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Phylogeography and population genetics of *Macronycteris commersonii* s.s. (Chiroptera: Hipposideridae), an endemic Malagasy bat

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ABSTRACT

Background. *Macronycteris commersonii* (Hipposideridae), a bat species endemic to Madagascar, is widespread across the island, utilising open woodland, degraded habitats, and forested areas from sea level to 1325 m.

Methods. We investigated the fine-scale phylogeographic history and relationships of populations occurring in the western half of the island using sequence data from two mitochondrial DNA regions and extensive geographical sampling.

Results. Our results indicated a highly supported monophyletic group of *M. commersonii*, in which the Northern Madagascar Clade C formed a single monophyletic clade. The most recent common ancestor of *M. commersonii* was dated to 0.82 million years ago (mid-Pleistocene). Population expansion events were inferred for Clade B from approximately 130,000 to 70,000 years BP. Bayesian clustering and AMOVA analyses inferred week population genetic structure and sequence data indicated that genetic subdivisions do not support an isolation-by-distance model. Lineage dispersal, genetic divergence, and expansion events of *M. commersonii* were likely to be associated with Pleistocene climate fluctuations.

Discussion. Our data suggested that the northern and the central western regions of Madagascar may have acted as refugia for this species during periods of cooler and drier climate conditions associated with the Pleistocene.

Keywords: bioclimate, diversification, geographical structure, *Macronycteris*, Madagascar.

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INTRODUCTION

The biota of Madagascar, with its numerous higher-level endemic taxonomic groups, as well as a large number of endemic genera and species (Myers et al., 2000), have been isolated from those of other continental landmasses for over 120 million years (Ali and Aitchison, 2008). The mechanisms driving this diversity are varied. Excluding vicariance, which can be applied to some living vertebrate lineages (Noonan and Chippindale 2006; Yoder and Nowak, 2006), different abiotic (e.g., ocean current direction or prevailing winds) and biotic (e.g. dispersal ability) filters have been in place over geological time limiting or promoting the colonization of the island by continental vertebrates (Ali and Huber, 2010; Samonds et al., 2012, 2013).

Given Madagascar's topographical, meteorological, and geological complexity, after successful colonization of this mini-continent by different ancestral forms, in many cases subsequent diversification took place, leading to endemic species as well as some of the most extraordinary adaptive radiations known in the world. Examples of such patterns of extensive speciation and morphological variation in volant vertebrates include birds of the families Vangidae and Bernieridae (Cibois et al., 2001; Jønsson et al., 2012; Reddy et al., 2012). In contrast, other adaptive radiations found on the island show distinctly less morphological differentiation and these groups contain numerous cryptic species, for example, bats of the genus *Miniopterus* (Christidis et al., 2014; Schoeman et al., 2015). Further, there are cases of presumed congenerics colonizing the island independently of one another, such as amongst bats of the families Molossidae (Lamb et al., 2011) and Rhinonycteridae (Foley et al., 2015; Russell et al., 2007). Regardless, the discerned periods of rapid cladogenesis amongst these different groups are not coincidental, yet no single unifying explanation can be presented to explain successful colonization and subsequent diversification patterns amongst extant volant vertebrates (Samonds et al., 2012, 2013).

Factors that may mediate colonization success include the period of initial colonization, ranging from the Mesozoic through the Cenozoic (Holocene), and life-history traits (e.g. large organisms with fast and efficient flight are more likely to colonize than small and slow flying organisms). Subsequent biogeographic and phylogeographic patterns of species are driven in part by the landscape and climatic heterogeneity of the island (Pearson and Raxworthy, 2009; Vences et al., 2009; Wilmé et al., 2006). Additionally, differences in modes of dispersal and habitat

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requirements amongst flying Malagasy vertebrates result in different biogeographic and phylogeographic patterns (e.g., for birds see Cruaud et al., 2011; Fuchs et al., 2007, 2013; for bats see Chan et al., 2011; Goodman et al., 2010a, 2010b, 2016; Lamb et al., 2012; Ratrimomanarivo et al., 2007, 2008, 2009a, 2009b; Russell, 2007, 2008a, 2008b; Weyeneth et al., 2011). These different factors make Madagascar an excellent model system for testing and contrasting the process of species diversification and fine-scale spatial patterning across different lineages.

Macronycteris commersonii (Family Hipposideridae), which feeds predominantly on Coleoptera, is widespread across Madagascar and utilizes open woodland, degraded habitats, and forested areas from sea level to 1325 m (Goodman and Ramasindrazana, 2013; Rakotoarivelo et al., 2009). It occupies day roosts in caves found in areas of eroded sedimentary rock, often forming colonies of several thousand individuals; individuals also roost under vegetation in areas of relatively non-degraded or heavily degraded forest vegetation (Goodman, 2006; Raharinantenaina et al., 2008). There is evidence that *M. commersonii* exhibits morphological and bioacoustic variation across its geographical range; this includes sexual dimorphism, where males are significantly larger than females; both of these parameters show a clinal pattern correlated with latitude (Ranivo and Goodman, 2007; Ramasindrazana et al., 2015). For details on the complex taxonomic history of *M. commersonii* sensu lato, which previously included some African populations, see Goodman et al. (2016). Herein, we consider this species endemic to Madagascar.

Examination of Quaternary fossils from Madagascar found in cave deposits at Anjohibe (Fig. 1) revealed that a species referable to *Macronycteris*, *H. besaoka*, morphologically similar to *H. commersonii*, went extinct in the Late Pleistocene or Holocene (Samonds, 2007); these two species probably occurred sympatrically at Anjohibe. One particularly striking aspect of the fossil record of the genus *Macronycteris* is that specimens dating from the second half of the Eocene of France (Sigé, 1988), the Miocene of Australia (Hand, 1993, 1997), and the Pliocene of Ethiopia (Wesselman, 1984) show remarkably consistent craniodental structure across tens of million years and are notably similar to living members of this genus. Given this conservative morphological pattern, it is assumed that molecular genetics will provide an important signal to

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the evolutionary history of members of this genus, and there are probably numerous unrecognized cryptic species.

Recent work on the molecular genetics of animals referred to as *M. commersonii* from different areas of Madagascar, particularly the western half of the island, has found the presence of several independently evolving lineages, some geographically structured (Rakotoarivelo et al., 2015). On the basis of these results, a cryptic endemic species (clade A in Rakotoarivelo et al., 2015) was identified, and subsequently named as *M. cryptovalorona* (Goodman et al., 2016). Rakotoarivelo et al. (2015) found *M. commersonii* sensu stricto (clades B and C therein) to be sister to African *M. vittatus* and *M. gigas*, with *M. cryptovalorona* basal to this grouping. On the basis of molecular clock estimates, clade A diverged from clades B and C during the Miocene, approximately 5.81 MYA and clades B and C last shared a common ancestor about 3.38 MYA. This indicates that large-bodied *Macronycteris* experienced two possible colonization events hypotheses. First, Clade A and Clade B-C could have originated from two independent eastward dispersals from Africa. The second hypothesis involves multiple, bidirectional dispersal; an early eastward dispersal to Madagascar, followed by a later back-dispersal to Africa.

Herein we focus on intra-population variability within *H. commersonii* sensu stricto, specifically clades B and C of Rakotoarivelo et al. (2015). Using sequence data from two mitochondrial genes and increased geographical sampling, we investigate the fine-scale phylogeographic history and relationships of populations occurring in the western half of the island.

MATERIALS AND METHODS

Sample collection

All of the tissue samples used herein were associated with specimens deposited in museums (Table S1) and no individual was specifically collected for this study. In total, 146 specimens of *Macronycteris commersonii* falling within clades B and C of Rakotoarivelo et al. (2015) and Goodman et al. (2016), from 29 localities were included: the majority spanning much of the latitudinal breadth and known distribution of the species in the western half of the island, including 140 specimens from the dry and subarid bioclimatic zones, five specimens from the humid or subhumid bioclimatic zones (Montagne d'Ambre, Nosy Be, Tampolo, Analalava,

Andringitra), and one specimen from the mid-western Central Highlands at the limit of the subhumid zone (Ambohijanahary) (Fig. 1; Table S1, Supporting information). Two African species, *M. gigas* and *M. vittatus,* were included as out-group taxa and used to root the phylogenetic trees. We did not include genetic data of *M. cryptovalorona*, which is basal to *M. commersonii* s.s., *M. gigas* and *M. vittatus*, and is presumed to represent a separate colonization event of members of the *M. commersonii* group on Madagascar (Goodman et al., 2016).

DNA extraction and amplification

Genomic DNA was isolated using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany), following the manufacturer's protocol for tissue samples. Two mitochondrial (mtDNA) markers were amplified: hypervariable control region (CR, 481 bp) using the primers P/E (Wilkinson and Chapman, 1991) and cytochrome *b* (*Cyt b*, 705 bp) using the primers JorF/H15553 (Irwin et al., 1991; Rakotoarivelo et al., 2015). PCR amplifications consisted of: ~20-150 ng template DNA, 2.5 µl 10 x KAPA buffer, 1 U KAPA Taq DNA polymerase, 200 µM dNTPs, 0.2 µM of each primer and 18.4 µl dH_2O to give a final reaction volume of 25 µl. The PCR cycle parameters for CR and *Cyt b* included an initial denaturation step at 95 °C for 3 min followed by 30 cycles at 95°C for 30 s, 50-55°C for 30 s, 72°C for 30 s, with a final extension step at 72°C for 5 min. PCR reactions included a negative control to check for possible contamination. PCR products were sent to the Central Analytical Facility at Stellenbosch University South Africa, for sequencing. Cycle sequencing was performed using the BigDye Chemistry, v3.1 and sequencing products were analyzed on an Applied Biosystems 3730xl Genetic Analyzer (Applied Biosystematics, Perkin Elmer). All sequences were first aligned using ClustalW (Thompson et al., 1997) as implemented in BioEdit (Hall, 1999), and thereafter manually optimized. All new sequences were deposited in GenBank (Table S1).

Phylogenetic analyses and molecular clock dating

The two markers (CR, *Cyt b*) were analyzed separately and then combined into a single data set. The number of variable sites, number of parsimony informative sites and nucleotide frequencies were estimated for each data matrix in MEGA 6 (Tamura et al., 2013).

Phylogenetic reconstruction was performed using both maximum likelihood (ML) and Bayesian (Bayes) approaches using the programmes Garli 2.0 (Zwickl, 2006) and MrBayes 3.2

(Ronquist et al., 2012), respectively. The most appropriate substitution model for each gene (CR - GTR+I+G, *Cyt b* - TrN+I+G; Fig. 2) was selected using the Akaike information criterion (AIC) as implemented in jModelTest (Darriba et al., 2012; Posada and Crandall, 1998). For the concatenated data set, partitioned analyses were conducted, with data partitioned by gene. The parameters of nucleotide substitution models were unlinked across partitions. Each ML analysis was initiated from a random starting tree, with nodal support assessed using 1000 bootstrap replicates. Two independent Bayes runs of 5 million generations each were performed; each run consisted of four Monte Carlo Markov chains (MCMC), with topologies sampled every 250 generations. The program Tracer 1.6 (Rambaut et al., 2014) was used to determine that the effective sample size (ESS) had reached > 200 for all parameters. A 50% majority rule consensus tree was constructed using the CONSENSE program in the PHYLIP package (Felsenstein, 2005). In each simulation, the first 20% of generations were discarded as burn-in, after a pilot run to determine that this was sufficient to achieve stationarity.

We built haplotype networks for visualization of the two mitochondrial markers (CR, *Cyt b*) genealogies by converting MP tree estimates with Haploviewer (http://www.cibiv.at/~greg/haploviewer; Salzburger et al., 2011).

The *Cyt b* was chosen to estimate the time of most recent common ancestor of major evolution lineages because of its moderate mutation rate. TMRCA was assessed using BEAST (Drummond and Rambaut, 2007) with a strict molecular clock, a coalescent prior (appropriate for intraspecific radiations), and the GTR + I + G model. A fixed mean substitution rate of 1.30×10−8 subs/site/year (Nabholz, Glemin & Galtier, 2008; Thong et al., 2012; Puechmaille et al., 2012; Rakotoarivelo et al., 2015; Liu et al., 2016) was applied as a fixed mean substitution rate. Several preliminary short runs were performed to adjust the prior parameters, including models and MCMC length, and to ensure sufficient mixing of chains. Tracer 1.6 was used to assess the convergence of the trace files (Rambaut et al., 2014). We ran three independent runs of 20 million generations, with sampling every 1,000 generations, and a burn-in of the first 10% of generations. Results were combined using Tracer 1.6 (Rambaut et al., 2014); effective sample size (ESS) values exceed 200 for all parameters.

Population structure analyses

To examine the fine-scale population structure of *M. commersonii*, without making *a priori*

assumptions about the partitioning of local populations, a Bayesian model-based approach to inferring hidden genetic population structures was implemented in the program BAPS 6 (Bayesian analysis of population structure; Cheng et al., 2013; Corander and Marttinen, 2006). BAPS potentially offers insight into the historical genetic connectivity of populations. Analyses were first performed on the entire data (including all sequenced individuals from across the latitudinal range of *M. commersonii*) and then repeated on subsections of the data, including only individuals assigned to the "northern group" and the "southern group". In each independent run the number of proposed clusters (K) ranged from 1 to 10, with 5 runs for each K. In each case, analyses were conducted using the concatenated mtDNA.

A Mantel test was used to determine the relationship between genetic and geographic distance across distribution of *M. commersonii* and significance was assessed by 1000 permutations using Alleles In Space (AIS) program (Miller, 2005).

The geographical pattern of genetic differentiation was evaluated using analysis of molecular variance (AMOVA) with Arlequin 3.5 (Excoffier and Lischer, 2010). We assessed population structure at three hierarchical levels of subdivision (among regions, among populations within regions, and within populations). Two separate regions have been defined, at least in part based on the transition between the subarid and dry bioclimatic zones (Fig. 1): "northern group" includes all animals obtained in the latitudinal swath to the north of Kirindy (CNFEREF) and "southern group" including those to the south of Kirindy (CNFEREF). To evaluate possible correlations of genetic differentiation with climatological aspects of Madagascar, we also used AMOVA to test significant genetic differentiation among four bioclimatic zones, following the classification of Cornet (1974): "Dry1" includes sites from Nosy Be to the northern most locality; "Dry2" from Marovaza to Bemaraha; "Subarid"; and "Humid-Subhumid" as delineated in Fig. 1.

Demographic analysis

Demographic analyses were performed separately for *M. commersonii* clades (clades B and C see Results) using the concatenated sequence data (CR+*Cyt b*). In addition to Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997), which may also be used to infer demography in neutrally evolving loci, demographic changes in both clades were also inferred from the observed mismatch distribution for each of the clades, calculating the raggedness index (R2; Harpending,

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1994) according to the population expansion model in DnaSP version 5.10 (Librado and Rozas, 2009). This measure quantifies the smoothness of the observed mismatch distribution, with lower raggedness characterizing population that experienced sudden expansion, whereas higher raggedness values suggest stationary or bottlenecked populations (Harpending et al., 1993; Harpending, 1994). Lastly, changes in effective population size were inferred using Bayesian Skyline Plots (BSP: Drummond et al., 2005). These plots utilize the coalescent properties of gene trees to plot population size changes over time, and were inferred using BEAST (Drummond and Rambaut, 2007). A mitochondrial substitution rate of 1.30×10−8 subs/site/year was used The lengths of the MCMC chains were set to 20 million to achieve effective sample sizes (ESS) and proper mixing of Markov chains. To account for biases due to genetic structure (Ho and Shapiro, 2011), we divided the data into clades B and C and reconstructed their demographic histories separately.

RESULTS

Genetic diversity and divergence

The nucleotide composition and levels of variation of the two mitochondrial genes differed with CR having the highest number of variable characters (132 variable sites), while *Cyt b* was more conserved (76 variable sites). The CR partition contained the highest number of parsimony informative characters, whereas the mutational rate of *Cyt b* was more conservative, containing 52 parsimony informative characters (Table 1).

For the CR, after analyses with DnaSP software, 92 unique haplotypes were identified. The haplotypic diversity for this dataset was high (Hd = 0.98), but the nucleotide diversity was low (p = 0.032). For the *Cyt b* gene, the same analysis identified 70 unique haplotypes, also with high haplotypic diversity (Hd = 0.98) and low nucleotide variability (π = 0.008).

Phylogeny and Time of most recent common ancestor

Maximum likelihood and Bayesian analyses produced consistent topologies. There was no significant conflict between the CR and *Cyt b* topologies, although most clades in phylogenetic trees generated from the CR data had posterior probability bootstrap support values below 50% (Fig. S2). Consequently, the Bayesian phylogram constructed from the concatenated mtDNA data set is presented as Fig. 2. The ML and Bayesian analysis of the concatenated data matrix

(CR + *Cyt b*; Fig. 2) recovered animals referred to as *M. commersonii* as a single monophyletic lineage, further supporting the conclusions of Rakotoarivelo et al. (2015). Two distinct clades were recovered: clade B (ML bootstrap, 59; Bayes' PP, 0.99) and clade C (ML bootstrap, 96; Bayes' PP, 1.0). Clade B is the most geographically widespread.

Haplotype network construction of *M. commersonii* implemented in HaploViewer yielded two connected haplotype sub-networks (clades B and C), similar to the topology described in the most concordant tree (Fig. 3). Clades B and C were distinguished by 13 mutational steps, whereas haplotype differences within clades are typically distinguished by only a few steps (Fig. 3).

The most recent common ancestor of all examined *M. commersonii* herein could be traced back to 0.82 (95% highest posterior density (HPD); 0.48–1.12) MYA. The TMRCA estimates suggest that the two sister clades B and C shared their last common ancestor back to the mid-pleistocene. The TMRCA estimates obtained for Clade B were 0.70 (95% HPD; 0.42–0.89) MYA. For Clade C, the TMRCA was 0.32 Ma (95% HPD; 0.13-0.46)MYA, which could be traced back to the late Pleistocene.

Bayesian clustering and population structure

The Bayesian clustering method of BAPS performed on the concatenated sequence data defined four genetically distinct clusters (P = 1, optimal partition, log likelihood = -5536.8; Fig. 4): two widespread clusters distributed throughout the range of *M. commersonii* (cluster 1 and cluster 3), the northern isolate (cluster 2 = Clade C), and that restricted to largely the subarid bioclimate zone (cluster 4).

Additional phylogeographical resolution was recovered through the independent analysis of the mtDNA of the southern and northern groups (Fig. 5). Four genetically distinct BAPS clusters were recovered within the southern group (P = 0.99, log likelihood of optimal partition = -2295.82), whereas within the northern group three distinct genetic clusters were recovered (P = 1, log likelihood of optimal partition = -2836.21). When all individuals were used, the Mantel test failed to support the isolation-by-distance (IBD) model (r = -0.009, $P > 0.05$).

Analysis of molecular variance (AMOVA) revealed that significant genetic variance was attributable to all three examined hierarchical levels (among regions, among populations within

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regions, and within populations). However, a large part of the total variation was found within populations (96.41% for geographical group and 94.62% for bioclimatic group, Table 2). The subarid bioclimatic region was the most differentiated region (Table 3). There was also differentiation between Dry1 and Dry2 but there was no differentiation between the dry regions (Dry1 and Dry2) and the humid-subhumid region (Table 3).

Historical Demography

Mismatch distribution analysis based on *Cyt b* revealed different historical demography. Clade B returned significant negative Fu's Fs and Tajima's D values, allowing a rejection of the neutrality/constant population size null hypothesis, as expected in cases of population expansion. Lower R2 and a unimodal mismatch distribution also indicate a population expansion. However, Clade C, failed to reject the neutrality/constant population size null hypothesis based on statistically non-significant indices (Table 4). High R2 value, non-significant Fu's Fs and Tajima's D values and bimodal mismatch distribution of Clade C suggests a stable population history.

The Bayesian Skyline plot analysis indicated that all individuals herein assigned to Clade B underwent a slow demographic expansion that started ~130,000 years ago, followed by stable growth ~ 70,000years ago up to the present time, with no sign of population decline during the evolutionary history of the clade. On the other hand, Clade C remained stable up to the present time (Fig. 6).

DISCUSSION

Phylogeography and demographic history

Paleoclimatic variation has played an important role in the distribution and speciation of organisms on continental landmasses and islands (Hewitt, 2000; Vences et al., 2009). Madagascar was cooler and drier during periods of late Tertiary-Quaternary glaciation, inducing habitat shifts that presumably forced certain taxa to retreat into refugia (Burney, 1995; Vences et al., 2009; Wilmé et al., 2006) such as the northern volcanic massif of Montagne d'Ambre or the central western massif of Isalo, from where they subsequently re-expanded during more favorable climatic periods.

We recovered some geographic genetic subdivision within *M. commersonii*, with Clade C restricted in the north of the island. Clades B and C appear to have diverged ~0.82 MYA, in the mid-Pleistocene. We suggest that initial intraspecific divergence within *M. commersonii* might be related to refugial isolation, with at least one of these refugia, having possibly harboured Clade C, located in the north of the island. The separation between the groups within clade B, which are distributed across the island, may have resulted from multi-directional dispersal during more favorable periods (see below). These findings suggest that divergence events within *M. commersonii* may have been associated with Pleistocene climatic fluctuations. Our results also reveal that expansion of the extant Clade B commenced approximately 0.70 MYA. There is a scarcity of published divergence dates of bat taxa below the family level, and most dates are reliant on a sparse fossil record (Teeling et al., 2005). The uncertainty in the mutation rate of the *Cyt b* dataset will affect the molecular clock dates presented here. These divergence dates should be considered preliminary until more precise calibration points can be added to the analyses.

At least three other Malagasy bat species, *Paratriaenops furculus* (Russell et al., 2007), *Chaerephon leucogaster* (Ratrimomanarivo et al., 2009), and *Myotis goudoti* (Weyeneth et al., 2011) show similar haplotypic segregation along a latitudinal gradient. However, the latitudinal distribution of different clades and the calculated expansion periods of the other species differ from late Pleistocene in *M. goudoti* to early Holocene in *C. leucogaster*, suggesting that no common historical process underlies the different demographic events between these taxa.

Based on demographic analysis, *M. commersonii* species forming the Clade B underwent a single historical population expansion event. A slow demographic expansion that started ~130,000 years ago, followed by stable growth ~ 70,000years ago up to the present time. It is possible that these demographic shifts were associated with the optimality of climatic conditions for *M. commersonii*; the more favorable conditions are hypothesized to have contributed to a population expansion and multi-directional dispersal associated with more suitable feeding habitats and abundant food resources. The mismatch distribution analyses revealed a similar pattern of demographic expansion in Clade B, as indicated by the significant negative Fu's Fs and Tajima's D values and the unimodal mismatch distribution.

Intriguingly, the extinct *M. besaoka* described from the late Pleistocene-Holocene of Anjohibe Cave, western lowland Madagascar was temporally sympatric with *M. commersonii*

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(Samonds, 2007). No clear hypothesis has been presented on the principal factor that led to the extinction of *M. besaoka*. Changes in vegetational types in lowland areas of the western half of the island in the late Pleistocene and Holocene of Madagascar saw a shift to drier climates and more arid natural vegetational types (Burney, 1997; Goodman and Jungers, 2014). These changes were most notable in the extreme southwestern portion of the island during the late Holocene with shift from forests and woodlands to drier wooded savanna (Burney, 1993; Goodman and Jungers, 2014). Similarly, northwestern Madagascar was the scene of vegetational changes from a mosaic of dry forest and wooded savanna from ~3500 years BP to a savanna formation from 1000-500 years BP (Matsumoto and Burney, 1994; Crowley and Samonds, 2013; Goodman and Jungers, 2014). What is unclear is why the factor(s) that led to the extinction of *H. besaoka* did not have the same impact on the presumed ecologically similar *H. commersonii*. Another possibility is competitive exclusion of the former by the latter.

Wesselman (1984) described fossil remains recovered from the Omo formation of Ethiopia and dated to the late Pliocene (2.08 MYA) as a distinct species, *H. kaumbului*. The author suggested that this taxon was morphologically similar to *M. commersonii*. From recent molecular research, it has been shown that different species included in the *M. commersonii* group (i.e. *M. gigas*, *M. vitattus*, *M. cryptovalorona*, and *M. commersonii*) are genetically distinct (Goodman et al., 2016; Rakotoarivelo et al., 2015). Apart from their size, these different species are morphologically similar to one another.

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355 **Population genetic structure**

356 The Bayesian clustering analyses revealed four groups, with cluster 2 referring to Clade C and
357 three others clusters (clusters 1, 3 and 4) belonging to Clade B. The outcome of the recovered
358 clusters withing Clade B could be attributed to a population level differentiation.

359 The haplotype network generated from mtDNA exhibited a split between the northern Clade C
360 and Clade B, as did the phylogenetic analyses, with a well-supported monophyletic Clade C.
361 Based on a previous study (Rakotoarivelo et al., 2015), the lack of haplotype sharing in the
362 OSTA5 gene for clades B and C supports the inferred genetic distinctness of these two lineages.

363 Although around 95% of the genetic variance occurred within populations, AMOVA
364 revealed that a relatively low although highly significant proportion of the variance occurred
365 among groups of *M. commersonii* based on latitude (north vs south) and climate (degree of
366 humidity). Significant genetic differences were observed between populations from the dry
367 region (Dry1 and Dry2) and subarid region (Table 4), possibly reflective of a lack of gene flow
368 (Xu et al., 2010). Mantel tests failed to support the isolation-by-distance (IBD) model for *M.*
369 *commersonii* across much of its distribution. This lack of relationship between genetic
370 differentiation and geographic distance is in accordance with the high dispersal ability (Norberg
371 and Rayner, 1987) of this species, which in turn may limit genetic differentiation among
372 populations.

373 African members of the *M. commersonii* species complex, specifically *M. vittatus* and *M.*
374 *gigas* undertake local seasonal movements associated with fluctuations in prey abundance
375 (McWilliam, 1982; Vaughan, 1977). Large hipposiderid bats have high wing loading and low to
376 medium aspect ratios (Norberg and Rayner, 1987), which may favor relatively quick, long-
377 distance movements, allowing individuals to track food resources (Jones and Rayner, 1989;
378 Bernard and Fenton, 2003).

379 On Madagascar, some apparent seasonal intra-island dispersal of *M. commersonii* has
380 been documented at Kirindy (CNFEREF) (Fig. 1) (Rakotondramanana and Goodman, 2011;
381 Ramasindrazana et al., 2015). This dispersal behaviour, particularly at a broad geographical
382 scale, may explain shallow phylogeographic structure in this species. On the other hand, there is
383 some evidence from the northwest, specifically the region of Anjohibe, that *M. commersonii*

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remains inactive in caves during times of resource shortage (A.R. Rakotoarivelo, unpublished results); this is the site where sequenced individuals falling within Clade B have been identified.

Furthermore, no apparent physical barrier in the western half of the island, such as a high mountain range, divides the latitudinal distribution of *M. commersonii* (Fig. 1), and the members of the Clade B overlap in their distributions (Fig. 2). Bioclimatic features might have direct effect on habitat structure and prey availability of bats that show seasonal variation in their diet (Razakarivony et al., 2005; Rakotoarivelo et al., 2007), but these aspects do not explain the distributions of the different clades. Hence, based on the mitochondrial markers used herein, the best explanation for the lack of genetic structure in this broadly distributed and apparently widely dispersing species, is that in the recent geological past populations were isolated in refugia and underwent some level of genetic differentiation. Subsequently, these populations - at least females - expanded and in many cases are now overlapping giving rise to the modern structure of the clades presented herein. However, because the inferred evolutionary relationships in the current study are based mainly on information from mitochondrial genes, a more informative assessment of relationships between clades could be performed using microsatellites allowing the detection of nuclear gene flow between lineages.

Conservation implications

Macronycteris commersonii is a beetle specialist (Razakarivony et al., 2005; Rakotoarivelo et al., 2009) and due to its diet, may be susceptible to habitat destruction associated with diminished food resources. Besides habitat loss, a major threat to different Malagasy bat species, particularly those of larger body-size, is hunting for bush meat (Jenkins and Racey 2008). In western Madagascar, considerable numbers of *M. commersonii* are harvested for food at day roost sites (Goodman, 2006; Jenkins and Racey, 2008), including an estimate in the extreme southwest of 140,000annually, particularly between January and March coinciding with the period of human food shortages (Goodman, 2006). This level of exploitation in certain areas of the island is certainly having a direct impact on the population dynamics of *M. commersonii*, reducing population size and presumably recruitment into the breeding population (Ramasindrazana and Goodman, 2012). Genetically divergent populations have been recognized in the literature as conservation priorities (Palsböll et al. 2007; Lu et al., 2013). Based on this and the results recovered in the current study, the populations of *M. commersonii* belonging to Clade C of

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Analamera, Ankarana, Montagne d'Ambre, and Marovaza (Fig. 1) represent those of conservation importance.

CONCLUSIONS

Our research has indicated that several lineages of the endemic Malagasy bat *M. commersonii* sensu stricto, largely represented in our sequenced samples across most of western Madagascar, have a single common ancestor, which is dated to 0.82 MYA. Lineage dispersal, genetic divergence, and expansion events of *M. commersonii* (at least females) are likely associated with Pleistocene climate fluctuations. Our data indicate the northern region (Montagne d'Ambre and neighbouring areas) and the central western area of the Isalo Massif may have acted as refugia for this species during the Plio-Pleistocene, specifically periods of cooler and drier climate conditions. These are the areas where this species shows high levels of genetic diversity and overlap, and these zones should be the focus of conservation efforts.

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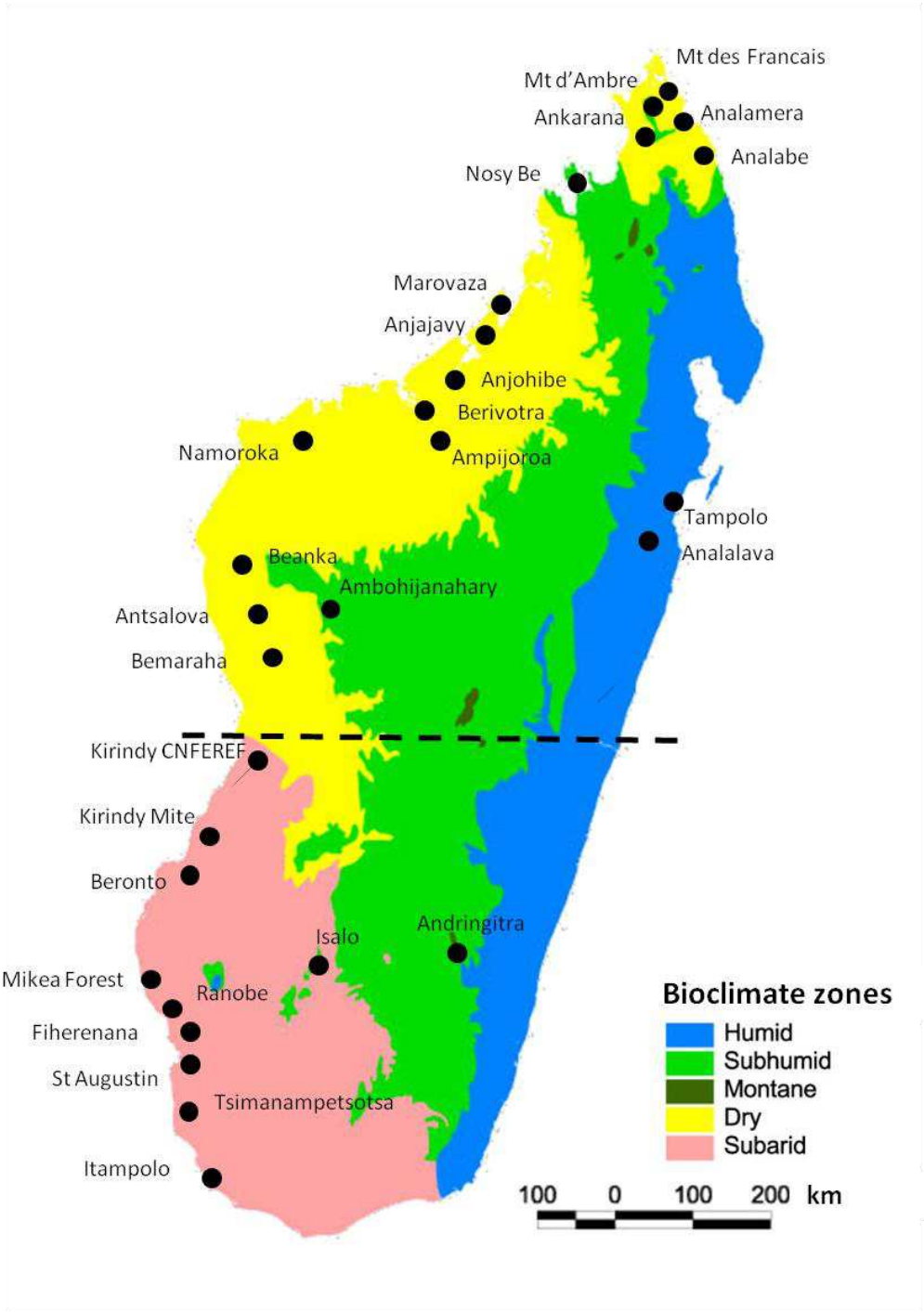

Figure 1: Map of the different collection localities of specimens of *Macronycteris commersonii* used in this study. The map overlay is the simplified bioclimatic zones classification proposed by Cornet (1974). The stippled line separates the "northern group" from the "southern group"

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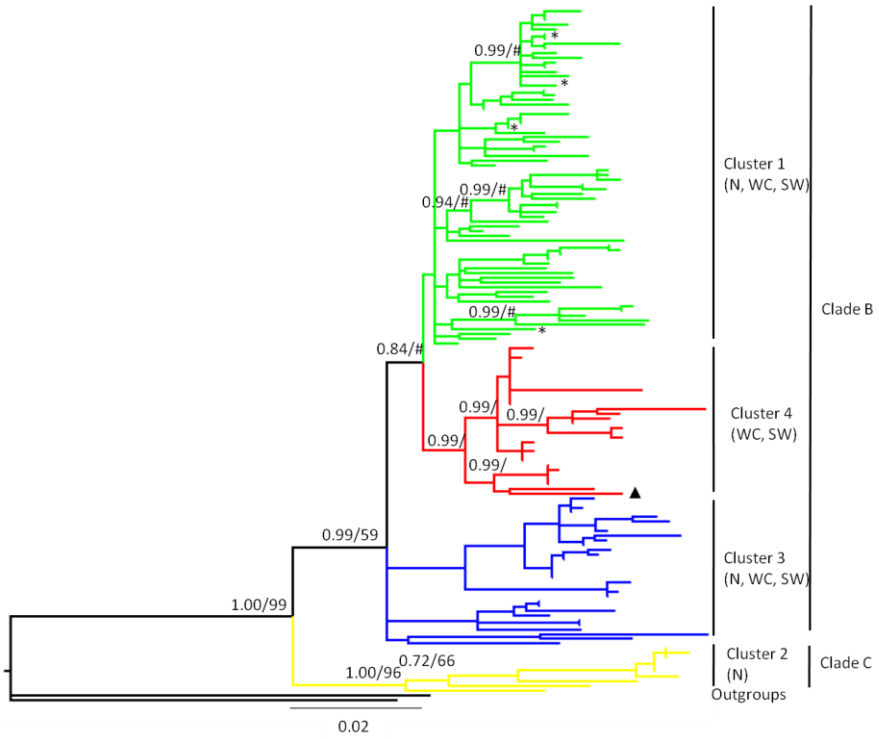

Figure 2: Bayesian phylogram based on the combined analysis of mtDNA control region and Cytochrome b data drawn from 146 individual *Macronycteris commersonii*. Nodal support values are represented as Bayesian posterior probability/likelihood bootstrap percent (# = values ≥ 50); numbers below branches are times to the most recent common ancestor (in MY) with 95% highest posterior density. Tree is color coded based on the result of the genetic mixture analysis in BAPS (Fig. 4) * = specimens from eastern Madagascar, specifically the sites of Analalava and Tampolo, ▲ = specimen from Andringitra, N = north, WC = west central, SW = southwest.

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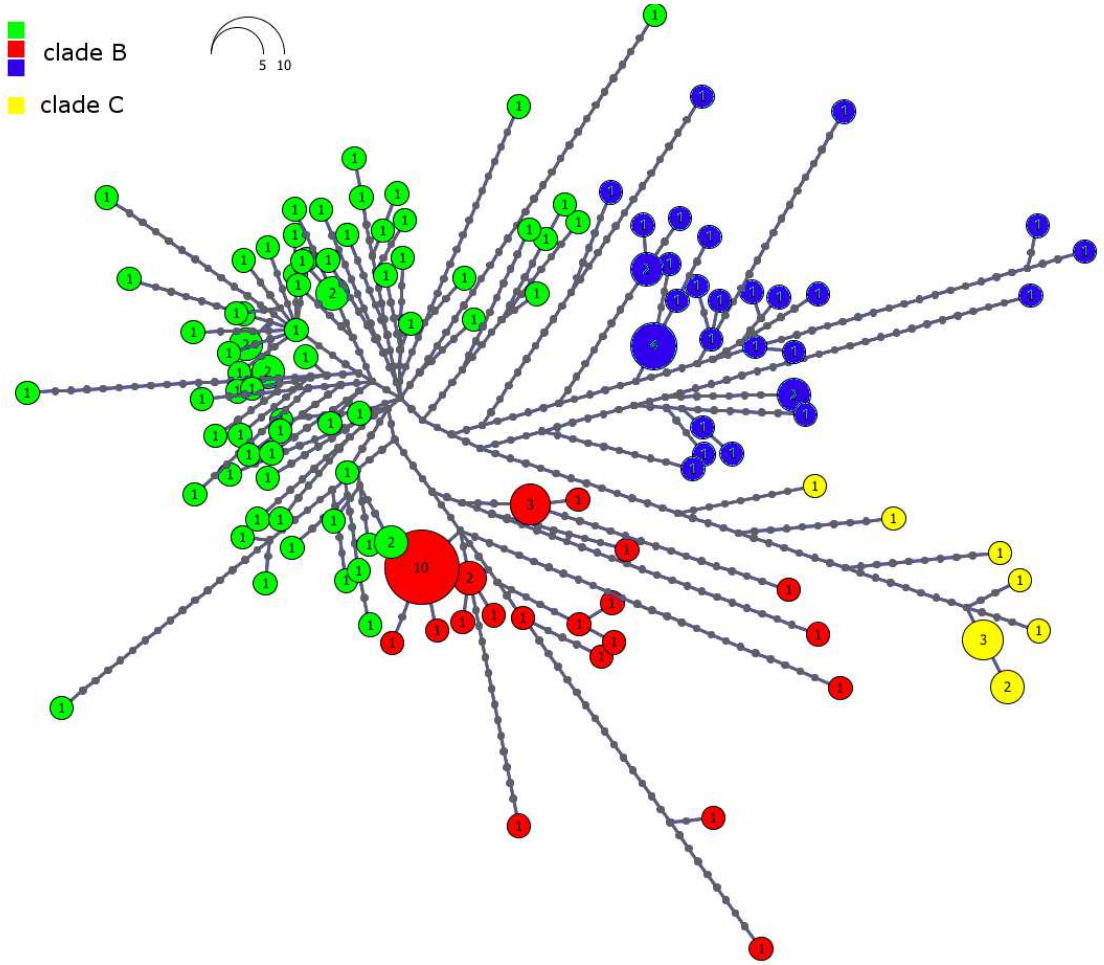

Figure 3: Haplotype network of the combined of mtDNA control region and Cytochrome b data drawn from 146 individual *Macronycteris commersonii* built with HaploViewer. Clades are color-coded based on the BAPS clustering results (as in Fig. 2). Numbers inside the proportionally sized circles represent the number of individuals sharing that particular haplotype.

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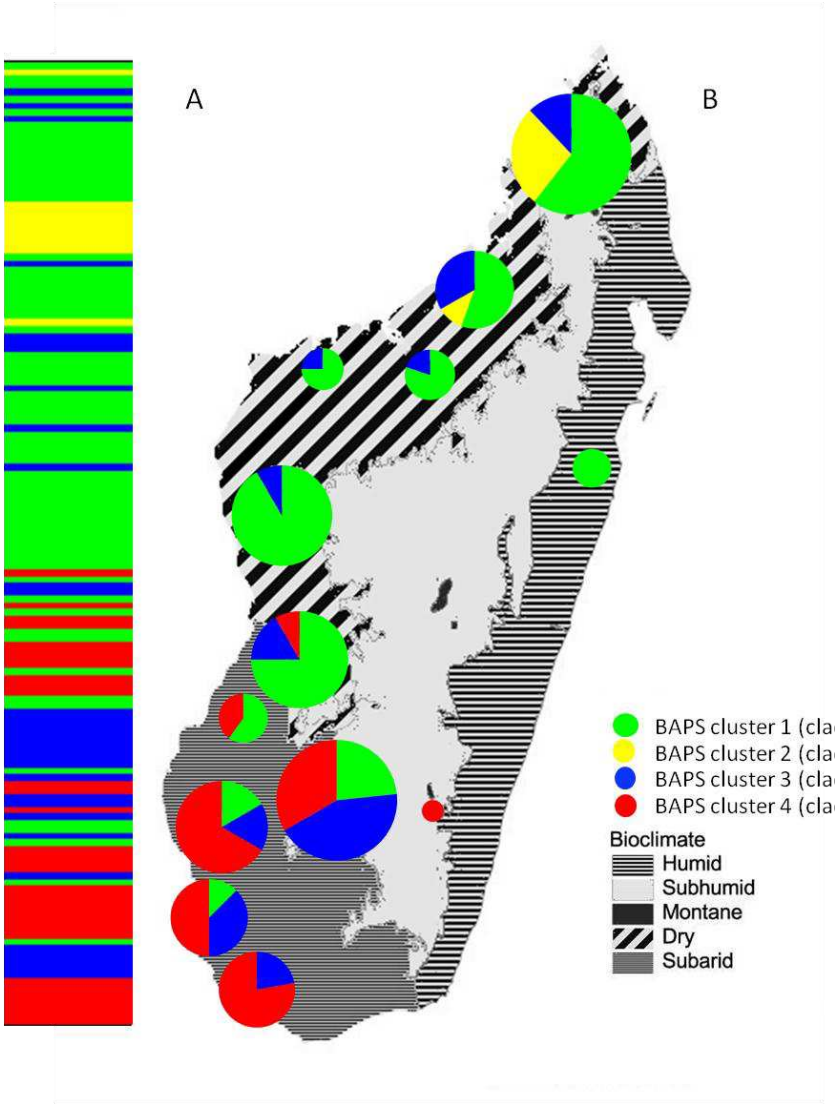

Figure 4: Posterior mode clustering of *Macronycteris commersonii* concatenated mtDNA data using the individual based genetic mixture analysis in BAPS (a). The 146 specimens are clustered by specific locality or grouped neighbouring localities. (b) Distribution of estimated BAPS cluster frequency for the complete concatenated mtDNA sequence data. The simplified bioclimatic zone classifications of the island in which lineages are located are on the maps. Reference to clades and groups are associated with information in Fig. 2.

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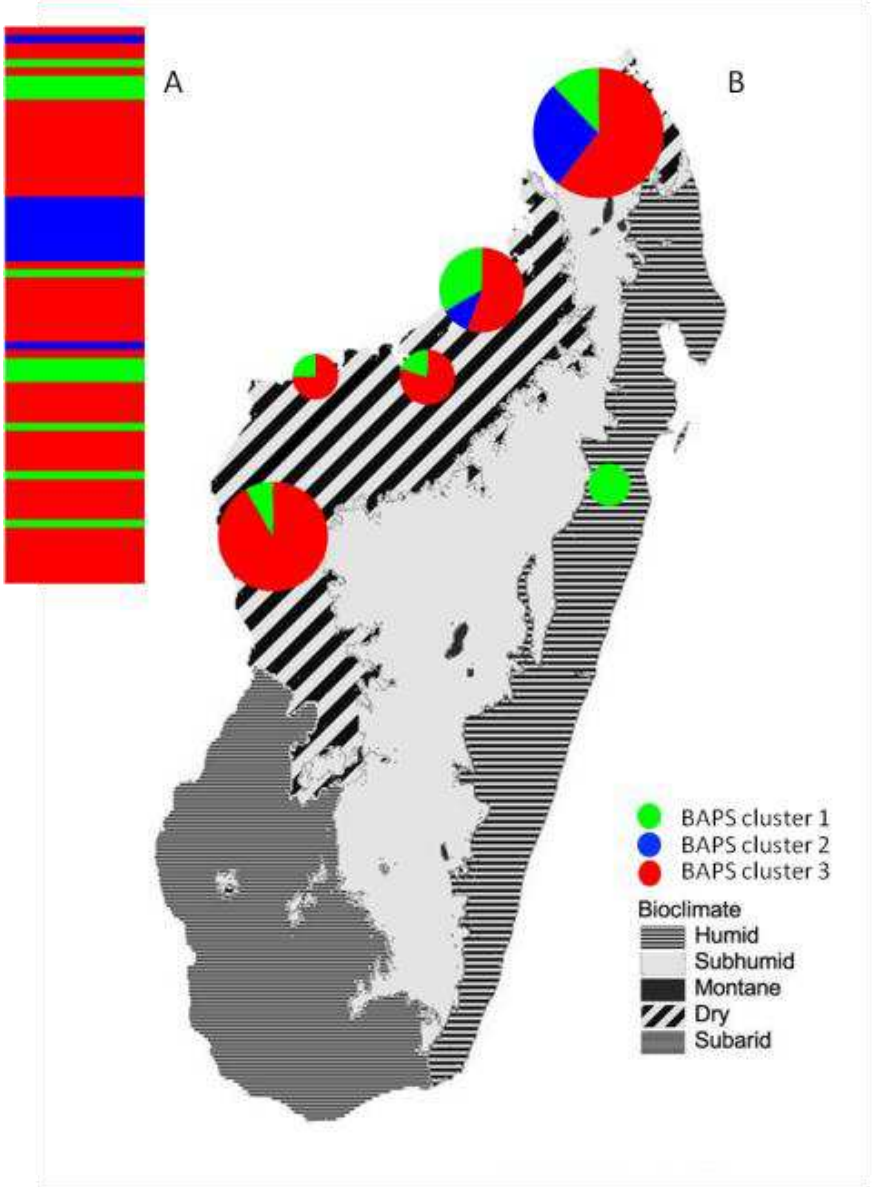

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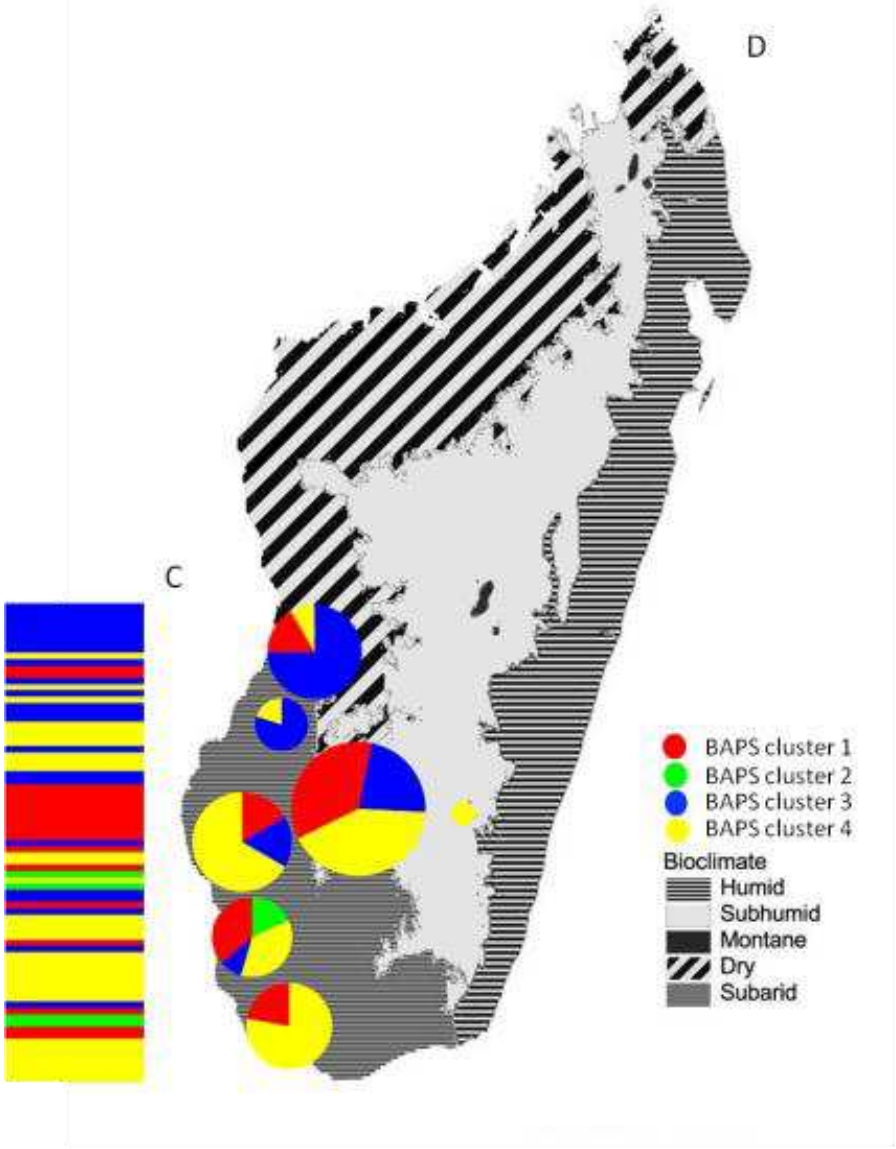

Figure 5: **Posterior mode clustering of (A) the northern group and (C) the southern group of *Macronycteris commersonii* using the genetic mixture analysis at the individual level in BAPS.** The 69 individuals in the northern group and 77 individuals in the southern group are clustered by specific locality or grouped neighbouring localities. Distributions of the estimated BAPS cluster frequency for the mtDNA sequence data of (B) the northern group and (D) the southern group are shown. The simplified bioclimatic zone classification of the island in which lineages are located are on the maps.

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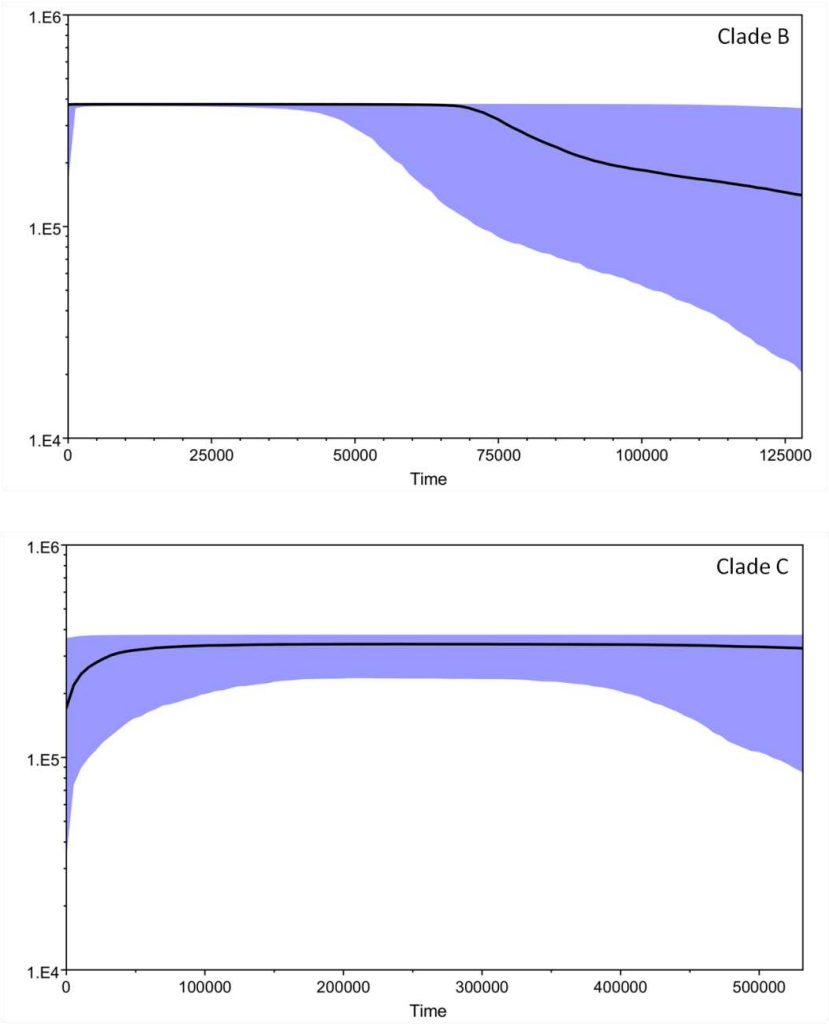

Figure 6: **Bayesian skyline plot for mtDNA with a strict clock and based on a generation time for *Macronycteris commersonii* of two years.** The y-axis shows the effective number of individuals N_e. The thick solid line is the estimated median and the shaded area shows the 95% HPD limits. The x-axis is time scaled in years.

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Table 1:

Characteristics of mtDNA datasets used in this study of *Macronycteris commersonii*. Patterns of sequence variability are presented for two mtDNA regions (CR and *Cyt b*) and the combined data matrix. The total number of nucleotide sites, variable and parsimony informative sites, as well as nucleotide frequencies are given for each partition and the combined data matrix.

Gene	Total number of individuals	Total sites	Variable sites	Parsimony informative sites	Nucleotide frequencies			
					%A	%T	%C	%G
CR	146	481	132	91	32.90	27.00	25.80	14.34
Cyt b	146	703	76	52	26.90	27.36	30.57	15.18
Combined	146	1184	208	143	29.90	27.18	28.18	14.76

Table 2:

Percentages from hierarchical analyses of molecular variance (AMOVA) for mtDNA control region of *Macronycteris commersonii* based on geographical groupings and bioclimatic zones.

Population groups	Among groups	Among populations within regions	Within populations
Northern/Southern	1.27***	2.32**	96.41***
Dry1/Dry2/Subarid/Humid-Subhumid	2.82***	2.56*	94.62***

Statistically significant results were indicated by asterisks: * $P < 0.05$;** $P < 0.01$; ***$P < 0.001$.

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718 **Table 3:**

719 **Pairwise ΦST values for mtDNA control region among populations of *Macronycteris***
720 ***commersonii*.**

	Dry1	Dry2	Subarid	Humid-Subhumid
Dry1	-			
Dry2	0.064*	-		
Subarid	0.151*	0.069*	-	
Humid-Subhumid	0.050	0.0617	0.155*	-

721 Statistically significant results were indicated by asterisks: *$P < 0.001$.

722 **Table 4:**

723 **Neutrality statistics for three defined major clades and groups of *Macronycteris***
724 ***commersonii* based on *Cyt b* sequences.**

	Clade B	Clade C
Nucleotide diversity (π)	0.007	0.003
Haplotype diversity (Hd)	0.958	0.911
Fu and Li's Fs	-63.055 ***	-2.671
Tajima's D	-1.811 *	-1.045
Ramos-Onsins and Rozas (2002) (R2)	0.0371	0.1253
Mismatch distribution	Unimodal	Bimodal

725 Statistically significant results were indicated by asterisks: * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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