps, consistent with an average divergence between haplotypes of 4.95%, (maximum 8.14%). *Myotis goudoti*, an endemic Malagasy bat species, shows similar genetic distances between populations (Weyeneth *et al.* 2011), as does the Australian ghost bat, *Macroderma gigas* Dobson, 1887, which showed up to 6% control region divergence and 87% variance among populations (Worthington-Wilmer *et al.* 1994). These authors postulate that the high divergence between populations is due to female philopatry leading to restricted female mediated gene flow between populations, as evidenced by this mitochondrial marker.

Genetic divergence in *C. atsinanana* falls within Avise *et al*. (1987) category 1, characterised by long-term barriers to gene flow, in which major haplotypes are limited to specific regions. The highly structured and diverse sub-populations of *C. atsinanana* may have been formed as a result of isolation caused by barriers to gene flow, in combination with genetic drift (Atartouch *et al.* 2006). The barrier to gene flow may have been due to a behavioural cause such as female philopatry, revealed here by the maternally-inherited control region marker, similar to the results

reported by (Worthington-Wilmer *et al.* 1994) for the Australian ghost bat. The relatively stable climate experienced by Madagascar during the Quaternary (Virah-Sawmy *et al*. 2009a) would have allowed retention of phylogeographic structure, as it would not have been consistent with the creation of climate-induced bottlenecks. Further, it is unlikely that barriers to gene flow would have been caused by vicariance, as *C. atsinanana* is a strong flying bat which has been found at high elevations on mountain passes and is therefore assumed to be able to traverse mountain ranges (Lamb *et al*. 2012).

Thus it appears likely that the high levels of genetic structure among populations of *C. atsinanana* may be a result of restricted maternal gene flow due to female philopatry. Studies based on nuclear sequence markers, which are biparentally-inherited and also reflect the male contribution to gene flow, are needed to ascertain the likelihood of the presence of male philopatry in *C. atsinanana.* Further, a study of the inheritance of biparentally-inherited nuclear microsatellite markers will be able to provide a finer-scale picture of population structure and levels of gene flow and possibly relatedness between populations. To conclude, *C. atsinanana* shows high levels of population structure, which are likely to be the result of low levels of gene flow due to female philopatry; this population structure is likely to have been maintained by the stable climatic conditions in eastern Madagascar during the Quaternary.

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APPENDIX

Appendix 1. Details of *Chaerephon atsinanana* samples used in this study. Université d'Antananarivo - Département de Biologie Animale - UADBA; Steven Goodman – SMG; Field Museum of Natural History - FMNH; field collection number - *; not available – NA; Lat. - Latitude; Long – Longitude; Hap. – Haplotype. Genbank accession numbers are listed for samples used in genetic analyses.

Name	Museum number	Locality in Madagascar	Lat.	Long.	Hap	Genbank #
C. atsinanana	UADBA43912	Ampasika	-19.019	48.349	2	JN867871
C. atsinanana	SMG 16901*	Ampasika	-19.019	48.349	2	JN867872
C. atsinanana	SMG 16902*	Ampasika	-19.019	48.349	2	JN867873
C. atsinanana	SMG 16903*	Ampasika	-19.019	48.349	2	JN867874
C. atsinanana	UADBA43913	Ampasika	-19.019	48.349	2	JN867875
C. atsinanana	UADBA43914	Ampasika	-19.019	48.349	2	JN867876
C. atsinanana	UADBA43915	Ampasika	-19.019	48.349	2	JN867877
C. atsinanana	UADBA43916	Ampasika	-19.019	48.349	2	JN867878
C. atsinanana	UADBA43917	Ampasika	-19.019	48.349	2	JN867879
C. atsinanana	UADBA43918	Ampasika	-19.019	48.349	2	JN867880
C. atsinanana	UADBA43919	Ampasika	-19.019	48.349	2	JN867881
C. atsinanana	UADBA43921	Ampasika	-19.019	48.349	2	JN867882
C. atsinanana	UADBA43913	Ampasika	-19.019	48.349	2	JN867883
C. atsinanana	FMNH 184677	Anjiro	-18.882	47.971	2	JN867884
C. atsinanana	FMNH 184678	Anjiro	-18.882	47.971	2	JN867885
C. atsinanana	FMNH 184680	Anjiro	-18.882	47.971	2	JN867886
C. atsinanana	FMNH 184491	Andasibe	-18.895	48.415	2	JN867887
C. atsinanana	FMNH 184492	Andasibe	-18.895	48.415	2	JN867888
C. atsinanana	FMNH 184493	Andasibe	-18.895	48.415	2	JN867889
C. atsinanana	FMNH 184494	Andasibe	-18.895	48.415	2	JN867890
C. atsinanana	FMNH 184495	Andasibe	-18.895	48.415	2	JN867891
C. atsinanana	FMNH 184496	Andasibe	-18.923	48.421	2	JN867892
C. atsinanana	FMNH 184499	Andasibe	-18.923	48.421	2	JN867893
C. atsinanana	FMNH 184500	Andasibe	-18.923	48.421	2	JN867894
C. atsinanana	FMNH 184509	Beforona	-18.889	48.578	7	JN867924
C. atsinanana	FMNH 184510	Beforona	-18.889	48.578	2	JN867895
C. atsinanana	FMNH 184511	Beforona	-18.889	48.578	7	JN867925
C. atsinanana	FMNH 184512	Beforona	-18.889	48.578	7	JN867926
C. atsinanana	FMNH 184513	Beforona	-18.889	48.578	7	JN867927
C. atsinanana	FMNH 184514	Beforona	-18.889	48.578	7	JN867928
C. atsinanana	FMNH 184515	Beforona	-18.889	48.578	7	JN867929
C. atsinanana	FMNH 184516	Beforona	-18.889	48.578	7	JN867930
C. atsinanana	FMNH 184522	Beforona	-18.889	48.579	7	JN867931
C. atsinanana	FMNH 188142	Marozevo	-18.983	48.617	2	JN867896
C. atsinanana	FMNH 188143	Marozevo	-18.983	48.617	2	JN867897

C. atsinanana	FMNH 188144	Marozevo	-18.983	48.617	2	JN867898
C. atsinanana	FMNH 188113	Moramanga	-18.933	48.200	2	JN867899
C. atsinanana	FMNH 188114	Moramanga	-18.933	48.200	2	JN867900
C. atsinanana	FMNH 188116	Moramanga	-18.933	48.200	2	JN867901
C. atsinanana	FMNH 188117	Moramanga	-18.933	48.200	2	JN867902
C. atsinanana	FMNH 185229	Vangaindrano	-23.355	47.596	1	JN867846
C. atsinanana	FMNH 185230	Vangaindrano	-23.355	47.596	1	JN867849
C. atsinanana	FMNH 185231	Vangaindrano	-23.355	47.596	1	JN867848
C. atsinanana	FMNH 185232	Vangaindrano	-23.355	47.596	1	JN867849
C. atsinanana	FMNH 185233	Vangaindrano	-23.355	47.596	1	JN867850
C. atsinanana	FMNH 185259	Farafangana	-22.821	47.831	1	JN867851
C. atsinanana	FMNH 185260	Farafangana	-22.821	47.831	1	GQ489119
C. atsinanana	FMNH 185261	Farafangana	-22.821	47.831	1	JN867852
C. atsinanana	FMNH 185262	Farafangana	-22.821	47.831	1	JN867853
C. atsinanana	FMNH 185263	Farafangana	-22.821	47.831	1	JN867854
C. atsinanana	FMNH 185265	Farafangana	-22.821	47.831	1	JN867855
C. atsinanana	FMNH 185283	Vohipeno	-22.367	47.837	1	JN867856
C. atsinanana	FMNH 185284	Vohipeno	-22.367	47.837	1	JN867857
C. atsinanana	FMNH 185285	Vohipeno	-22.367	47.837	1	JN867858
C. atsinanana	FMNH 185286	Vohipeno	-22.367	47.837	1	GQ489120
C. atsinanana	FMNH 185287	Vohipeno	-22.367	47.837	1	JN867859
C. atsinanana	FMNH 185288	Vohipeno	-22.367	47.837	1	JN867860
C. atsinanana	FMNH 185290	Vohipeno	-22.367	47.837	1	JN867861
C. atsinanana	FMNH 185291	Vohipeno	-22.367	47.837	1	JN867862
C. atsinanana	FMNH 185292	Vohipeno	-22.367	47.837	1	JN867863
C. atsinanana	FMNH 185295	Vohipeno	-22.367	47.837	1	JN867864
C. atsinanana	FMNH 185307	Vohipeno	-22.367	47.837	1	JN867865
C. atsinanana	FMNH 185313	Manakara	-22.157	48.017	1	JN867866
C. atsinanana	FMNH 185314	Manakara	-22.157	48.017	1	JN867867
C. atsinanana	FMNH 185315	Manakara	-22.157	48.017	1	GQ489121
C. atsinanana	FMNH 185316	Manakara	-22.157	48.017	1	JN867868
C. atsinanana	FMNH 185317	Manakara	-22.157	48.017	1	JN867869
C. atsinanana	FMNH 185318	Manakara	-22.157	48.017	1	JN867870
C. atsinanana	FMNH 185319	Ifanadiana	-21.307	47.636	4	JN867907
C. atsinanana	FMNH 185320	Ifanadiana	-21.307	47.636	4	JN867908
C. atsinanana	FMNH 185321	Ifanadiana	-21.307	47.636	4	JN867909
C. atsinanana	FMNH 185322	Ifanadiana	-21.307	47.636	4	JN867910
C. atsinanana	FMNH 185323	Ifanadiana	-21.307	47.636	4	JN867911
C. atsinanana	FMNH 185324	Ifanadiana	-21.307	47.636	4	JN867912
C. atsinanana	FMNH 185326	Ifanadiana	-21.307	47.636	4	JN867913
C. atsinanana	FMNH 185335	Ifanadiana	-21.298	47.638	4	JN867914
C. atsinanana	FMNH 185336	Ifanadiana	-21.298	47.638	4	JN867915
C. atsinanana	FMNH 187797	Toamasina	-18.141	49.378	3	GQ489122
C. atsinanana	FMNH 187799	Toamasina	-18.141	49.378	3	GQ489123
C. atsinanana	FMNH 187801	Toamasina	-18.141	49.378	3	JN867903
C. atsinanana	FMNH 187803	Toamasina	-18.141	49.378	3	JN867904

C. atsinanana	FMNH 187804	Toamasina	-18.141	49.378	3	JN867905
C. atsinanana	FMNH 187805	Toamasina	-18.141	49.378	3	JN867906
C. atsinanana	FMNH 187816	Fanandrana	-18.252	49.268	7	JN867932
C. atsinanana	FMNH 187817	Fanandrana	-18.252	49.268	7	JN867933
C. atsinanana	FMNH 187820	Fanandrana	-18.252	49.268	6	JN867923
C. atsinanana	FMNH 187822	Fanandrana	-18.252	49.268	7	JN867934
C. atsinanana	FMNH 187823	Brickaville	-18.822	49.072	7	JN867935
C. atsinanana	FMNH 187835	Ranomafana Atsinanana	-18.961	48.847	7	JN867936
C. atsinanana	FMNH 187836	Ranomafana Atsinanana	-18.961	48.847	7	GQ489125
C. atsinanana	FMNH 187837	Ranomafana Atsinanana	-18.961	48.847	7	JN867937
C. atsinanana	FMNH 188082	Ranomafana Ifanadiana	-21.258	47.456	4	JN867916
C. atsinanana	FMNH 188083	Ranomafana Ifanadiana	-21.258	47.456	4	JN867917
C. atsinanana	FMNH 188084	Ranomafana Ifanadiana	-21.258	47.456	4	JN867918
C. atsinanana	FMNH 188085	Ranomafana Ifanadiana	-21.258	47.456	4	JN867919
C. atsinanana	FMNH 188086	Ranomafana Ifanadiana	-21.258	47.456	4	JN867920
C. atsinanana	FMNH 188088	Ranomafana Ifanadiana	-21.258	47.456	4	GQ489126
C. atsinanana	FMNH 188089	Ranomafana Ifanadiana	-21.258	47.456	4	GQ489127
C. atsinanana	FMNH 188090	Ranomafana Ifanadiana	-21.258	47.456	4	JN867921
C. atsinanana	FMNH 188091	Ranomafana Ifanadiana	-21.258	47.456	5	JN867922
C. atsinanana	FMNH 184651	Ambatondrazaka	-17.900	48.483	8	JN867938
C. atsinanana	FMNH 184652	Ambatondrazaka	-17.900	48.483	8	JN867939
C. atsinanana	FMNH 184653	Ambatondrazaka	-17.900	48.483	8	JN867940
C. atsinanana	FMNH 184654	Ambatondrazaka	-17.900	48.483	8	JN867941
C. atsinanana	FMNH 184655	Ambatondrazaka	-17.900	48.483	8	JN867942
C. atsinanana	FMNH 184659	Ambatondrazaka	-17.900	48.483	8	JN867943

CHAPTER 2

FURTHER NUCLEAR INSIGHTS INTO THE PHYLOGENY OF AFRO-MALAGASY MOLOSSIDAE (CHIROPTERA)

2.1 ABSTRACT

This study of phylogenetic relationships among five genera and 13 species of Afro-Malagasy Molossidae is based on five nuclear gene regions (FES, GHR, PNPO-3, RHO1 and PRKC1). PNPO-3 is a novel nuclear intron marker, previously unused in phylogenetic studies of bats. It is more variable at intergeneric level and in some interspecific comparisons than the other nuclear markers (above). It provides resolution primarily at the genus level, with more limited interspecific resolution. We added mitochondrial cytochrome *b* (Cyt *b*) and nuclear Rag2 sequences to the study, retrieved from the NCBI Genbank (appendix).

Individual and concatenated datasets were analysed using Bayesian inference, neighbor-joining and parsimony methods. There is no support for the monophyly of either of the genera *Chaerephon* or *Mops*, or for the generic affiliation of *Chaerephon jobimena*. *Mops midas*, *M. leucostigma* and *M. condylurus* do not form a monophyletic clade. In contrast, there is support for the monophyly of taxa belonging to *Mops* (*M. leucostigma* and *M. condylurus*) and *Chaerephon* (*C. atsinanana*, *C. leucogaster*, *C. pusillus* and *C. pumilus* s. l.) in a clade with a crown age of 10.46 (4.24-18.66) million years. This study provides strong support for the monophyly of the *Chaerephon* and *Mops* taxa included, with a crown age of 14.82 (6.44-25.54) million years, or 21.97 (12.16-33.44) million years if *C. jobimena* is included. Based on this there is a case for the inclusion of *Mops* and *Chaerephon* in a single combined genus. As there is good nuclear support for the previously mitochondrially-defined subclades of *Otomops martiensseni*, which last shared a common ancestor 4.18 (1.08-9.96) MYA, it appears that these may be distinct species. We[1] propose that the clade from north east Africa and Arabia be named as a new species of *Otomops*, as the clade from southern and western Africa includes the type locality. This study provides weak support based on individual gene regions for associations of *Sauromys* with *Otomops* and *Mormopterus*, although these do not stand up in the concatenated datasets which offer better resolving power, indicating that *Sauromys* may be independent of

[1] This chapter will be submitted for publication. As there will be more than one author on this paper, collective nouns are used where appropriate in the text.

Chaerephon, *Mops*, *Otomops* and *Mormopterus*. These results provide some support for the membership of *Mormopterus* in the proposed Old World Molossid tribe, Tadarini; however, they also support its position as a basal genus within the Molossidae, consistent with its designation as a separate tribe, Mormopterini.

Keywords: Chiroptera, nuclear DNA, phylogenetics, mitochondria, Malagasy, *Chaerephon*, Molossidae.

2.2 INTRODUCTION

Molossidae are strong flying bats, with long narrow wings and have the ability to maintain flight for long periods at high elevations, as noted in their presence at altitudes ranging from sea level to 1100m (Altringham 2011; Lamb *et al*. 2012). Unlike most other bats, Molossids have a tail that is not enclosed in a membrane (Ammerman *et al*. 2012) hence their common name, free-tailed bats. There are still many unresolved questions in molossid phylogenetics. Conflicting inferences have been derived from morphological and molecular studies (Freeman 1981; Lamb *et al*. 2011; Agnarsson *et al*. 2011; Ammerman *et al*. 2012) and taxonomic sampling remains a problem in this family of high-flying bats which are difficult to trap, especially in less accessible regions of their pan-tropical ranges.

The family Molossidae Gervais, 1856 consists of 16 genera and approximately 100 species (Simmons 2005). It is further divided into two subfamilies; the Tomopeatinae comprises the genus *Tompeas* Miller, 1900 (Sudman *et al*. 1994) while the Molossinae consists of 15 genera. These include seven Old World genera (*Myopterus* Geoffroy, 1818; *Cheiromeles* Horsfield, 1824; *Mops* Lesson, 1842; *Chaerephon* Dobson, 1874; *Platymops* Thomas, 1906; *Otomops* Thomas, 1913; *Sauromys* Roberts, 1917), six new world genera (*Molossus* Geoffroy, 1805; *Promops* Gervais, 1856; *Molossops* Peters, 1865; *Nyctinomops* Miller, 1902; *Eumops* Miller, 1906; *Cynomops* Thomas, 1920) and two genera represented in both the Old World and New World (*Mormopterus* Peters, 1865; *Tadarida* Rafinesque, 1814) (Simmons 2005).

Whilst earlier systematic studies of Molossidae (e.g. Peterson 1965; Freeman 1981) were founded on morphological data, molecular phylogenies based on mitochondrial and nuclear sequences have recently been published (Lamb *et al*. 2011, Ammerman *et al*. 2012). Although these studies, which had overlapping taxonomic representation, were based on analysis of different genome regions, their findings were largely congruent.

Both studies supported the monophyly of the sub-family Molossinae. The monophyly of the genus *Otomops* and sister relationship of Malagasy *O. madagascariensis* Dorst, 1953 and Afro-Arabian *O. martiensseni* Matschie, 1897 were strongly supported by Lamb *et al*. (2008)

based on mitochondrial data. *Otomops martiensseni* formed two strongly-supported geographically-circumscribed mitochondrial clades, one from north east Africa and Yemen, and the other from southern and western Africa.

Both Lamb *et al*. (2011) and Ammerman *et al*. (2012) recovered significant support for a monophyletic *Mops*/*Chaerephon* clade and paraphyly involving *Chaerephon* and *Mops* taxa. There was general support for a sister relationship between the mainland African *Mops condylurus* Smith, 1833 and the Malagasy form, *Mops leucostigma* Allen, 1918. Rosevear (1965) proposed on morphological grounds that *M. condylurus* should be included in *Chaerephon*, consistent with Peterson *et al*. (1995), who reported *Chaerephon*-like morphological attributes in *M. condylurus* and *M. leucostigma*.

At issue is the phylogenetic position of the Malagasy form, *C. jobimena* Goodman & Cardiff, 2004, recently described based on morphological characters (Goodman & Cardiff 2004). *Chaerephon jobimena* is distinct from the well supported *Mops/Chaerephon* clade reported by Lamb *et al*. (2011) based on nuclear Rag 2 and Cyt *b* seqences, and included in a strongly-supported clade with *Tadarida aegyptiaca* Geoffroy, 1818. Ammerman *et al*. (2012) report a moderate- to weakly-supported affinity between *C. jobimena* and *C. plicatus* based on mitochondrial ND1 sequences.

Lamb *et al*. (2011) reported significant support for a *Tadarida brasiliensis* Geoffroy, 1824, *T. aegyptiaca*, and *C. jobimena* clade while Ammerman *et al*. (2012) report a supported *T. aegyptiaca*, *T. brasiliensis* and *S. petrophilus* Roberts, 1917 clade, distinct from other species of *Tadarida*, and recommend inclusion of *Sauromys* in *Tadarida*, consistent with Freeman (1981).

Lamb *et al*. (2011) report *Mormopterus* as paraphyletic, with the American *M. kalinowskii* Thomas, 1893 distinct from a well-supported western Indian Ocean *Mormopterus* clade which occupies a relatively basal position with the Molossinae.

A suggestion by Simmons (1998) that the molossid subfamilies, Molossinae and Tomopeatinae, be further subdivided into tribes was adopted by Ammerman *et al*. (2012) who proposed that Molossinae be further divided into four tribes: Tadarini (Old World taxa: *Otomops*, *Tadarida*, *Chaerephon*, *Mops*, *Platymops*, *Sauromys* and *Myopterus*); Molossini (New World taxa: *Molossus*, *Eumops*, *Molossops*, *Cynomops*, *Neoplatymops*, *Nyctinomops*, and *Promops*); Cheiromelini (*Cheiromeles*) and Mormopterini (*Mormopterus*).

Recently-described *C. atsinanana* (Goodman *et al.* 2010) shows very high levels of phylogeograhic and genetic structure over its range in south eastern Madagascar based on analyses of the mitochondrial Cyt *b* gene and control region (Lamb *et al*. 2012). These authors hypothesised that this may be due to female philopatry as revealed by these maternally-inherited markers. One of the initial aims of this study was to evaluate levels of population structure in *C.*

atsinanana using biparentally-inherited nuclear sequence markers, in order to assess the possible presence of male philopatry. We therefore attempted to amplify and sequence candidate nuclear regions in order to identify a suite which was variable in *C. atsinanana.* Of the five nuclear markers which were successfully optimised, none were sufficiently variable to study nuclear genetic structure in *C. atsinanana.* These nuclear sequences were used to provide additional insight into phylogenetic relationships among Afro-Malagasy Molossidae.

This study focuses on phylogenetic relationships among five genera and 13 species of Afro-Malagasy Molossidae, namely: *Mops leucostigma* Allen, 1918, *M. midas* Sundevall, 1843, *M. condylurus* Smith, 1833, *Mormopterus jugularis* Peters, 1865, *M. acetabulosus* Hermann, 1804, *Otomops martiensseni* Matschie, 1897 (SA and NA forms), *O. madagascariensis* Dorst, 1953, *Chaerephon atsinanana* Goodman *et al*. 2010, *C. lecuogaster* Grandidier, 1869, *C. pumilus* sensu lato (s. l.) from south eastern Africa, *C. pusillus* Miller, 1902, *C. jobimena* Goodman & Cardiff, 2004 and *Sauromys petrophilus* Roberts, 1917. Molecular methods were based on five nuclear markers, in the hope of revealing more information on the relationships of African and Malagasy Molossid bats. We added mitochondrial Cyt *b* and nuclear Rag2 sequences to the study, retrieved from the NCBI Genbank (appendix).

We aim to provide dates for major divergences within the Afro-Malagasy Molossidae, and to evaluate the levels of support provided by our analyses of five nuclear gene regions for: (a) The monophyly of the *Chaerephon* taxa included in this study; (b) the generic status of *C. jobimena*; (c) the monophyly of the *Mops* taxa included here; (d) the monophyly of *Chaerephon* and *Mops* taxa jointly; (e) the two major mitochondrially-defined clades of *Otomops martiensseni*, from north east Africa and Yemen (NA) and southern and western Africa (SA) respectively; (g) the phylogenetic position of *Sauromys*; (h) the proposed tribes Tadarini (Old World bats) and Mormopterini (*Mormopterus*) (Ammerman *et al*. 2012).

2.3 MATERIALS AND METHODS

TAXON SAMPLING

Tissue samples were acquired from several institutions or individuals, in the form of wing punches, previously isolated DNA samples and heart, liver, kidney or muscle tissue preserved in 80% ethanol. Samples included representatives of five genera and 13 species of the Molossidae. Outgroups, comprising sequences from the family Vespertilionidae and Mustelidae were either downloaded from the NCBI Genbank or sequenced directly (Appendix 1 & 2). Mustelidae was used as an outgroup sequence in some cases where outgroup Chiropteran sequences were unavailable for a particular gene marker.

GENERATION OF SEQUENCE DATA

DNA was extracted from tissue samples using a DNeasy® DNA isolation kit (QIAGEN) according to the manufacturer's instructions. Amplicons were generated from a set of five nuclear primers (Table 1). All of the regions amplified were introns; these included FES, GHR, RHO1 (Venta *et al*. 1996), PRKC1 (Matthee *et al*. 2001).and PNPO-Intron 3 (Igea *et al*. 2010), a novel marker previously unused in phylogenetic studies. Mitochondrial Cyt *b* and nuclear Rag2 sequences added to the study were retrieved from the NCBI GenBank (appendix).

PCR amplifications were performed in 25µl reaction volumes containing 9 µl of a DNA solution (3 ng μl^{-1}), 0.8 µl PCR water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl 25 mM $MgCl_2$ (Super-Therm), 0.5 µl 10 mM deoxynucleoside-triphosphate mixture (dNTPs) (Roche), 0.2 µl *Taq* polymerase (5 u/µl) (Super-Therm, Southern Cross Biotech, SA) and 4 µl of each primer (6 µM) (forward and reverse). The thermal cycling parameters used were similar for all five nuclear markers, except that the annealing temperature varied: 95°C for 5 min, followed by 39 cycles of [95°C for 30 s, 55-60°C (varies among markers, Table 1) for 30 s, 72°C for 2 min] followed by 72°C for 10min. Amplified fragments were separated by agarose gel electrophoresis, using a 100bp molecular weight marker (Roche Applied Science) to size the bands. DNA was purified from excised gel bands using a QIAquick PCR Purification Kit (QIAGEN) and sequenced at Inqaba Biotec (Hatfield, Pretoria, South Africa). Sequences were edited and aligned in BioEdit Version 7.0.9.0 for Windows 95/98/NT (Hall 1999), and further adjustments were made manually

Table 1. Primers and annealing temperatures of DNA markers used in this study. Ta = annealing temperature.

Acronym	Gene name	Ta (°C)	Primer sequence
FES	feline sarcoma protooncogene	58	F: GGGGAACTTTGGCGAAGTGTT [a] R: TCCATGACGATGTAGATGGG
GHR	growth hormone receptor	60	F: CCAGTTCCAGTTCCAAAGAT[a] R: TGATTCTTCTGGTCAAGGCA
RHO1	rhodopsin	55	F: TACATGTTCGTGGTCCACTT [a] R: TGGTGGGTGAAGATGTAGAA
PRKC1	protein-kinase C1	55	F: GGGTAATAGGAAGAGGAAGTT[b] R: CCAACAAGGAAAGGATGAT
PNPO-3	Pyridoxine 5'-phosphate oxidase intron 3	55	F: GATGGCTTCCRHTTCTWCACTAACTT[c] R: GGYTCCCARTAGAAGACMAKSGA

F- forward: R- reverse;[a] Venta *et al*. 1996;[b] Matthee *et al*. 2001;[c] Igea *et al*. 2010

SEQUENCE ANALYSES

Phylogenetic analyses were carried out on each of the five DNA regions and on Cyt *b* and Rag 2 data (retrieved from the NCBI GenBank) separately. Incongruence of phylogenetic signal between datasets was evaluated by determining whether there were any nodes which were strongly supported (70% bootstrap support, ≥ 95% Bayesian posterior probability) in one dataset that conflicted with strongly-supported nodes in another (De Queiroz, 1993; Eick *et al*. 2005). The absence of such instances allowed for the concatenation of sequences. In addition to a phylogeny based on concatenation of all five datasets, three further concatenated datasets were created to provide phylogenetic information on taxonomic units for which certain sequences were not obtained. The FES, RHO1, Cyt b and Rag 2 datasets were combined to evaluate the placement of *C. jobimena*, and FES, RHO1, PNPO-3, RHO1, Cyt b and Rag2 were combined to evaluate the position of *M. jugularis* and *S. petrophilus*. Mitochondrial Cyt *b* and nuclear Rag2 datasets were concatenated for comparison to other datasets. We used mitochondrial DNA as it has a higher rate of evolution than nuclear DNA, thus the analyses can be compared and be more reliable. Rag2 nuclear DNA was added for comparison to the other nuclear genes, as it has been used more frequently in other studies. In a few cases it was necessary to concatenate different samples of the same species.

The AKAIKE information criterion was applied in jModelTest 0.1.1 (Posada 2008) to identify the appropriate nucleotide substitution model (Table 2) for each dataset analysed. We analysed all datasets using parsimony and neighbor-joining methods in PAUP 4.0b10 (Swofford, 2002) and Bayesian Inference as implemented in MrBayes v.3.2.1 (Ronquist and Huelsenbeck 2003). Parsimony trees were based on heuristic searches with starting trees obtained through stepwise addition with random addition of sequences. The number of replicates was 10 and one tree was retained at each step during the stepwise addition. The branch-swapping algorithm was based on tree-bisection-reconnection (TBR). Nodal support was estimated using 100 bootstrap replicates for parsimony analyses and 1000 replicates for neighbor joining analyses.

In the Bayesian inference of phylogenetic relationships, four Markov chains were run for 5million generation such that the standard deviation of the split frequencies was less than 0.01. Trees were sampled every 100 generations. The chains were heated using a temperature scaling factor of T=0.02. We discarded the first 50,000 trees as burn-in, in each case having checked in a preliminary run that this was more than sufficient to achieve stationarity, and constructed a 50% majority rule consensus tree from the remaining trees.

In order to compare the variability of the five nuclear markers used in this study, MEGA v. 5 (Tamura *et al*. 2011) was used to calculate mean uncorrected p-distances within

species and between species, genera and families. Polymorphic sites were determined in DnaSP v.5.0 (Librado and Rozas 2009).

DIVERGENCE TIME

Concatenated dataset 3 (1470 nt) (Table 2) was used to produce a chronogram estimating the dates of the major divergences within the dataset. Analyses were carried out using Bayesian inference implemented in Beast 1.6.1 (Drummond & Rambaut 2007). The data was calibrated on an estimate of 35-38 MYA for the crown divergence for Molossidae (Jones *et al*. 2005). Bayesian analyses were carried out using the GTR substitution model as determined in jModelTest 0.1.1 (Guindon & Gascuel 2003), using a Yule tree prior. Divergence times and their 95% credibility intervals were estimated using a relaxed clock model with branch rates drawn from an uncorrelated lognormal distribution. Two independent runs of 2 million generations each with burnins of 1 million were performed.
MCMC chains were run for a sufficient time to achieve convergence [accessed using TRACER 1.2.1 (Rambaut & Drummond 2009)]. The trace analysis tab was selected, this showed the trace of negative log likely hood against generation number and allowed assessment of convergence of chains. The annotated tree was viewed in FigTree v1.3.1 (Rambaut 2009).

2.4 RESULTS

SEQUENCE ANALYSES

Generally low homoplasy indices (HI) ($\leq$0.384) and high retention indices (RI) ($\geq$ 0.805) indicated that there was no significant homoplasy in all the datasets (Table 2). For the seven individual genes segments analysed, the RI ranged from 0.815-0.968 and the HI from 0.040-0.384 (Table 2). In the concatenated data sets the RI ranged from 0.805-0.867 and the HI from 0.090-0.305 (Table 2).

For the five nuclear regions, among species *p*-distances (Table 3), were generally lower (0.000-0.043) than those among genera (0.028-0.087), which were in turn lower than those among families (0.066 -0.464). In most cases Cyt *b* genetic distances among species were higher than nuclear distances (Table 3).

PNPO-3, optimised for this study and previously unused in phylogenetic studies of bats, was more variable than the other nuclear markers (FES, GHR1, PRKC1 and RHO1) in comparisons among molossid genera and in comparisons between *Mops* species and between *Otomops* species (Table 3). This marker provides good resolution at the genus level and can resolve the phylogenetic position of taxa at the species level.

THE MONOPHYLY OF Chaerephon

The nine datasets contained different taxonomic samples of *Chaerephon*. In three of these data sets represented *Chaerephon* taxa were monophyletic. Analysis of the FES region showed unsupported monophyly of *C. lecuogaster*, *C. pumilus*, *C. pusillus*, *C. atsinanana* and *C. jobimena*. Concatenation 1 (2747 nt) provides strong support (1.00/100/100) for the monophyly of *C. atsinanana*, *C. leucogaster* and *C. pumilus* s. l. from south eastern Africa, and moderate support (-/89/81) for that of *C. lecuogaster* and *C. pumilus*. In concatenation 4 (2455 nt) the *C. atsinanana*, *C. leucogaster*, *C. pumilus* s. l. clade is again strongly supported (1.00/100/100), and there is weak support (-/93/75) for the association of *C. leucogaster* and *C. pumilus* s. l. Concatenation 2 (828 nt) provides moderate support (-/100/84) for a clade comprising *C. atsinanana*, *C. leucogaster*, *C. pumilus* s. l. and *C. pusillus*. Six of our datasets do not support the overall monophyly of the *Chaerephon* taxa included, but do support certain clades. RHO1 provides strong support (1.00/99/100) for a *C. atsinanana*, *C. leucogaster*, *C. pumilus* s. l. clade, as does Concatenation 3 (1470 nt), which also shows strongly supports (1.00/100/100) for a *C. atsinanana*, *C. leucogaster*, *C. pumilus* s. l. clade and moderately supports (-/93/86) a *C. leucogaster*, *C. pumilus* s. l. clade.

Table 2. Characteristics of data sets used in this study. Concat. = Concatenation.

Dataset	FES	GHR	PNPO3	PRKC1	RHO1	Cyt *b*	Rag2	Concat. 1	Concat. 2	Concat. 3	Concat. 4
No. genera	5	5	5	4	5	5	5	3	5	5	5
No. of species	13	11	11	9	12	12	12	8	12	11	10
Model of nucleotide substitution	HKY+G	GTR+G	GTR+G	K80	HKY+I	GTR+I+G	GTR+G	GTR+G	GTR+I+G	SYM+G	GTR+G
Length of alignment	339	623	362	274	286	268	559	2747	828	1470	2455
Variable characters	38	161	90	38	33	92	431	626	164	321	554
Parsimony informative characters	19	135	45	34	22	66	33	204	92	137	206
Retention index	0.914	0.915	0.909	0.922	0.968	0.815	0.879	0.867	0.845	0.822	0.805
Homoplasy index	0.057	0.074	0.146	0.048	0.133	0.384	0.040	0.090	0.305	0.230	0.159
MP tree length	159	190	185	168	45	203	500	777	292	505	776

Table 3. Mean uncorrected pairwise sequence distances for the DNA regions analysed in this study.

Comparison		FES	GHR	PNPO-3	PRKC1	RHO1	Cyt *b*	Rag2
Within species		0.00038 ± 0.00433	0.00044± 0.00045	0.00363± 0.01051	0.00003± 0.00075	0.00016± 0.00249	0.00499± 0.00819	0.06272± 0.21615
Among species within genera	*Chaerephon*	0.00787± 0.00561	0.00256± 0.00122	0.00111± 0.00096	0.00000	0.01992± 0.01426	0.06310± 0.07084	0.00936± 0.01209
	Mops	0.02083± 0.01218	0.01948± 0.01687	0.04304± 0.01880	0.01953± 0.01057	0.01988± 0.01428	0.07213± 0.04324	0.25466± 0.76400
	Mormopterus	0.00000	0.03074	0.054	/	0.007	/	/
	Otomops	0.01300	0.00812	0.024	0.067	0.004	0.039	0.001
Among genera		0.03083± 0.01039	0.04601± 0.01479	0.08763± 0.01848	0.06699± 0.02788	0.02892± 0.01386	0.13450± 0.01820	0.07060± 0.06370
Among families	Molosside/ Vespertilionidae	/	0.16786	0.138	0.464	0.066	0.223	0.125

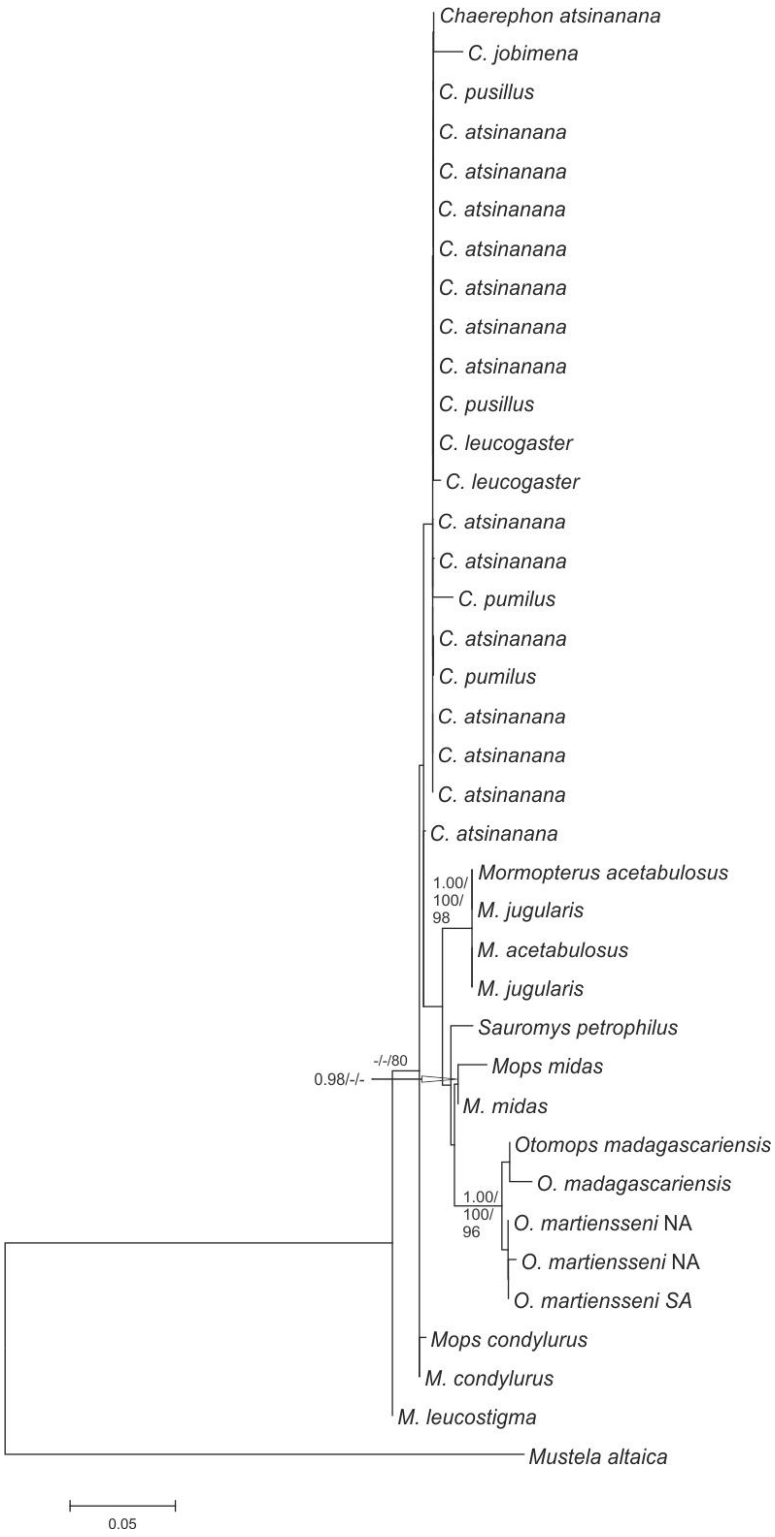

Figure 1. Bayesian inference tree inferred from the analysis of 339 nucleotides of the nuclear FES gene region displaying evolutionary relationships among Afro-Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability/ neighbor-joining bootstrap %/ parsimony bootstrap %. A dash indicates that the node is either not supported or not present. NA – northern Africa; SA – southern Africa.

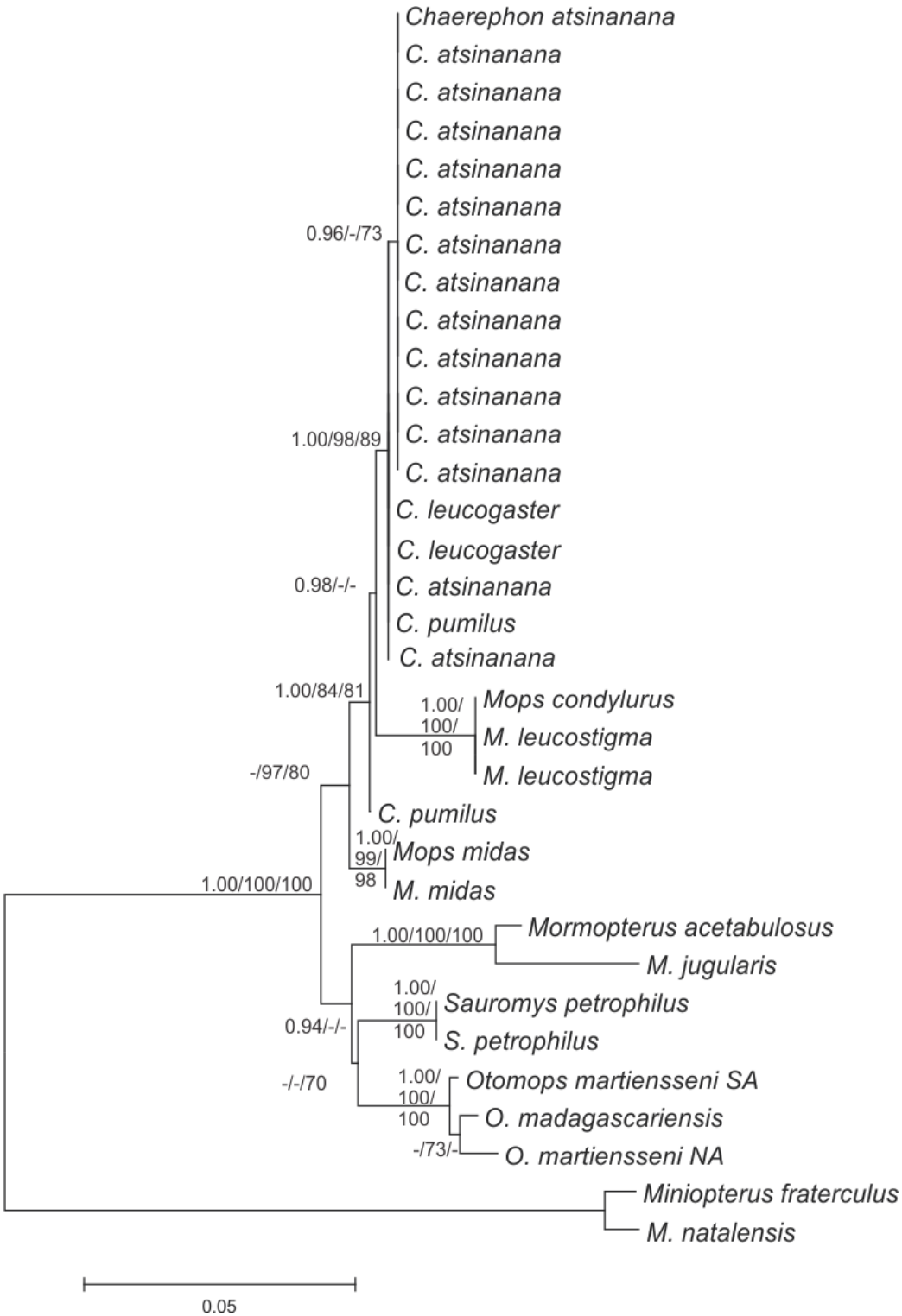

Figure 2. Bayesian inference tree inferred from analysis of 623 nucleotides of the nuclear GHR gene region displaying evolutionary relationships among Afro-Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability followed by neighbor-joining bootstrap % and parsimony bootstrap %. A dash indicates that the node is either not supported or not present.

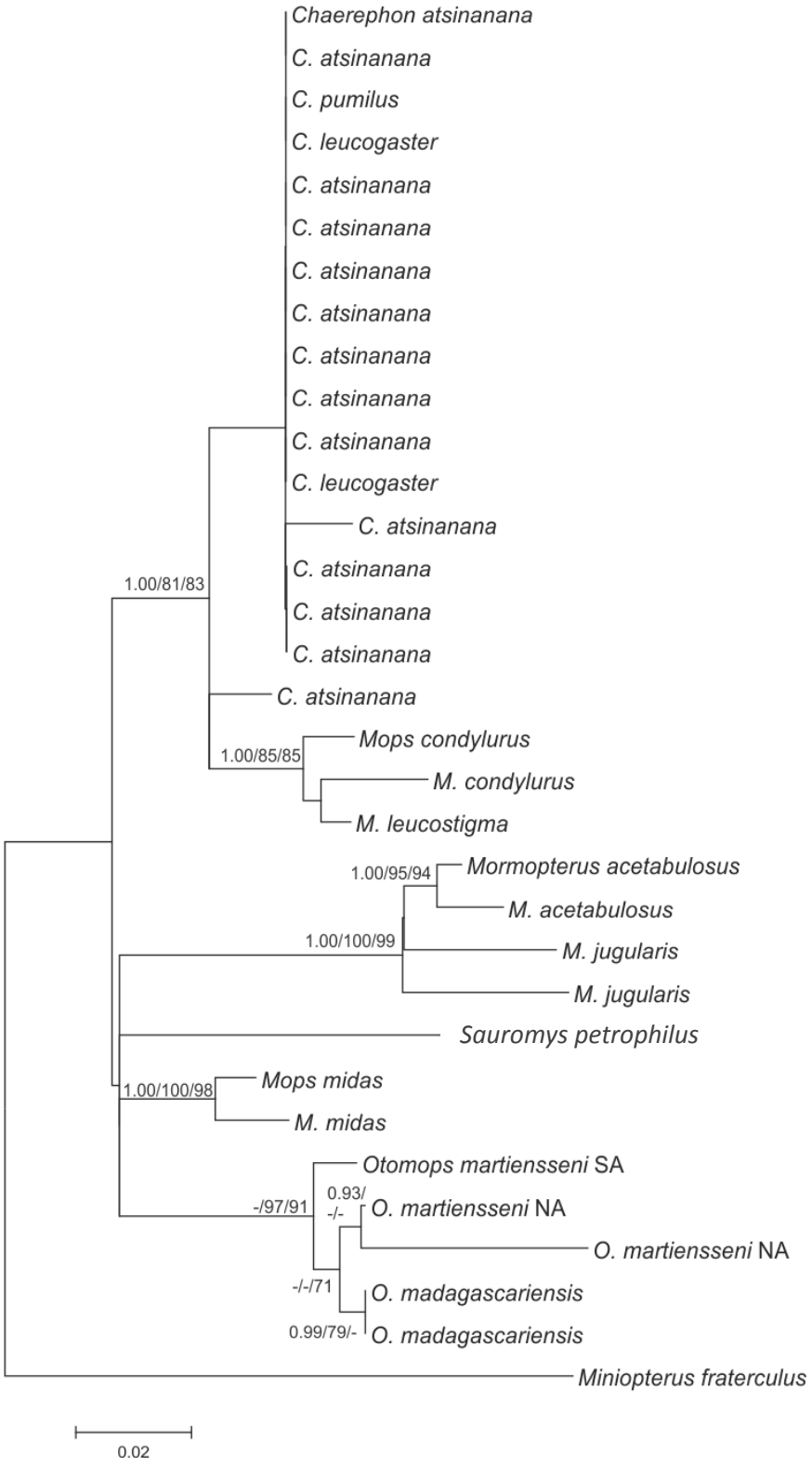

Figure 3. Phylogram based on Bayesian inference of the analysis of 362 nucleotides of the nuclear PNPO-3 gene region displaying evolutionary relationships among Afro Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability/ neighbor-joining bootstrap %/ parsimony bootstrap %. A dash indicates that the node is either not supported or not present.

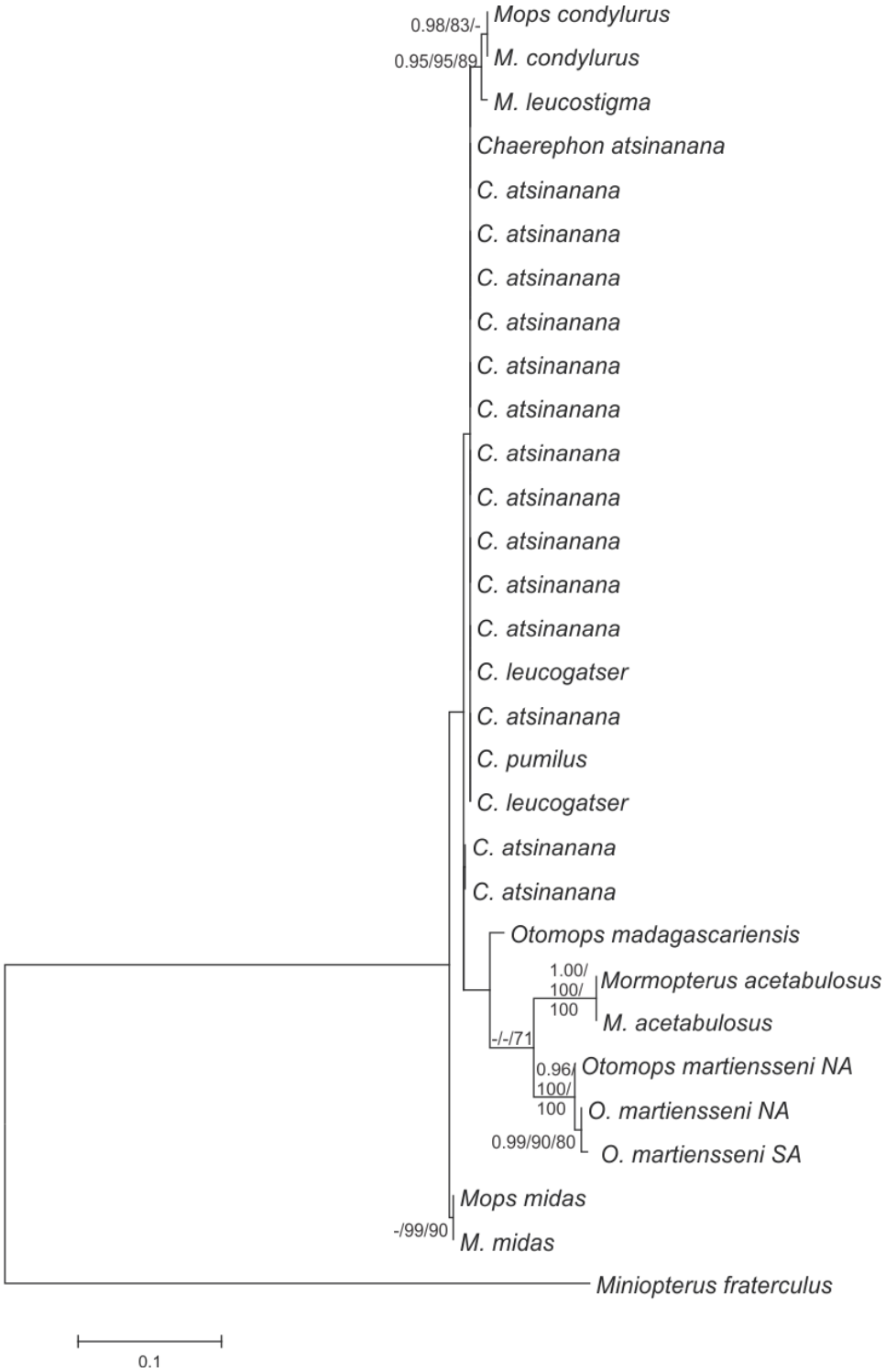

Figure 4. Bayesian inference tree inferred from the analysis of 274 nucleotides of the nuclear PRKC1 gene region displaying evolutionary relationships among Afro-Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability followed by neighbor-joining bootstrap % and parsimony bootstrap %. A dash indicates that the node is either not supported or not present.

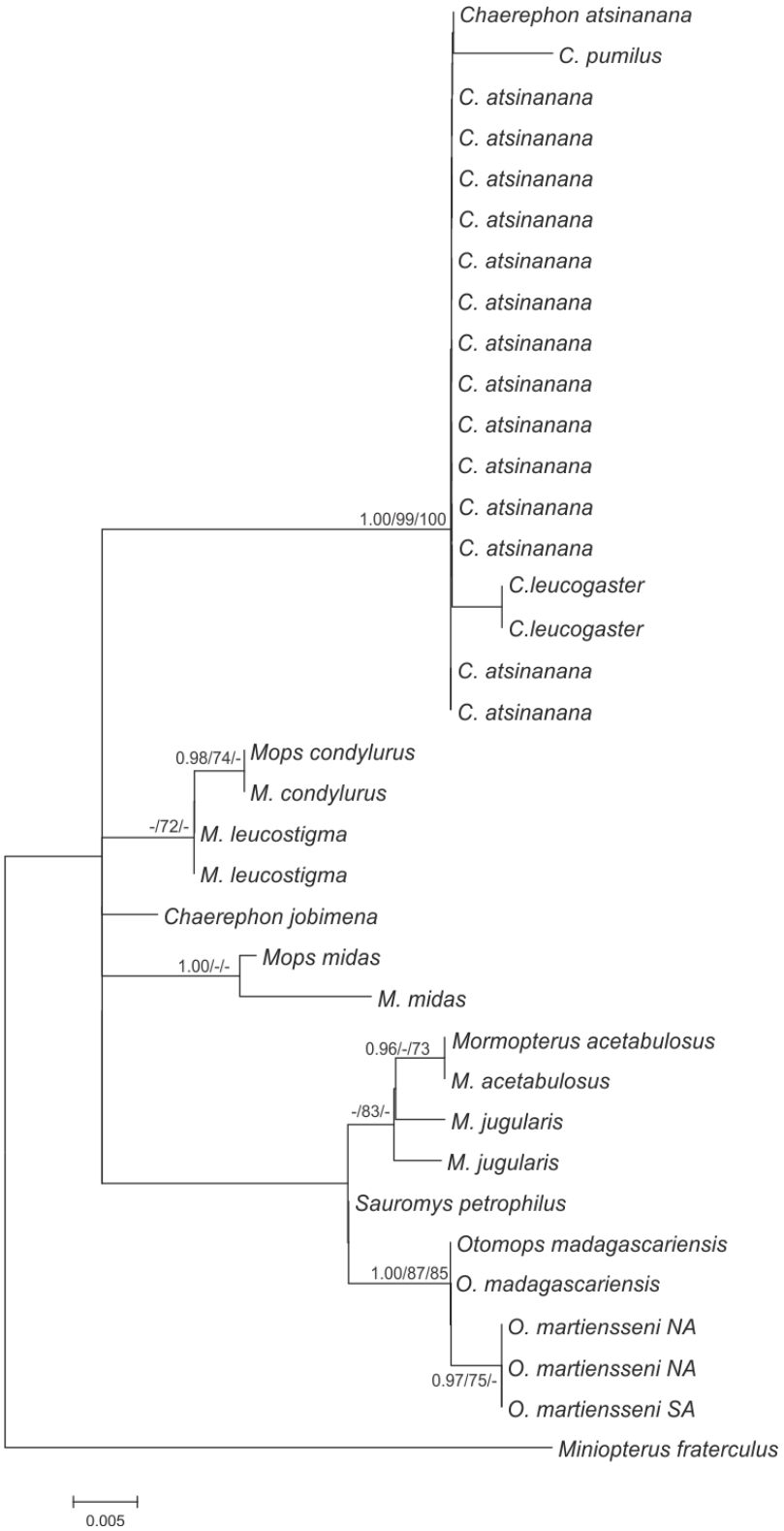

Figure 5. Bayesian inference tree inferred from the analysis of 286 nucleotides of the nuclear RHO1 gene displaying evolutionary relationships among Afro-Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability followed by neighbor-joining bootstrap % and parsimony bootstrap %. A dash indicates that the node is either not supported or not present.

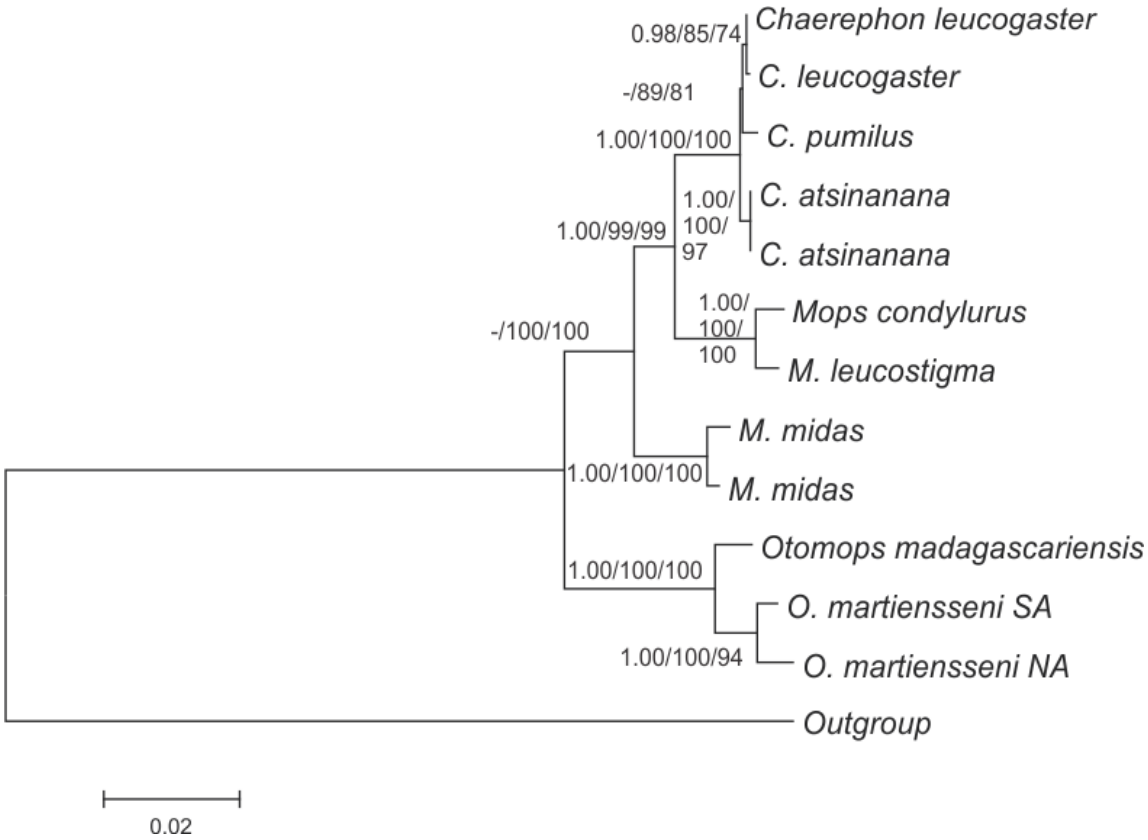

Figure 6. Bayesian inference tree inferred from the analysis of 2747 nucleotides of the concatenation of all 7 genes (Concatenation 1), displaying evolutionary relationships among Afro-Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability followed by neighbor-joining bootstrap % and parsimony bootstrap %. A dash indicates that the node is either not supported or not present.

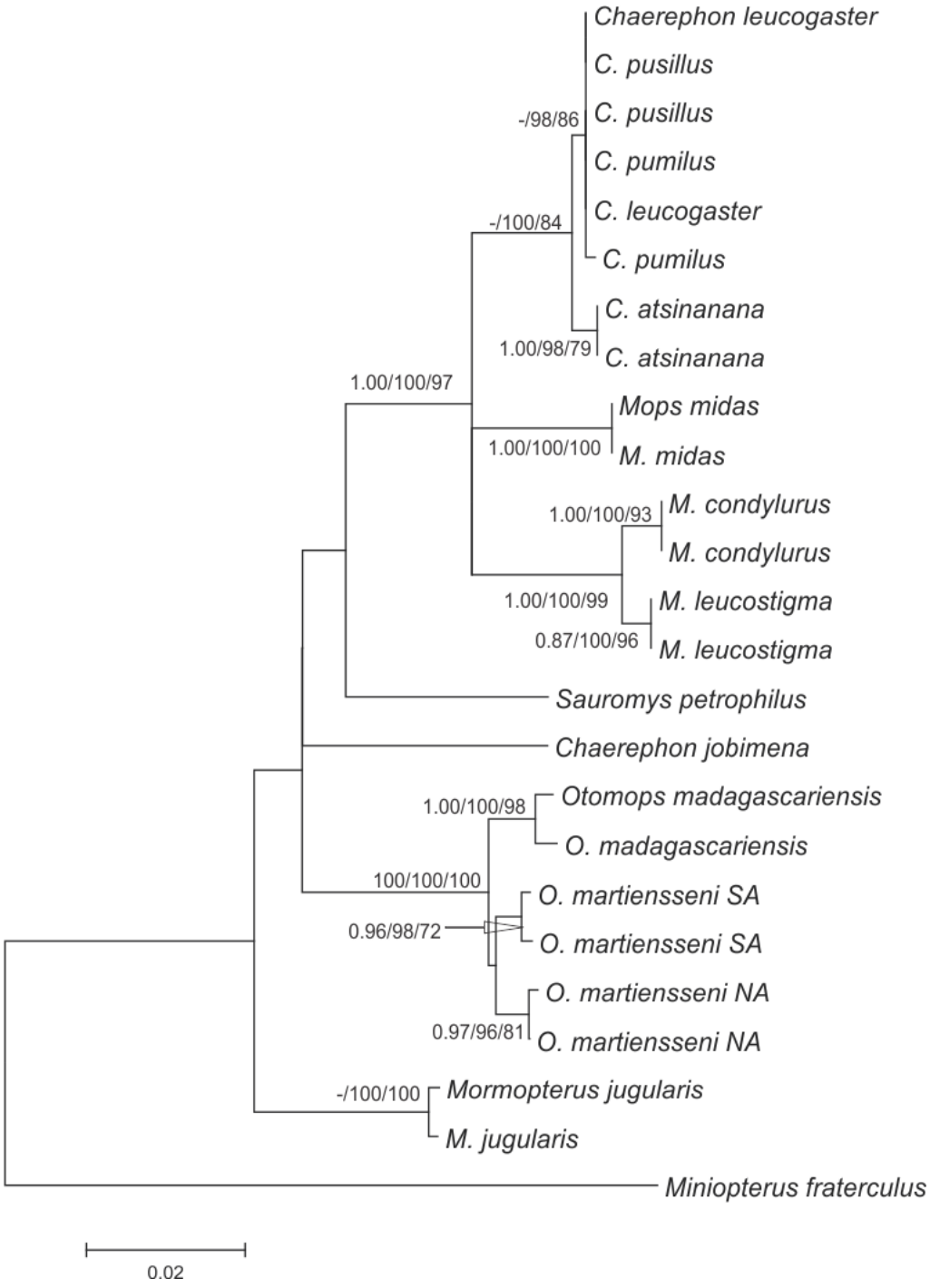

Figure 7. Bayesian inference tree based on the analysis of 828 nucleotides of the concatenated mitochondrial Cyt *b* and nuclear RAG2 genes (Concatenation 2), displaying evolutionary relationships among Afro-Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability followed by neighbor-joining bootstrap % and parsimony bootstrap %. A dash represents an unsupported node (<0.95/70/70). A dash indicates that the node is either not supported or not present.

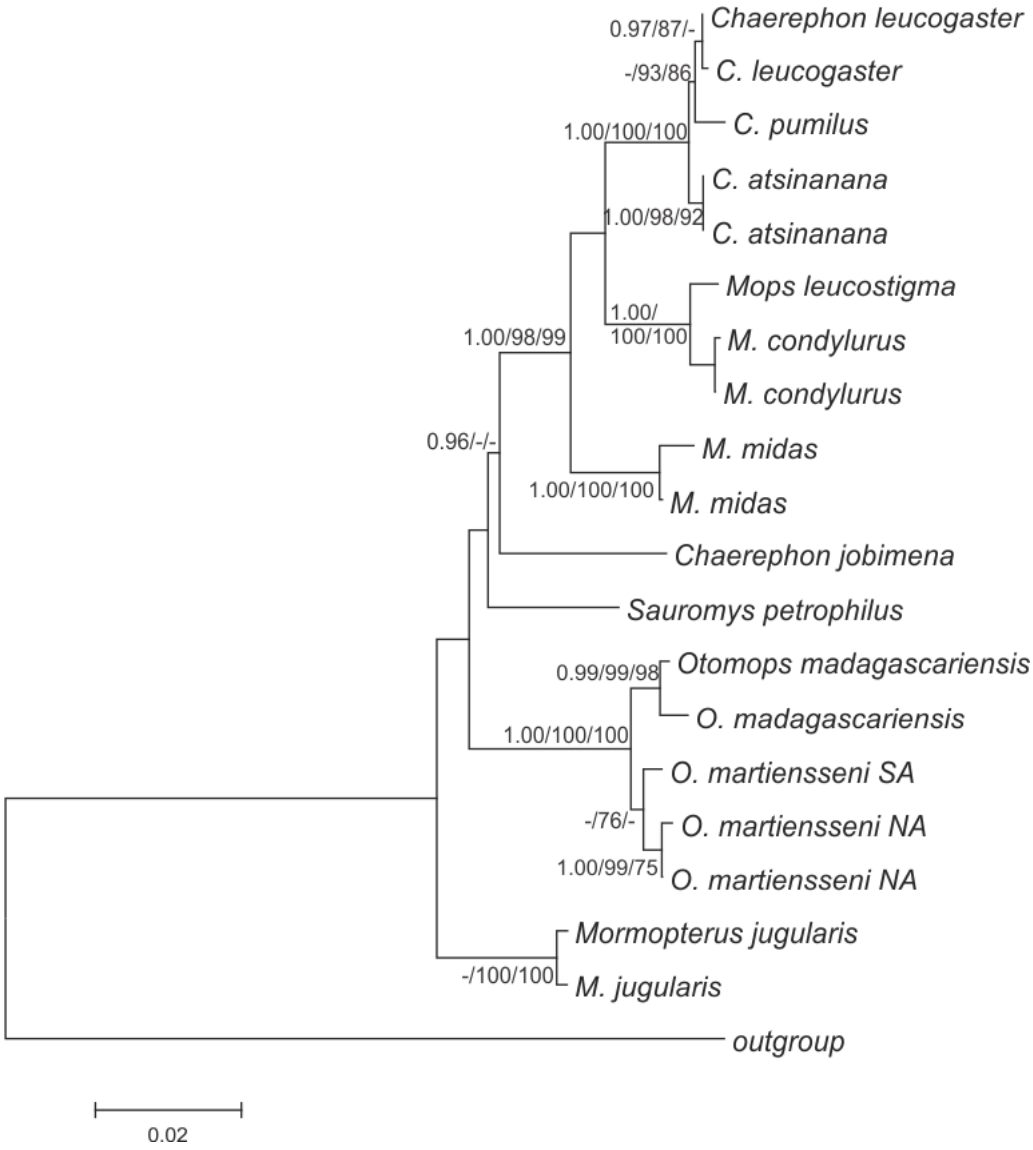

Figure 8. Bayesian inference tree based on the analysis of 1470 nucleotides of the concatenated Cyt *b*, RAG2, FES and RHO1 genes (Concatenation 3), displaying evolutionary relationships among Afro-Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability/ neighbor-joining bootstrap % / parsimony bootstrap %. A dash indicates that the node is either not supported or not present.

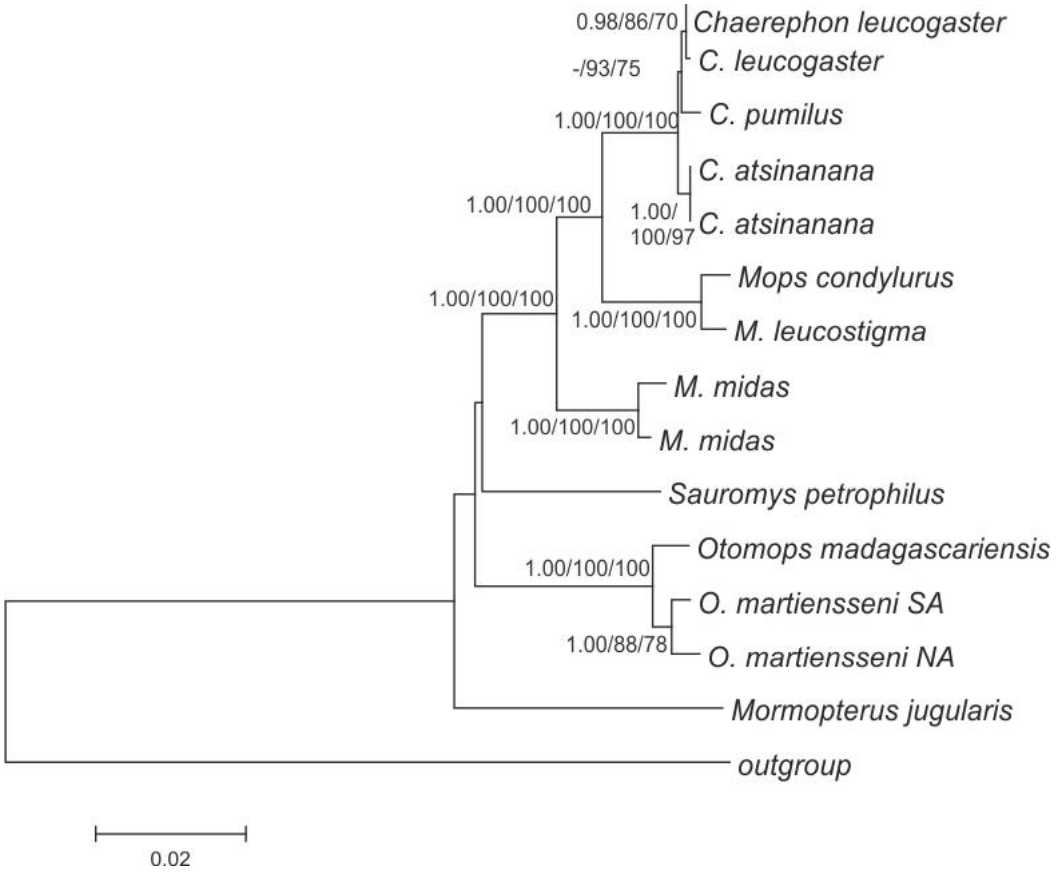

Figure 9. Bayesian inference tree based on the analysis of 2455 nucleotides of the concatenated Cyt *b*, RAG2, FES, RHO1, GHR and PNPO-3 genes (Concatenation 4), displaying evolutionary relationships among Afro-Malagasy molossid bats. Values at nodes are represented as Bayesian posterior probability/ neighbor-joining bootstrap % / parsimony bootstrap %. A dash indicates that the node is either not supported or not present.

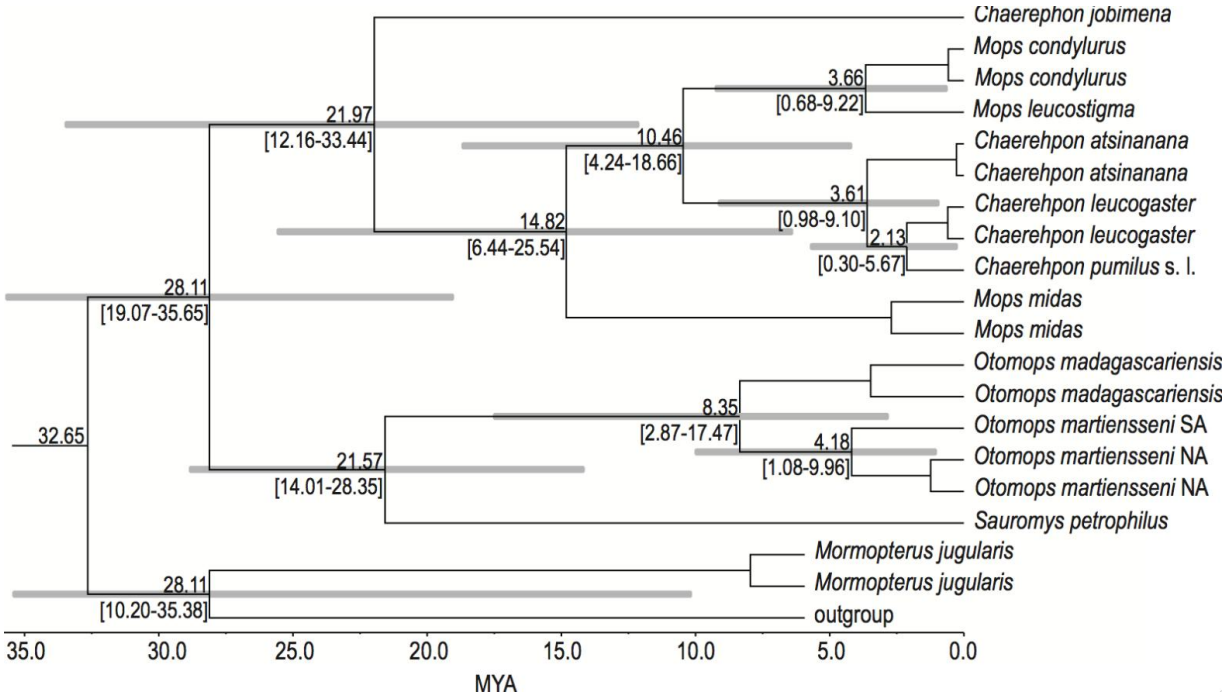

Figure 10. Dated maximum clade probability tree generated in Beast, based on 1470 nucleotides of the concatenated Cyt *b*, RAG2, FES and RHO1 genes (Concatenation 3), displaying evolutionary relationships among Afro-Malagasy molossid bats. Divergence times and 95 % HPD credibility intervals are indicated at the nodes. MYA = million years ago.

GENERIC STATUS OF *Chaerephon jobimena*

Chaerephon jobimena, represented in four of the datasets, is part of an unsupported monophyletic *Chaerephon* clade based on the FES dataset (Fig. 1) and is distinct from the *Chaerephon* clade in the other three data sets, (Figs 5, 7 and 8). In Concatenation 3 (Fig. 8), *C. jobimena* occupies a weakly-supported position (0.96/-/-) sister to a strongly-supported clade (1.00/98/99) comprising all other *Chaerephon* and *Mops* taxa. In analyses of the RHO1gene region (Fig. 5) and Concatenation 2 (Fig. 7), *C. jobimena* is not allied with any other genus or species group.

THE MONOPHYLY OF Mops

Whilst there is very strong support for the monophyly of *M. leucostigma* and *M. condylurus*, there is no support for the association of these taxa with *M. midas* (Figs 1-9), and therefore for the monophyly of the three *Mops* taxa represented in this study.

The monophyly of *M. lecuostigma*, *M. condylurus* and all *Chaerephon* taxa present in the analyses (*C. atsinanana*, *C. pumilus* s. l. and *C. lecuogaster*) is strongly supported (1.00/99/99; 1.00/100/100) by analyses of concatenated datasets 1and 4 respectively (Figs 6 and

9) and moderately supported (0.98/-/- ; 1.00/81/83) by analyses of the GHR and PNPO-3 datasets respectively (Figs 2 and 3).

Our analyses fail to provide support for the association of *M. midas* with the strongly supported *M. leucostigma – M. condylurus* clade (Figs 1-9). *Mops midas* is present in supported *Mops*/*Chaerephon* clades in the following datasets (Figs 2, 6, 7, 8, 9); GHR (moderate support, 0.98/-/-), Concatenations 1, 2, 4 (strong support, 1.00/99/99;1.00/100/97;1.00/100/100) and Concatenation 3 (weak support, <60%). In these analyses, *M. midas* is generally sister to a supported clade comprising all other *Mops* and *Chaerephon* taxa (Figs 2, 6, 8, 9, Concatenations 1, 3, 4). In other analyses, *M. midas* is a member of unsupported clades comprising; *Otomops* (FES, Fig. 1), *Otomops* and *Sauromys* (FES, Fig. 1), and *Otomops*, *Sauromys* and *Mormopterus* (FES, Fig. 1; PNPO-3, Fig. 3).

MONOPHYLY OF Mops and *Chaerephon*

We recover strong support for the monophyly of all *Mops* and *Chaerephon* taxa, except *C. jobimena*, in analyses based on the GHR gene region (1.00/84/81, Fig. 2) and concatenated datasets 1-4 (Figs 6-9). *Chaerephon jobimena* is weakly supported (0.96/-/-) as sister to this group in concatenated dataset 3 (Fig. 8).

GENUS *Otomops*

Otomops martiensseni and *O. madagascariensis* form a monophyletic clade in all except one dataset (PRKC1, Fig. 4); this is strongly supported in all four concatenated datasets and the GHR dataset, and moderately supported in the PNPO-3 and RHO1 datasets (Figs 2, 3, 5, 6,7,8 and 9). *Otomops madagascariensis* is monophyletic in all datasets except GHR, PRKC1, Concatenation 1 and 4; this is strongly supported (1.00/100/98; 0.99/99/98) in Concatenations 2 and 3 respectively, and moderately supported (0.99/79/-) in the PNPO-3 dataset. *Otomops martiensseni* is monophyletic in all datasets except PNPO-3, with weak (Concatenation 3) to moderate (PRKC1, RHO1, and Concatenation 4) support (Figs. 3, 4, 5, 8 and 9). The major clades of *O. martiensseni*, from north east Africa and Yemen (NA) and southern and western Africa (SA) are moderately to strongly supported in the concatenated datasets (Fig. 6-9).

THE PHYLOGENETIC POSITION OF *Sauromys*

Based on the GHR dataset, *S. petrophilus* forms a weakly supported clade (-/-/70) with *Otomops*; this clade is at best moderately supported (Bayesian posterior probability 0.94) associated with a clade comprising *M. jugularis* and *M. acetabulosus* (Fig. 2). *Sauromys petrophilus* is also part of unsupported associations with *Otomops* (FES, Fig. 1), and *Mormopterus*, *Otomops* and *M. midas* (FES, Fig. 1; PNPO-3, Fig. 3).

PROPOSED MOLOSSID TRIBES

All datasets yield a monophyletic molossid clade, although this is strongly supported only in analyses of the GHR region (Fig. 2). *Mormopterus* is nested within or sister to clades comprising Old World molossid taxa in the case of the FES, GHR, PNPO-3, PRKC1, and RHO1datasets; however it is basal to other Molossidae in Concatenated datasets 2, 3 and 4. The GHR dataset provides low levels of support (0.94/-/-) for a clade comprising *Otomops*, *Sauromys* and *Mormopterus*.

The presence of and support for monophyly in selected Afro-Malagasy molossid clades in Figs 1-9 is represented in Table 4.

DATING OF MAJOR DIVERGENCES

95 % HPD credibility intervals are relatively wide. The time to the most recent common ancestor of *C. atsinanana*, *C. leucogaster* and *C. pusillus* s. l. is 3.61 (0.98-9.10) MYA; these taxa last shared a common ancestor with *M. leucostigma* and *M. condylurus* 10.46 (4.24-18.66) MYA (Fig. 10). The combined *Chaerephon*/*Mops* clade (*C. atsinanana*, *C. leucogaster*, *C. pusillus* s.l, *M. leucostigma*, *M. condylurus* and *M. midas)* has a crown age of 14.82 (6.44-25.54) million years. The clade last shared a common ancestor with *C. jobimena* 21.97 (12.16-33.44) MYA. *Otomops* and *S. petrophilus* last shared a common ancestor 21.57 (14.01-28.35) MYA (Fig. 10).

Table 4. The presence of and support for monophyly in selected Afro-Malagasy molossid clades.

TAXON GROUP		PRESENCE OF MONOPHYLY IN TAXON GROUP								
		Nuclear region					Concatenation			
		FES	GHR	PNPO3	PRKC	RHO	1	2	3	4
Molossidae	Molossidae in tree	Y	Y***	Y	Y	Y	Y	Y	Y	Y
Chaerephon	*Chaerephon* taxa present in tree	Y	N	N	N	N	Y***	N	N	Y***
	C. leucogaster-C. pumilus s. l.	N	N	N	N	N	Y**	N	Y**	Y*
	C. leucogaster-C. atsinanana	N	N	N	N	N	N	N	N	N
	C. leucogaster-C. atsinanana-C. pumilus s. l.	N	N	N	N	Y***	Y***	N	Y***	Y***
	C. leucogaster-C. pumilus s. l.-*C. pusillus*	N	-	-	-	-	-	Y**	-	-
	C. leucogaster-C. atsinanana-C. pumilus s. l.-*C. pusillus*	N	-	-	-	-	-	Y**	-	-
	C. leucogaster-C. atsinanana-C. pumilus-C. pusillus s. l.-*C. jobimena*	Y	-	-	-	-	-	N	-	-
Mops	*M. condylurus-M. leucostigma-M. midas*	N	N	N	N	N	N	N	N	N
	M. condylurus-M. leucostigma	N	Y***	Y**	Y**	Y*	Y***	Y***	Y***	Y***
Chaerephon & Mops	*M. condylurus-M. leucostigma*- all *Chaerephon* present in tree	N	Y**	Y**	N	N	Y***	N	Y	Y***
	Chaerephon & Mops taxa present in tree	N	Y**	N	N	N	Y***	Y***	Y*	Y***
Otomops	*Otomops* taxa present in tree	Y	Y***	Y**	N	Y**	Y***	Y***	Y***	Y***
	O. madagascariensis	Y	-	Y**	-	Y	-	Y***	Y***	-
	O. martiensseni	Y	-	N	Y**	Y**	Y	Y	Y*	Y**
	O. martiensseni NA	-	-	Y*	N	N	-	Y**	Y***	-
	O. martiensseni SA	-	-	-	-	-	-	Y**	-	-
Mormopterus	*M. acetabulosus-M. jugularis*	Y***	Y***	Y***	Y***	Y*	-	Y**	Y**	-
Supra-generic associations	*Otomops-Mormopterus*	Y	N	N	Y	N	-	N	N	N
	Otomops-Mops midas	Y	N	N	N	N	-	N	N	N
	Otomops-Mops midas-Sauromys	Y	N	N	-	N	-	N	N	N
	Otomops-Sauromys	N	Y*	N	-	N	-	N	N	N
	Otomops-Mormopterus-Sauromys	N	Y*	N	-	Y	-	N	N	N
	Otomops-Mormopterus-Sauromys-M. midas	Y	N	Y	-	N	-	N	N	N

Y=taxa are monophyletic in the specified dataset; N=taxa not monophyletic; s. l.=sensu lato; - = cannot assess monophyly as only one taxon is present, or not all taxa in the group are present in the dataset; ***=strongly supported; **=moderately supported; *=weakly supported (support levels are accurately indicated on the trees).

2.5 DISCUSSION

This study of phylogenetic relationships among Afro-Malagasy Molossidae based on five nuclear gene regions provides greater resolution than a previous study based on one nuclear and one mitochondrial region (Lamb *et al*. 2011). It also complements work by Ammerman *et al*. (2012) on an overlapping taxonomic sample with more extensive representation of New World Molossidae, and with different genomic sampling including one mitochondrial and three nuclear regions. The use of a combined data set offers an improved estimate of relationships among taxa (Gadagkar *et al*. 2005), and as expected, our analyses based on concatenated data showed little homoplasy and provided greater resolving power than those based on individual genes. The PNPO-3 intron marker, optimised and used here based on Igea *et al*. (2010), is relatively novel and has not previously been used in phylogenetic studies, certainly of bats. It provides good resolution at genus level, and some resolution at species level.

THE MONOPHYLY OF Chaerephon

Overall, our analyses do not support the monophyly of the genus *Chaerephon* as currently described. This is due to a general lack of support for the association of *C. jobimena* with the other *Chaerephon* taxa represented and to paraphyletic nesting of *M. condylurus*/ *leucostigma* with *Chaerephon* groupings. Our analyses do, however, support a clade comprising the Malagasy endemic, *C. atsinanana* sister to *C. leucogaster* (Madagascar), *C. pumilus* s. l. (south eastern Africa) and *C. pusillus* (Comoros and Aldabra) from which it diverged 3.61 (0.98-9.10) MYA (Fig. 10)

GENERIC STATUS OF *Chaerephon jobimena*

Chaerephon jobimena was reported by Ammerman *et al*. (2012) to have a weak to moderately supported association with *C. plicatus*. Lamb *et al*. (2011) recovered a strongly supported relationship of *C. jobimena* with *Tadarida aegyptiaca* and *T. brasiliensis*, but not with any *Chaerephon* taxon, although *C. plicatus* was not included in their dataset. However this study based on five nuclear regions raises further questions as to the generic affiliation of *C. jobimena*, as we failed to recover support for its monophyly with the four other *Chaerephon* species included here. The referral of *C. jobimena* to *Chaerephon* was based on morphology (Goodman & Cardiff 2004), and may have been confounded by homoplasy, which has been shown to be a problem in classifications based on morphological traits which play a functional or adaptive role (Freeman 1981). The status of *C. jobimena* may only be resolved pending studies with more complete taxonomic representation. However our analysis based on

Concatenation 3 weakly recovers *C. jobimena* as sister to all other *Mops* and *Chaerephon* taxa, from which it split 21.97 (12.16-33.44) million years ago (Figs 8 and 10). Thus there is some molecular evidence to support membership of *C. jobimena* in a conflated *Mops*/ *Chaerephon* genus (see discussion below).

THE MONOPHYLY OF Mops

Our failure to recover monophyly in *M. midas*, *M. condylurus* and *M. leucostigma* raises questions about the generic assignment of these taxa and/or the validity of the genus *Mops*. Rosevear (1965) proposed on morphological grounds that *M. condylurus* be included in *Chaerephon* and Peterson *et al*. (1995) observed that *M. condylurus* and *M. leucostigma* share many *Chaerephon*-like morphological attributes. Consistent with this, we recovered strong support for the monophyly of these two *Mops* taxa with the *Chaerephon* species in our sample.

Although analysis of some individual gene regions places *M. midas* as part of unsupported associations with *Otomops*, *Sauromys* and *Mormopterus*, these associations disappear in the concatenated datasets which have more resolving power. The sister association of *M. midas* with all other *Mops* and *Chaerephon* taxa (excluding *C. jobimena*) is well supported in the concatenated datasets. Also, as previously mentioned, there is weak support for *C. jobimena* as sister to the *Mops*/*Chaerephon* group.

MONOPHYLY OF Mops and *Chaerephon*

This study concurs with the results of previously-published studies (Lamb *et al*. 2011, Ammerman *et al*. 2012) in finding that neither of the genera *Mops* nor *Chaerephon* are monophyletic and valid as currently described, whereas *Mops* and *Chaerephon* are monophyletic, possibly exclusive of *C. jobimena*. Within this clade, *Mops* taxa tend to be basal (*M. midas, M. condylurus, M. leucostigma* and *M. bakarii*) (Lamb *et al*. 2011) and *Chaerephon* taxa (excluding *C. jobimena*) derived (*C. atsinanana, C. lecuogaster, C. pumilus* s. l. and *C. pusillus*). Although wider taxonomic sampling is necessary, we propose that the genera *Chaerephon* and *Mops* be synonymized.

GENUS *Otomops*

Otomops formed a strongly supported monophyletic clade with a stem age of 21.57 (14.01-28.35) MYA, consistent with its status as a valid genus. In accordance with Lamb *et al*. (2008, 2011) we find *O. madagascariensis* to have a sister relationship to *O. martiensseni*, rather than

the subspecific relationship suggested by Koopman (1993). Our nuclear data uphold the division by Lamb *et al*. (2008, 2011) of *O. martiensseni* into two mitochondrial clades, from south and west Africa, and from north east Africa and Yemen. This points to the need to describe a new species of *Otomops* from north east African and Yemen (in preparation), as the south and west African clade includes the type locality of *O. martiensseni*.

THE PHYLOGENETIC POSITION OF *Sauromys*

This study provides weak support based on individual gene regions for associations of *Sauromys* with *Otomops* and *Mormopterus*, although these do not stand up in the concatenated datasets which offer better resolving power. Thus *Sauromys* may be independent of *Chaerephon*/*Mops*, *Otomops* and *Mormopterus*. This is consistent with Lamb *et al*. (2011), who recovered a clade with *Sauromys* basal to *Tadarida fulminans*, and Freeman (1981) and Ammerman *et al*. (2012), who suggest the incorporation of *S. petrophilus* in *Tadarida* based on a clade comprising *S. petrophilus*, *T. fulminans* and *T. aegyptiaca.* Resolution of the taxonomic position of *S. petrophilus* will depend on studies with greater taxonomic sampling.

PROPOSED MOLOSSID TRIBES

The results of this study are equivocal with respect to support for the proposed Molossid tribe, Mormopterini (Ammerman *et al*. 2012). Three of the concatenated datasets recover *Mormopterus* as basal to the Old World genera *Chaerephon*, *Mops*, *Otomops* and *Sauromys*, consistent with its membership of a separate tribe, Mormopterini. Analysis of individual gene regions, however, weakly support an *Otomops-Sauromys-Mormopterus* clade, or show *Mormopterus* either nested within or sister to Old World molossid genera, consistent with its membership of the Old World Tribe, Tadarini.

2.6 CONCLUSIONS

This study, based overall on data from six nuclear gene regions (FES, GHR, PNPO-3, RHO1, PRKC1 and Rag2) and one mitochondrial region (Cyt *b*), provides strong support for the monophyly of the *Chaerephon* and *Mops* taxa included, but does not support the monophyly of either *Chaerephon* or *Mops*. Within the *Chaerephon*/*Mops* clade, *Mops* taxa (*M. leucostigma*, *M. condylurus* and *M. midas*) tend to be basal and *Chaerephon* taxa (*C. leucogaster*, *C. pusillus*,

C. pumilus s. l. and *C. atsinanana*, but excluding *C. jobimena*) derived. We recover no support for the current generic affiliation of *C. jobimena*, and limited support for its basal position within the combined *Chaerephon*/*Mops* clade. We suggest that *Mops* and *Chaerephon* be combined into a single genus, and that the membership of *C. jobimena* in this genus be further evaluated with wider taxonomic and genomic sampling.

We find good nuclear support for two previously mitochondrially-defined clades of *O. martiensseni*. Based on this we propose that the clade from south and west Africa retains the name *O. martiensseni*, as the type locaility is included in this region (Tanzania) and that the clade from north east Africa and Arabia be designated a new species of *Otomops* (in preparation).

There is very weak support for an association between *S. petrophilus*, *Mormopterus* and *Otomops*, although the balance of the evidence suggests that *Sauromys* is an independent taxonomic unit within our dataset. This is not inconsistent with the proposal of Ammerman *et al*. (2012) that *Sauromys* be included in *Tadarida*. Our analyses suggest but do not strongly support a basal position for *Mormopterus* relative to the Molossidae represented in this study (*Chaerephon*, *Mops*, *Otomops*, *Sauromys*), consistent with the proposal by Ammerman *et al*. (2012) that *Mormopterus* be designated as a distinct tribe, Mormopterini, within the Molossidae.

2.7 ACKNOWLEDGEMENTS

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APPENDICES

Appendix 1. Details of samples and outgroups used in this study. Durban Natural Science Museum - DM; National Museum of Kenya - NMK; Steven Goodman - SMG; Field Museum of Natural History - FMNH; RHF- Fanja Ratrimomanarivo field collection number; not available – NA.

Species	Geographic locality	Field Collection Number
Chaerephon atsinanana	Madagascar: Anjiro	RHF 517
Chaerephon atsinanana	Madagascar: Andasibe	RHF 63
Chaerephon atsinanana	Madagascar: Ambatondrazaka	RHF 453
Chaerephon atsinanana	Madagascar: Ambatondrazaka	RHF 456
Chaerephon atsinanana	Madagascar: Beforona	RHF 88
Chaerephon atsinanana	Madagascar: Vangaindrano	RHF 1020
Chaerephon atsinanana	Madagascar: Vohipeno	RHF 1155
Chaerephon atsinanana	Madagascar: Ifanadiana	RHF 1296
Chaerephon atsinanana	Madagascar: Ranomafana/Ifanadiana	RHF 1653
Chaerephon atsinanana	Madagascar: Tamatave ville	RHF 1442
Chaerephon atsinanana	Madagascar: Tamatave ville	RHF 1443
Chaerephon atsinanana	Madagascar: Fanandrana	RHF 1480
Chaerephon leucogaster	Madagascar: Toliara	RHF 167
Chaerephon leucogaster	Madagascar: Mahajanga	RHF 380
Chaerephon pumilus	South Africa: Durban	DM 7363
Chaerephon pumilus	South Africa: Durban	DM 7851
Chaerephon pusillus	NA	CP 15708
Chaerephon pusillus	NA	CP 15709
Chaerephon jobimena	Madagascar: Province d'Antsiranana	FMNH 177395
Mops leucostigma	Madagascar	N3
Mops leucostigma	Madagascar	N5
Mops midas	Madagascar: Sakaraha	RHF 263

Mops midas	Madagascar: Ankazomborona	RHF 926
Mops condylurus	South Africa	DM 6332
Mops condylurus	South Africa	DM 6291
Mormopterus jugularis	Madagascar: Ankazobe	FMNH 184834
Mormopterus jugularis	Madagascar: Ankazobe	FMNH 184835
Mormopterus acetabulosus	La Réunion	SMG 15339
Mormopterus acetabulosus	La Réunion	SMG 15340
Otomops madagascariensis	Madagascar: Antsiranana	S6
Otomops madagascariensis	Madagascar: Toliara	S18
Otomops martiensseni SA	Southern Africa: South coast	DP2
Otomops martiensseni NA	Africa: Kenya	NMK 15461
Otomops martiensseni NA	Africa: Kenya	NMK 15462
Sauromys petrophilus	Southern Africa	DM 8612
Sauromys petrophilus	Southern Africa	DM 8613
Outgroups		
Miniopterus fraterculus	South Africa: Melmoth	DM 8381
Miniopterus natalensis	South Africa: Mkuze game reserve	DM 9533

Appendix 2. Details of Molossidae samples downloaded from GenBank. Durban Natural Science Museum - DM; Field Museum of Natural History - FMNH; National Museum of Kenya - NMK; Steven Goodman - SMG; Transvaal Museum - TM; Université d'Antananarivo - Département de Biologie Animale - UADBA. Not available - NA; U – museum unknown.

Species	Geographic locality	Museum no.	GenBank accession no.	
			Cyt b	RAG2
Chaerephon atsinanana	Madagascar: Vohipeno	FMNH 185294	HQ384479	HQ 384487
Chaerephon atsinanana	Madagascar: Ambatondrazaka	FMNH 184654	JN 867844	JN 867941
Chaerephon leucogaster	Madagascar: Toliara	FMNH 184237	HM802905	HM 631634
Chaerephon leucogaster	Madagascar: Toliara	FMNH 184239	EU 716036	HM 631635

Chaerephon pusillus	France: Mayotte	FMNH194031	HQ 384481	HM 631644
Chaerephon pusillus	France: Mayotte	FMNH194032	HQ 384482	HM 631645
Chaerephon pumilus	South Africa: KwaZulu-Natal	DM 7377	HM802906	HM 631637
Chaerephon pumilus	South Africa: KwaZulu-Natal	DM 7371	HM802908	HM 631640
Chaerephon jobimena	Madagascar: Isalo National Park	FMNH 175992	HM 802932	HM 631627
Mops leucostigma	Madagascar: Mahajanga,	FMNH 184698	HM802914	HM631649
Mops leucostigma	Madagascar: Ampitabe	FMNH 188009	HQ 384484	HQ 384489
Mops midas	Madagascar:Toliara	FMNH 184306	HM802915	HM631650
Mops midas	Madagascar: Ankazomborona	FMNH 185187	HM802916	HM631652
Mops condylurus	South Africa: KwaZulu-Natal	DM 6291	HM802912	HM631647
Mops condylurus	South Africa: KwaZulu-Natal	DM 6332	HM802913	HM631648
Mormopterus jugularis	Madagascar: Andasibe	FMNH 184576	HM802920	HM631656
Mormopterus jugularis	Madagascar: Fianarantso	FMNH 184445	HM802921	HM631657
Otomops madagascariensis	Madagascar: Bisihiko Cave	FMNH 172944	HM802922	HM631658
Otomops madagascariensis	Madagascar: Isalo National Park	UADBA SMG 10996	HQ 384485	HQ 384490
Otomops martiensseni (SA)	South Africa: KwaZulu-Natal	DM 7909	HM802923	HM631659
Otomops martiensseni NA	Kenya: Ithundu Caves	NMK 15461	HM802927	HM631663
Otomops martiensseni NA	Kenya: Ithundu Caves	NMK 15462	HM802926	HM631662
Sauromys petrophilus	South Africa: Cedarberg	DM 8613	HM802931	HM631664
Outgroups				
Miniopterus fraterculus	NA	TM47722	EU091246	NA
Miniopterus fraterculus	NA	U 98058	NA	GU328067

SUMMARY AND CONCLUSIONS

The first data chapter has focused on genetic variability and structure in *C. atsinanana*, a newly-described molossid bat (Goodman *et al*. 2010) found in the mid to southern region of the eastern watershed of Madagascar. As *C. lecuogaster*, endemic to western Madagascar, has been reported to display relatively low levels of genetic structure based on analyses of mitochondrial DNA, one might expect a similar pattern in *C. atsinanana*. The aim of this study was to examine the historical demography and genetic structure of *C. atsinanana* populations, based on analyses of the mitochondrial control region.

Contrary to expectation, there were high overall levels of genetic structure among six major *C. atsinanana* haplotype groups. These highly structured and diverse sub-populations, separated by control region genetic distances of up to 8%, may have been formed as a result of isolation, in combination with genetic drift (Atartouch *et al.* 2006). As found for the Australian ghost bat, *Macroderma gigas* (Worthington-Wilmer *et al.* 1994), it appears that *C. atsianana* populations may have been isolated by behavioural barriers to gene flow such as female philopatry, revealed here by the maternally-inherited control region marker. Mismatch distributions and Bayesian skyline analyses indicated that ancient stable *C. atsinanana* populations had not undergone expansions or contractions associated with Pleistocene era glacial cycling. This was consistent with evidence from fossil pollen cores that the eastern portion of Madagascar, to which *C. atsinanana* is endemic, experienced a relatively stable climate during the Quaternary; this would have allowed retention of phylogeographic structure, as it would not have been consistent with the creation of climate-induced bottlenecks. Further, it is unlikely that barriers to gene flow would have been caused by vicariance, as *C. atsinanana* is capable of flight at high elevations and is known to be able to traverse the mountain ranges within its habitat. To conclude, *C. atsinanana* shows high levels of population structure as a result of low gene flow due to female philopatry, which has been maintained by the stable climatic conditions of eastern Madagascar during the Quaternary.

A second aim was to complement the study of population genetic structure based on maternally inherited mitochondrial DNA by evaluating relationships among *C. atsinanana* samples using biparentally-inherited nuclear sequence markers, which would also have reflected the male contribution to gene flow. A finding of similar structure based on nuclear markers might have indicated the presence of male philopatry. Of the 12 nuclear regions initially investigated, the markers PNPO-3 (Igea *et al*. 2010), FES, GHR, RHO1 (Venta *et al*. 1996) and PRKC1 (Matthee *et al*. 2001) were successfully amplified and sequenced. However they showed no variability and very little polymorphism, and were therefore unsuitable for testing

hypotheses related to genetic variability and possible population structure in *C. atsinanana*. The five nuclear markers were used in combination with already published mitochondrial Cyt *b* and nuclear Rag2 data (Lamb et al. 2011) to further investigate phylogenetic relationships among the Molossid species of the Western Indian Ocean region and south eastern Africa. This study of nuclear phylogenetic relationships among five genera and 13 species of Molossidae from the WIO regions islands and south eastern Africa also included the C. *atsinanana* samples reported on in the first data chapter of this dissertation.

The second data chapter of this dissertation, based on analysis of 5 nuclear gene regions, provides strong support for the monophyly of the *Chaerephon* and *Mops* taxa included, but do not support the monophyly of either of the genera *Chaerephon* or *Mops*, as currently described. The results support the amalgamation of *Mops* and *Chaerephon* into a single genus, exclusive of *C. jobimena*. This study provides no support for the current generic affiliation of *C. jobimena*, and limited support for its inclusion as a basal member of a combined *Chaerephon*/*Mops* clade.

The genus *Otomops* is strongly supported, as are the genera *O. madagascariensis* and *O. martiensseni*. The two recognised mitochondrial clades of *O. martiensseni* are also well supported by our nuclear data; we propose that the clade from south and west Africa retain the name *O. martiensseni*, and that the clade from north east Africa and Arabia be designated a new species of *Otomops* (in preparation).

There is very weak support for an association between *S. petrophilus*, *Mormopterus* and *Otomops*, although the balance of the evidence suggests that *Sauromys* is an independent taxonomic unit within our dataset. This is not inconsistent with the proposal of Ammerman *et al*. (2012) that *Sauromys* be included in *Tadarida*. Our analyses suggest but do not strongly support a basal position for *Mormopterus* relative to the Old World Molossidae represented in this study (*Chaerephon*, *Mops*, *Otomops*, *Sauromys*), consistent with the proposal by Ammerman *et al*. (2012) that *Morpmoterus* be designated as a distinct tribe, Mormopterini, within the Molossidae.

This study has increased our limited knowledge of phylogenetic relationships among Afro-Malagasy members of the Molossidae by providing additional insight based on data from five nuclear sequences, one of which, PNPO-3 had not previously been used in phylogenetic studies of bats. This marker is more variable at the intergeneric level and in some cases at interspecific level than the other nuclear markers used here. It is primarily useful in resolving intergeneric relationships, but also resolves some interspecific relationships.

Future research should involve a study of the inheritance of biparentally-inherited nuclear microsatellite markers to provide a finer-scale picture of population structure and levels

of gene flow in *C. atsinanana*. Future phylogenetic studies should be carried out on an increased taxonomic sample. Unfortunately members of this high-flying pan-tropical family of bats are difficult to trap and are often found in relatively inacessible areas. One approach would be to try to facilitate collaboration between the laboratories of Lamb et al. (2011) and Ammerman et al. (2012) with a view to combining samples to form a more complete sample set, and to sequence all samples with the same set of primers, providing increased taxonomic and genomic sampling.

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