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The evolutionary history of the African fruit bats (Chiroptera: Pteropodidae)

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Bats of the family Pteropodidae, also known as megabats or Old World fruit bats, are widely distributed in tropical areas of Africa, Asia, and Oceania. Of 45 genera in the family, 12 are endemic to the Afro-tropical region and two others have representative species on the African continent. African megabats inhabit wooded habitats and are nearly ubiquitous on the mainland and nearby islands with the exception of desert areas. Some species have been implicated as possible reservoirs of the Ebola Zaire virus. We studied the phylogenetic relationships of mainland African megabats using both mitochondrial and nuclear loci in separate and combined analyses. The phylogenetic trees obtained showed four main African clades: *Eidolon*, Scotonycterini (including two genera), African *Rousettus* (three species), and the previously identified 'endemic African clade' (nine genera). The latter three lineages form a clade that also includes the Asian species of *Rousettus* and the Asian genus *Eonycteris*; *Eidolon* does not show close relationships to other African genera, instead nesting elsewhere in the megabat tree. Although our results confirm many of the conclusions of previous studies, they challenge the taxonomic status and placement of *Epomops dobsonii* and *Micropteropus*, and provide evidence indicating that a new classification at subfamilial and tribal levels is highly desirable. The principal clades we detected represent four independent colonizations of Africa from most probably Asian ancestors. Estimates of divergence dates suggest that these events occurred in different periods and that although local diversification appears to have started in the late Miocene, the more extensive diversification that produced the modern fauna occurred much later, in the Pleistocene.

Key words: phylogenetic analysis, Africa, Epomophorinae, molecular systematics, molecular clock, pteropodids, *Rousettus*, classification

INTRODUCTION

Megabats (Mammalia: Chiroptera: Pteropodidae) represent a monophyletic group of mostly non-echolocating, phytophagous bats specialized for consumption of fruit and flower products (nectar and pollen — Kunz and Pierson, 1994). Pteropodids are distributed along the Old World tropics from western Africa to eastern Polynesia (Kunz and Pierson, 1994), and within this range Sub-Saharan Africa represents the largest land area where they occur. The African megabat fauna includes species from several clades that may have arrived, or evolved, independently on the continent and adjacent islands.

Epomophorinae sensu Bergmans (1997) is the largest group of African Pteropodidae, comprising 11 genera almost exclusively found in continental Africa. Bergmans' (1997) Epomophorinae included species previously widely separated in the traditional taxonomy, i.e., taxa included by Andersen (1912) in the subfamilies Cynopterinae (*Myonycteris*), Rousettinae (*Lissonycteris*) and Macroglossinae (*Megaloglossus*). Bergmans (1997) subdivided the epomophorines into four tribes: Epomophorini (genera *Epomophorus*, *Epomops*, *Hypsignathus*, *Micropteropus*, and *Nanonycteris*), Myonycterini (*Myonycteris*, *Lissonycteris*, and *Megaloglossus*), Plerotini (with the monotypic *Plerotes*), and Scotonycterini (*Scotonycteris* and *Casinycteris*). Early molecular phylogenetic analyses were in agreement with the existence of an endemic African clade that included the former two tribes, although the other tribes were not sampled in those studies (Hollar and Springer, 1997; Juste B. *et*

al., 1999; Álvarez *et al*., 1999). Subsequent phylogenies, with more species and additional character data (e.g., Giannini and Simmons, 2003, 2005), found support for the endemic African clade both in molecular and morphological data sets, but anticipated a more complex scenario of relationships, later confirmed with the recovery of Scotonycterini as a branch separated from other epomophorines (Almeida *et al*., 2011). In the phylogeny recovered by Almeida *et al*. (2011), typical rousettine megabats (*Eonycteris*, *Rousettus*, and *Stenonycteris*) appeared nested between Scotonycterini and the remainder of epomophorines, a result confirmed in subsequent analyses (e.g., Hassanin, 2014). Indeed, Nesi *et al*. (2013) proposed that *Stenonycteris* is best placed in a separate tribe, related to the endemic African clade. Therefore, rousettines and eonycterines are integral to the understanding of the evolution of African megabats, even though some of them are distributed in the Australasian tropics.

Other lineages of megabats are distributed in Africa in addition to epomophorines. Among the eight species of *Rousettus*, three have African-Malagasy distributions (*R. aegyptiacus*, *R. madagascariensis*, and *R. oblivious*), with *R. aegyptiacus* restricted to the mainland. Another African lineage is represented by the genus *Eidolon,* which includes two species and three subspecies (Simmons, 2005). This genus has proven extremely difficult to place within the pteropodid family tree. It seems likely that *Eidolon* represents an independent lineage not closely related to any other megabat clades (Almeida *et al*., 2011), although associations with rousettines (Andersen, 1912; Bergmans, 1997) and pteropodines (Giannini and Simmons, 2005) have been suggested. The latter comprise the flying foxes (*Pteropus*), a speciose, chiefly Australasian genus, that includes a few species distributed on islands of the western Indian Ocean and offshore African islands; nevertheless, no *Pteropus* species is found in continental Africa. O'Brien *et al*. (2009) and Almeida *et al*. (2014) have shown that *Pteropus* species from islands of the western Indian Ocean, including Madagascar, Aldabra, Seychelles, Comoros, Mascarene, and islands off the Tanzanian coast, belong to several clades mainly associated to the *P. vampyrus* species groups (sensu Almeida *et al*., 2014) and are deeply nested within pteropodines. Since none of these lineages have been linked neither to the 'endemic African clade' nor to the scotonycterines in recent phylogenies, they represent yet other independent instances of pteropodid colonization of Afro-Malagasy region.

In recent years, members of Pteropodidae have been identified as natural reservoirs of a number of viruses that cause zoonotic diseases including the Nipah, Hendra, Marburg, and Ebola viruses (Epstein *et al*., 2008; Halpin *et al*., 2011; Drexler *et al*., 2012; Olival *et al*., 2013; Wynne and Wang, 2013; Alexander *et al*., 2015; Razanajatovo *et al*., 2015; Schaer *et al*., 2015). More specifically, in Africa, five pteropodids (*Epomops franqueti*, *Hypsignathus monstrosus*, *Myonycteris torquata*, *Rousettus aegyptiacus*, and *Eidolon helvum*) have shown signs of infection (viral RNA and specific antibodies) with the Ebola subtype Zaire virus, which caused the 2014 epidemics in West Africa (Pourrut *et al*., 2009; Hayman *et al*., 2010; Vogel, 2014; Leroy *et al*., 2014). These bats have been shown to be seropositive without manifesting the disease. In at least one case, the long-term survival along a typical migration cycle was recorded for a female of *E. helvum* with antibodies for both Ebola subtype Zaire and Lagos bat viruses (Hayman *et al*., 2010). Nearby in Madagascar, novel coronaviruses have been detected in congeneric *E. dupreanum* and also in the endemic *Pteropus rufus* (Razanajatovo *et al*., 2015). Better understanding of the diversity and transmission of these viruses depends on not only studies of the viruses themselves, but also on gaining a better understanding of the biology of the bats including their evolutionary history.

The focus in the present study is determining phylogenetic relationships among taxa within the well-supported endemic African clade (Epomophorini and Myonycterini) and their closely-related allies (Scotonycterini, Rousettini, and Stenonycterini) as recovered in Almeida *et al*. (2011). The study by Almeida *et al*. (2011) focused on relationships at the generic level and, in terms of breadth of diversity sampled, is the most comprehensive molecular phylogenetic study of pteropodids to date. However, that study lacked breadth of sampling within many African genera and some of the recovered relationships lacked statistical support. Some subsequent studies have sampled much more densely within selected African taxa (e.g., Nesi *et al*., 2013; Hassanin, 2014), but their samples have never been comprehensively analyzed in concert with broader taxonomic and locus sampling, nor have some key taxa (e.g., *Plerotes anchietae*) been included in prior analyses. To address these issues, we conducted a comprehensive set of analyses based on sequence data from eight nuclear and mitochondrial markers, and generated a robust phylogeny of the pteropodids that occur in continental Africa. Our sample was

designed to include all the species that have been identified as potential reservoirs of hemorrhagic-fever viruses that infect humans and other primates.

Material and Methods

Samples

We sampled members of all genera and most species in the scotonycterine, rousettine, and epomophorine clades (the latter inclusive of Epomophorini, Plerotini, and Myonycterini). In addition to taxa studied in Almeida *et al*. (2011), we sampled 16 extra species of African genera by sequencing new tissue samples available to us and gathering published sequences available in GenBank. Among the new data that we generated are sequences of *Plerotes anchietae* (Plerotini, historically placed within Epomophorinae — Andersen, 1912), which has never before been included in a molecular phylogeny. Additionally, we included sequences from another African genus, *Eidolon*, which does not appear to be closely related to the remaining ingroup genera. In Almeida *et al*. (2011), *Eidolon* was recovered as an independent and perhaps basal lineage without close affinities to any other pteropodid lineage from inside or outside of Africa. Although determining the phylogenetic position of *Eidolon* within Pteropodidae as a whole was not a focus of this study (as that would require very broad sampling of Pteropodidae as a whole and more nuclear markers than employed in the current study; Almeida *et al*., 2011) inclusion of this genus completes our matrix with sampling all megabat genera that occur in continental Africa. For each genus, we included all species for which sequences of at least one of the eight chosen loci (see below) were available, totaling 31 species (but possibly 32 — see Discussion; Appendix I). Three other pteropodid species were included in the matrix as outgroups: *Pteropus medius* (= *giganteus*; Pteropodinae), *Cynopterus sphinx* (Cynopterinae), and *Nyctimene albiventer* (Nyctimeninae). This choice of outgroups was based on the phylogeny presented by Almeida *et al*. (2011), who convincingly demonstrated that these species are not closely related to any of the ingroup taxa sampled here. Whenever possible we included up to three sequences per species to make sure that samples had been correctly identified. In case of doubt, extra sequences were obtained from the Genbank for comparison, although some were not included in the trees shown here. The number of loci sequenced per species was variable (between one and seven; Appendix II — Table S1). For some species for which we could not obtain tissue samples, we acquire sequences from GenBank, usually represented by one or two genes (Appendix II — Table S1). For *Epomops dobsonii*, a fragment of the 12S gene was sequenced from a skin biopsy of a museum specimen collected in 1938. The procedures for collecting the skin biopsy, DNA extraction, sequencing, and sequence curation were carried out as described in Almeida *et al*. (2014). All tissue samples used herein were preserved and deposited in museum collections, and, therefore, no animals were collected or sacrificed specifically for this study.

Molecular Data

Four nuclear (RAG1, RAG2, vWF, and BRCA1) and four mitochondrial (Cyt*b*, 12S-rRNA, tRNA-val, and 16S-rRNA) markers were included in the matrix as in Almeida *et al*. (2011). The aligned nuclear matrix had 4410 bp, while the mitochondrial matrix comprised of 3673 bp. Sequences from 22 specimens were newly generated for this study (Appendix I). Primers, molecular methods, and sequence editing followed those previously described in our prior papers on pteropodid relationships (Almeida *et al*., 2009, 2011; Giannini *et al*., 2009).

Phylogenetic Analysis

Alignments were done with MAFFT version 7 (Katoh and Standley, 2013), although coding fragments, with the exception of BRCA1, could be aligned by eye since they did not include indels. Each gene was first analyzed individually to check for unexpected results (e.g., individuals of the same species that did not cluster together) to check for misidentifications or contamination (Appendix II — Figs. S1–S6). Three concatenated matrices (mitochondrial loci, nuclear loci, and all loci) were then constructed for analysis. In all analyses, the datasets were partitioned by gene and codon position (first + second and third) for the coding genes, a method previously suggested to represent a good compromise between likelihood gain and partition size (Almeida *et al*., 2011). The contiguous fragment containing the 12S-rRNA, tRNA-val, and 16S-rRNA genes was treated as a single partition.

Before concatenating the nuclear and the mitochondrial datasets, we ran an Incongruence Length Difference test (ILD — Farris *et al*., 1994) in PAUP* (Swofford, 2002) to test for phylogenetic incongruence between mitochondrial and nuclear genes. The ILD test was run 500 times, with 10 random species addition per search. We additionally used the Shimodaira-Hasegawa (SH — Shimodaira and Hasegawa, 1999) test to compare tree likelihoods given different datasets (mitochondrial, nuclear, all loci combined). In this test, incongruence is detected when a tree obtained with a dataset has a significantly higher likelihood than alternative trees obtained with other datasets. The SH test was run on a reduced dataset of 26 taxa where missing data was minimized as follows: we built chimeric sequences to complete the data for each species combining sequences of two or more individuals when possible and necessary, and removed all taxa for which only a few sequences were available (Appendix II — Table S1). The test was done with the RAxML program version 8.0.0. (option *-f H* — Stamatakis, 2014)

Maximum likelihood (ML) tree searches were also carried out with the program RAxML. The analyses employed the GTRGAMMA substitution model and were run 10 times independently to obtain the best tree. Statistical support for clades was measured with 200 standard bootstrap replicates (commands: *-f d –b 1234 -# 200*). Maximum Parsimony (MP) tree searches with the three datasets were carried out with TNT (Goloboff *et al*., 2008). Bayesian inference (BI) trees were obtained with MrBayes (Ronquist and Huelsenbeck, 2003) in two runs with 10^6 generations and 6 chains each. Trees were sampled every 2000 generations. All data partitions were analyzed under the GTRGAMMA model with unlinked parameter estimation. Convergence was evaluated based on ESS values (>200) as visualized with Tracer v1.6 (Rambaut *et al.*, 2003). Tree illustrations were made with FigTree (Rambaut, 2009) and rooted at the midpoint.

Divergence Time Estimates

Species divergence times were estimated with the program BEAST2 (Bouckaert *et al*., 2014), which uses a Bayesian

framework and allows for the use of relaxed clocks. Due to a lack of ingroup fossils, we based this analysis on the substitution rate estimated for the Cyt*b* gene in bats (Ruedi and Mayer, 2001; Hulva *et al*., 2004). The Cyt*b* alignment was trimmed so that only one individual per species was included. The sites were partitioned by codon position, which were unlinked for the estimation of the parameters of the GTR+Γ+I model. We assumed a relaxed lognormal clock and the Yule speciation model with a Gamma distribution prior. For the mean substitution rate, the prior was set as a lognormal distribution with mean of 0.023 subs/site/My and a standard deviation of 0.3 to match the rate estimated based on fossil data (Ruedi and Mayer, 2001; Hulva *et al*., 2004). The chain was run for 10^7 generations and sampled every 10,000 generations. The convergence of parameters was checked with Tracer (Rambaut *et al*., 2003).

Biogeography

Previous studies have suggested that pteropodids had an Australasian origin (Butler, 1984; Aguilar *et al*., 1986) and have colonized the African continent on several independent occasions (Juste B. *et al*., 1999; Almeida *et al*., 2011). To further investigate the history of the African colonization by pteropodids, we analyzed our data using DIVA (Ronquist, 1997) as implemented in RASP v 3.1 (Yu *et al*., 2015). For this analysis, we increased the number of non-African (Australasian) pteropodids in the data set and included two non-pteropodid taxa (*Hipposideros vittatus* and *Megaderma lyra)* as additional outgroups (Appendix II — Table S1) and decreased the number of ingroup terminals to one sample per species. The ML tree used in the DIVA was obtained as described above.

Results

Phylogenetic analyses of mitochondrial and nuclear datasets agreed in most relationships within and among the main clades and each recovered Scotonycterini, *Eonycteris*, *Rousettus*, and the endemic African clade as monophyletic groups (Figs. 1 and 2, Appendix II — Figs. S1–S6). The two partitions disagreed, however, on the positions of *Rousettus* and *Eonycteris*, with the nuclear dataset favoring *Rousettus* as the second ingroup clade to split, while the mitochondrial dataset favoring *Eonycteris* in this position instead (Figs. 1 and 2, Appendix II — Figs. S1–S6). In neither case was there statistical support for the respective placements. Another difference between results based on the two datasets involved basal relationships within the endemic African clade, though again in neither case did alternative relationships receive statistical support (Figs. 1 and 2). This result mirrors the fact that there was disagreement (without statistical support) between different mitochondrial genes (Cyt*b* and 12S16S) with respect to basal relationships of the endemic African clade (Appendix II — Figs. S5 and S6). Finally, there was lower resolution in the nuclear tree with respect to relationships among the *Epomophorus*/*Micropteropus* and *Myonycteris* species. This seems to be due to lack of phylogenetic information in slowly-evolving nuclear genes; very few substitutions were observed within these clades and even fewer were phylogenetically informative (e.g., only 15 out of 4410 sites were variable among *Myonycteris* species, and only three of these were phylogenetically informative).

No significant incongruence between datasets was detected with the ILD test. The SH test did not reject congruence between either datasets (mitochondrial or nuclear) and the tree obtained with the all-loci matrix (nuclear: D = 4.07; mitochondrial: D = 6.27). The SH test also did not detect incongruence between the nuclear dataset and the mitochondrial tree (D = -11.46). When considering the mitochondrial dataset, however, the mitochondrial tree was significantly better than the nuclear tree (D = -80.46). These results point to a higher likelihood of the mitochondrial tree, which is probably an effect of the lower number of variable and informative characters of the nuclear dataset (5.7% of the nuclear sites were informative, in comparison with 13% of the mitochondrial sites). In fact, the topology of the ML tree based on the concatenated matrix is more similar to the one based on the mitochondrial dataset than it is to the tree based only on nuclear genes. It is possible that the mitochondrial genes (maternally inherited) and nuclear genes (biparentally inherited) have different evolutionary histories as seems to be the case in some bat groups (e.g., Larsen *et al*., 2010; Almeida *et al*., 2014) but we did not detect evidence of this.

A tree summarizing the analyses done with the all-loci matrix (including both mitochondrial and nuclear loci) is shown in Fig. 3. The three reconstruction methods employed yielded highly congruent trees with high statistical support for all major clades and most subclades. *Eidolon*, as previously observed (Almeida *et al*., 2011), is not closely related to the other African taxa (ingroup taxon set). The first ingroup clade to split from the others was Scotonycterini. The second clade to split within the ingroup in all analyses was *Eonycteris*, but only the BI tree provided statistical support for this relationship. In this way, the genus *Rousettus* was found to be sister to the endemic African clade (Fig. 3). Relationships within the endemic African clade were inconsistent across analytical methods (BI and MP trees are illustrated in Appendix II — Figs. S7 and S8). Most higher-level clades within the ingroup received high statistical support in all analyses.

The main exceptions were the sister relationship between *Rousettus* and the endemic African clade and the basal relationships within the endemic African clade. Similar results were obtained in the Almeida *et al*. (2011) analyses, which included a subset of the ingroup species sampled in the present study.

To rule out missing data as an explanation for some of the low support values observed, we reran the ML analysis on matrices with minimal missing data (Appendix II — Fig. S9). These matrices were built by forming chimeras of terminals belonging to the same species when not all gene sequences were available for a particular sample (e.g., *Epomophorus*

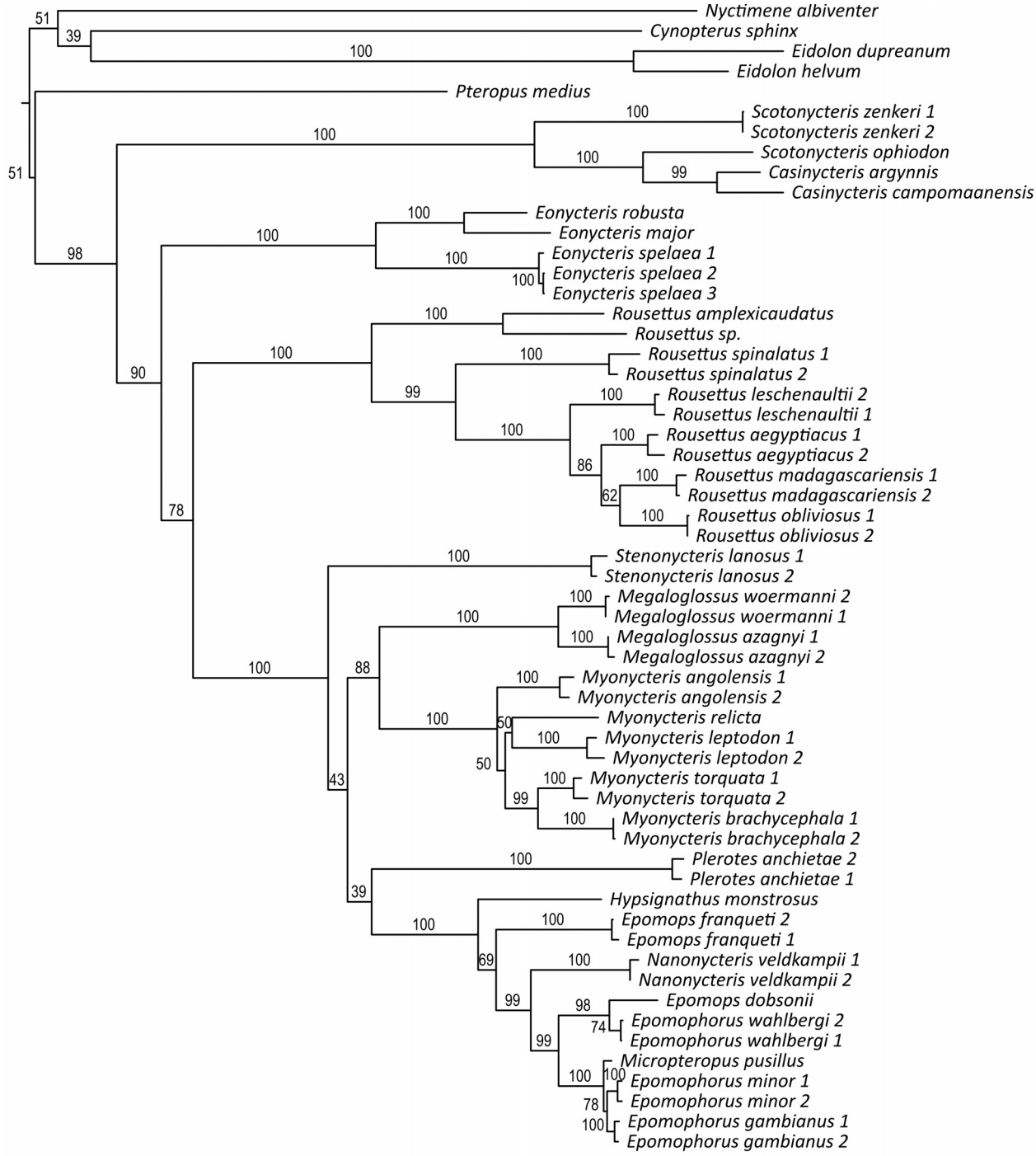

FIG. 1. Maximum likelihood phylogeny based on mitochondrial DNA data (Cyt*b*, 12S rRNA, and 16S rRNA genes). ML bootstrap support values are shown above branches

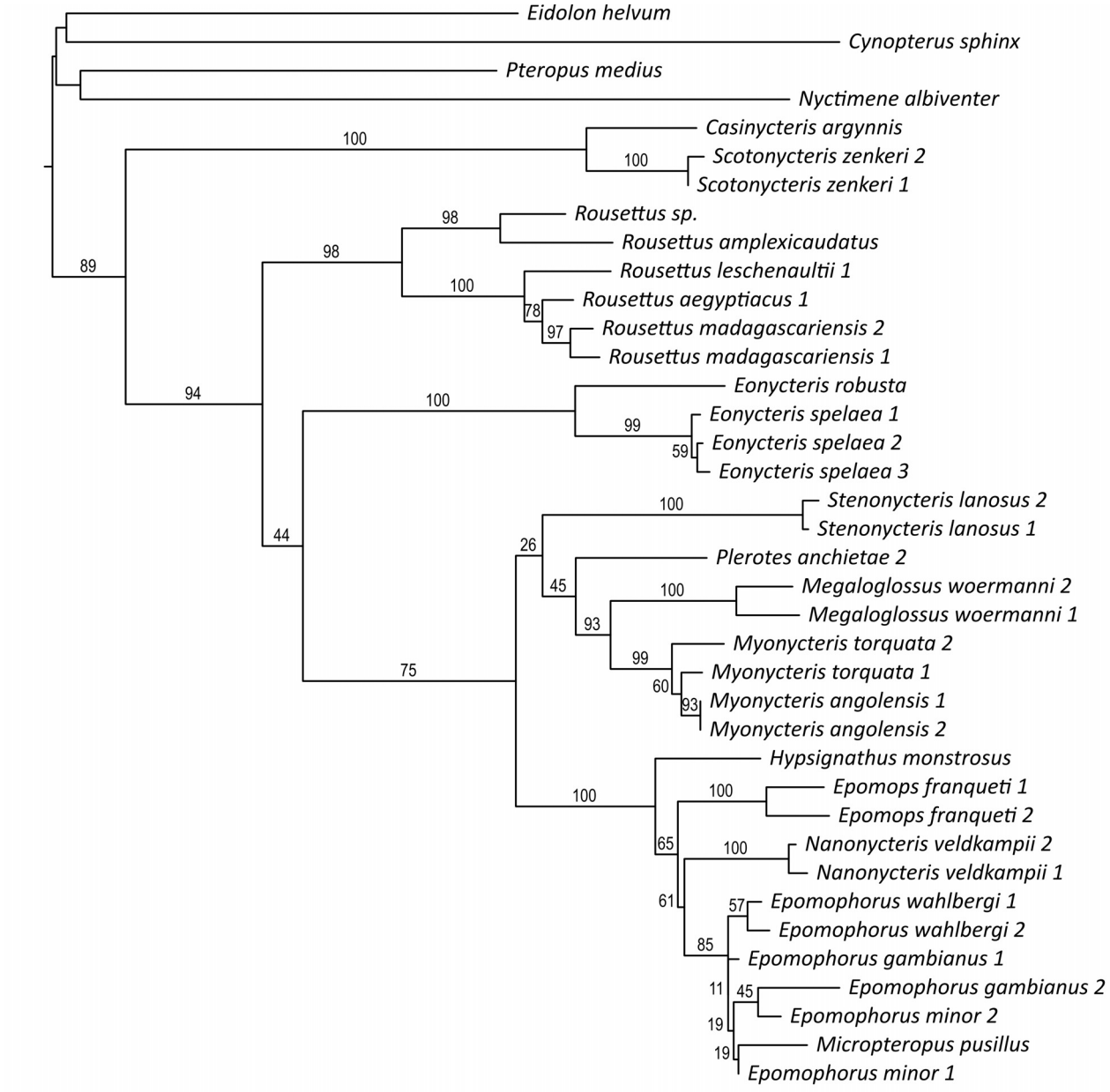

FIG. 2. Maximum likelihood phylogeny based on nuclear data (RAG1, RAG2, vWF, and BRCA1 genes). ML bootstrap support values are shown above branches

wahlbergi) and excluding all terminals missing more than two of the seven genes analyzed here. An exception was made for *Plerotes anchietae*, which was included in one of the low-missing-data matrices. We did not find significant differences either in topology or levels of perceived support between trees obtained with these reduced matrices and those obtained our complete matrix (Fig. 3). This finding is in accordance with the lack of empirical evidence that missing data have a strong influence in phylogenetic inference and/or statistical support of clades (Wiens, 2006).

Another piece of evidence suggesting that missing data is not a major issue across our matrix as a whole was the high support found for the position of *Scotonycteris ophiodon* in all analyses (Fig. 3), even though this species was represented in the combined matrix by only the Cyt*b* gene and a fragment of the 12S gene (ca. 1000 bp). However, missing data may have produced uncertainty in other parts of the tree. Low support values were obtained for some intraspecific relationships in the genera *Rousettus* and *Myonycteris*. In both cases, some of the involved species had few sequences available

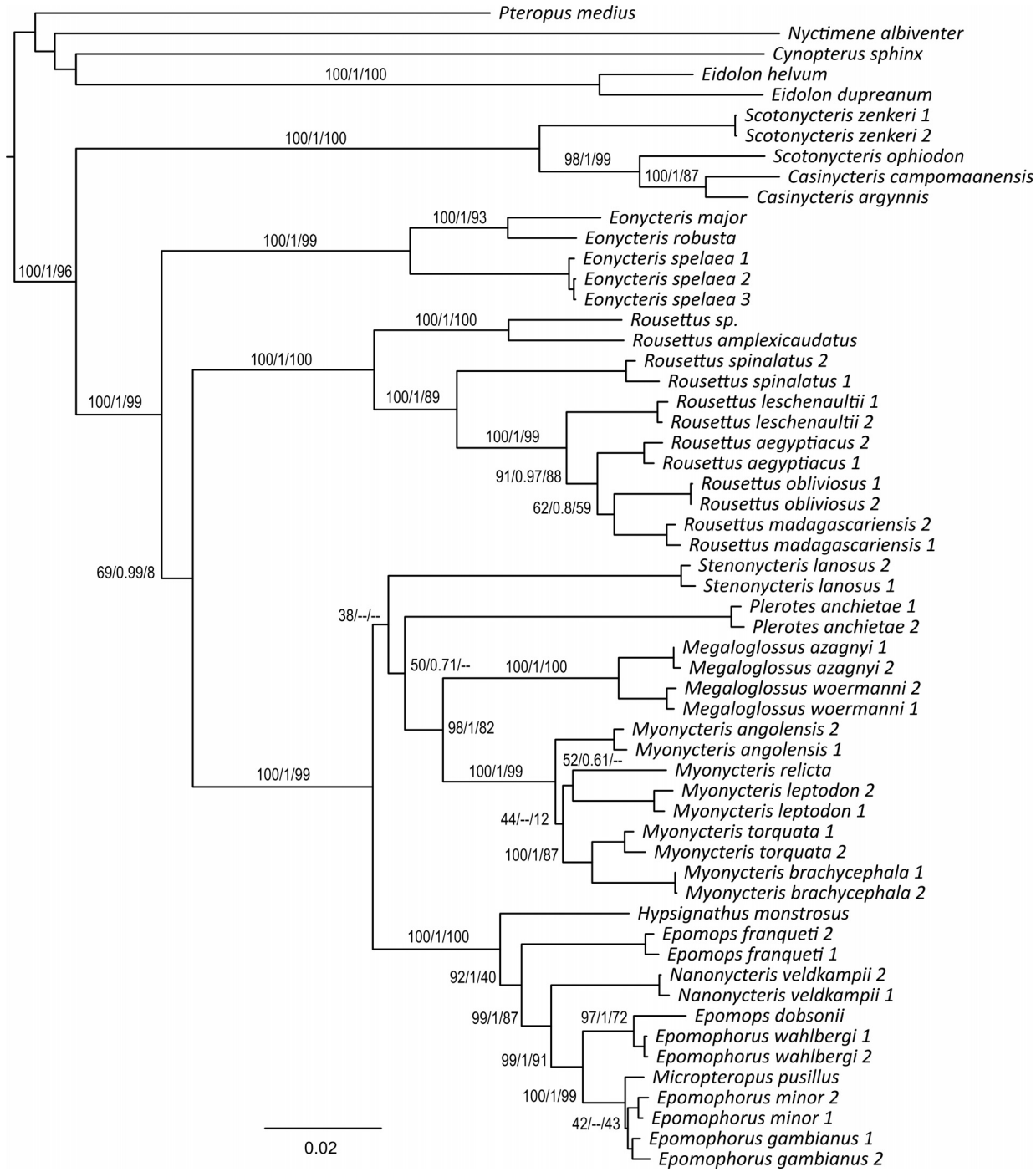

FIG. 3. Maximum likelihood phylogeny based on total evidence (mitochondrial + nuclear genes concatenated). Above branches are shown maximum likelihood bootstrap support values / Bayesian posterior probabilities / maximum parsimony bootstrap values

(e.g., for *R. obliviosus* and *M. leptodon* only the Cyt*b* gene was available). In these particular cases, which appear to involve short branches, it is possible that sequences from more loci could help improving statistical support for phylogenetic relationships.

Divergence time estimates based on the substitution rates of the Cyt*b* gene are illustrated in Fig. 4. Biogeographic reconstruction using DIVA revealed four independent colonizations of Africa/western Indian Ocean islands (maroon color in circles of Fig. 5). Changes in the dataset for the DIVA analysis (see methods) did not change the main phylogenetic results (Appendix II — Fig. S10).

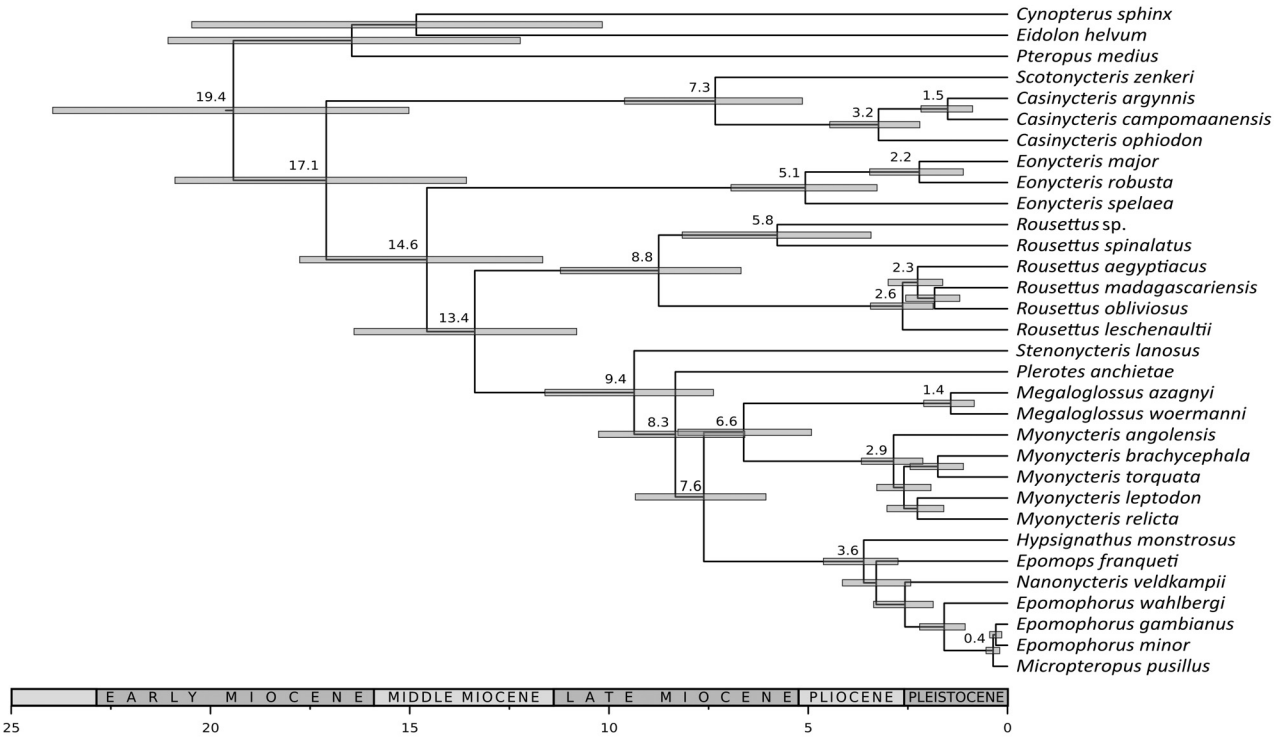

FIG. 4. Time-calibrated phylogeny bars on nodes represent the 95% confidence intervals of the estimated split dates

DISCUSSION

Scotonycterini

This tribe was included in the Epomophorinae subfamily by Bergmans (1997), but cumulative evidence from phylogenetic analyses (Almeida *et al*., 2011; Hassanin, 2014) has shown that such inclusion makes Epomophorinae a non-monophyletic grouping. Scotonycterini sensu Bergmans is composed of two genera, *Casinycteris* and *Scotonycteris*, of small to medium-sized pteropodids (18–95 g) endemic to either one or both the Guinean and Congo Basin rainforests blocks (Nowak, 1994). These bats have characteristic small patches of white hair on the nose and behind the eyes. Until recently, two species of *Scotonycteris* and one of *Casinycteris* were known to science (Simmons, 2005). An analysis of the cytochrome *b* gene revealed a closer relationship of *S. ophiodon* to *C. argynnis*, rather than to *S. zenkeri*, prompting the transfer of *ophiodon* to the genus *Casinycteris* (Hassanin, 2014). In the same study a new species, *C. campomaanensis* was described from Cameroon. In all our analysis, Scotonycterini was recovered as monophyletic with high statistical support, but did not share an immediate common ancestor with the endemic African clade. More recently, based on genetic discontinuity in both mitochondrial and nuclear loci, *S. zenkeri* has been split into three species: *S. zenkeri*, *S. occidentalis*, and *S. bergmansi* (Hassanin *et al*., 2015).

Eonycteris

Bergmans (1997) grouped *Rousettus* and *Eonycteris* together with *Eidolon* in the tribe Rousettini; likely, this arrangement reflected the morphological resemblance of *Eonycteris* to *Rousettus*, as well as the traditional association of *Eidolon* with *Rousettus* as described in Andersen (1912). In none of our analyses, however, did *Rousettus* clustered with *Eonycteris*, which suggests that *Rousettus* and *Eonycteris* should each be in their own tribe (see below).

Rousettus

This is a megabat genus that includes medium-sized, apparently unspecialized pteropodids. Several other relatively unspecialized pteropodid taxa (*Boneia*, *Lissonycteris*, and *Stenonycteris*) were previously included in *Rousettus* (e.g., Andersen, 1912; Bergmans and Rozendaal, 1988) but these were subsequently removed (Bergmans, 1997; Giannini and Simmons, 2003; Giannini *et al*., 2009; Nesi *et al*.,

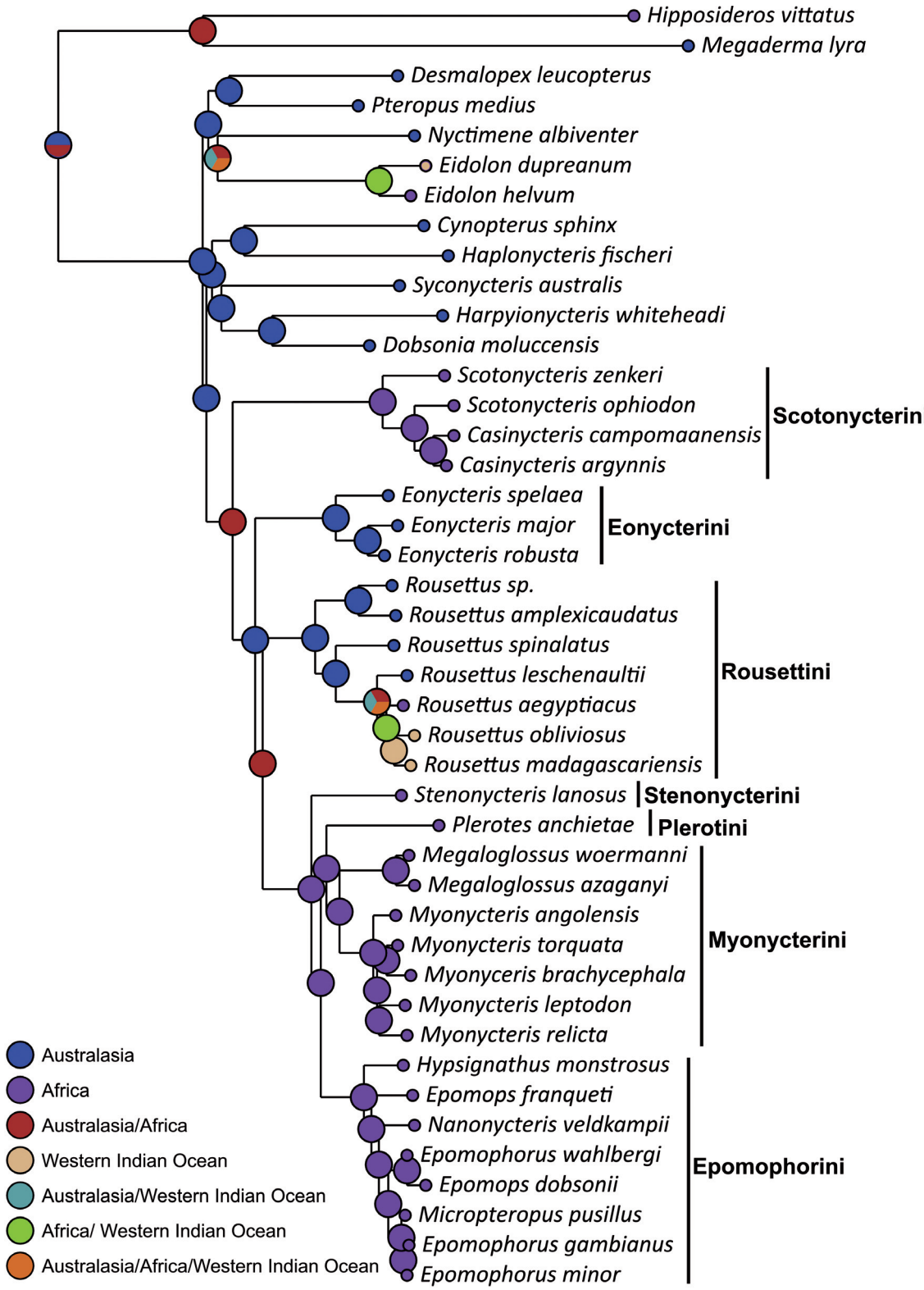

FIG. 5. Biogeographic reconstruction obtained with DIVA

2013). Remarkably, this is the only genus of Pteropodidae found both in mainland Africa and eastern Asia. In our analysis, we uncovered an interesting problem with the identification of *Rousettus* specimens from Indochina, where the distributions of *R. leschenaultii* and *R. amplexicaudatus* overlap

(see Appendix II — text and Fig. S4). Two Asian *Rousettus* species are unfortunately missing from our analyses (*R. celebensis* and *R. linduensis*, both from Sulawesi) because neither samples nor published sequence were available.

In the trees presented herein (Figs. 1–3), a basal split within *Rousettus* separates *R. amplexicaudatus* from the remaining species in the late Miocene. The second species to split is *R. spinalatus* (recorded in Borneo and Sumatra, Simmons, 2005). *Rousettus leschenaultii* appears as sister of a monophyletic clade that contained all the African species. This is a new and interesting result, since previous studies based on the cytochrome b gene alone were not able to resolve the relationships between African *Rousettus* and *R. leschenaultii* (Goodman *et al.*, 2010). Our results indicate that *Rousettus* colonized Africa only once, in the late Pliocene or early Pleistocene, and shortly thereafter diversified into three species: one on the continent (*R. aegyptiacus*) and two on islands (*R. madagascariensis* in Madagascar and *R. obliviosus* in the Comoros archipelago). Topology of our trees suggests that the first split was between *R. aegyptiacus* and the lineage leading to the island forms. A second split, occurring in the island lineage, subsequently resulted in the differentiation of *R. madagascariensis* and *R. obliviosus*, also in the Early Pleistocene.

The Endemic African Clade

This clade includes a diverse set of species, classified into nine genera (*Stenonycteris*, *Plerotes*, *Myonycteris*, *Megaloglossus Hypsignathus*, *Nanonycteris*, *Epomops*, *Epomophorus*, and *Micropteropus*). According to our results, the clade has a basal split into four main lineages corresponding to *Stenonycteris, Plerotes*, Myonycterini, and Epomophorini. The relationships among these lineages were variously resolved by our sequence data, with differences across methods and a general lack of statistical support for any single branching pattern among the 4 lineages.

Plerotes and *Stenonycteris* are each monotypic; while the former inhabits dry forests and savannas, the latter is found in montane forests. *Plerotes* is known from only about a dozen specimens, and the holotype has been lost (a neotype has been designated since then, Bergmans, 1989). Examination of a recently-captured specimen from Malawi (Senckenberg Museum SMF 85.744), included in our molecular data set and so far the only adult male in collections, revealed interesting morphological features that apparently link *Plerotes* to myonycterines. Particularly, the specimen exhibits a ruff of enlarged, clustered glandular hairs much longer (> 10 mm) than the surrounding pelage and slightly paler in coloration. This ruff appears in a few distantly related megabats but it is typical of myonycterines; thus, locally it represents a potential morphological synapomorphy supporting a close relationship of *Plerotes* and myonycterines. The latter includes two genera: *Megaloglossus* and *Myonycteris*. Recently, in a comprehensive molecular analysis of this tribe, *Lissonycteris angolensis* (previously included in *Rousettus*) was included in an expanded *Myonycteris* (Nesi *et al*., 2013). In the same study, genetic discontinuity correlated with allopatric distribution prompted the erection of a new species, *Megaloglossus azagnyi*, and the recognition of *Myonycteris leptodon* Andersen, 1908 as a distinctive species, both from West Africa (Nesi *et al*., 2013).

The species of Epomophorini are characterized by conspicuous white hair patches at the base of the ears. With the exception of *Hypsignathus monstrosus*, males also possess ‘epaulets’, i.e. tufts of white hair on the shoulders that are used in display (Bergmans, 1988). *Hypsignathus monstrosus* and *Nanonycteris veldkampii* inhabit the rainforests of West and Central African, while the other epomophorine species inhabit the adjacent deciduous forests, savanna woodlands, and montane forests. Within Epomophorini, we found the only polytypic genera included in our study that were not monophyletic: *Epomophorus* and *Epomops*. The genus *Epomops* has three currently recognized species (Simmons, 2005): *E. buettikoferi*, *E. dobsonii* and *E. franqueti*. The inclusion of *dobsonii* in *Epomops*, however, has been questioned (Bergmans, 1989). Although we could not obtain tissue samples for DNA extraction of the latter species, we were able to sequence a 776 bp fragment of the 12S gene from a museum skin of *E. dobsonii*. In our analyses (Fig. 1 and 3), the *E. dobsonii* sample clustered with *Epomophorus wahlbergi* samples with high statistical support. The identification of all three samples based on morphology and measurements was double-checked upon these results and confirmed. Bergmans (1989) noticed the distinctiveness of *E. dobsonii* as compared to other ‘typical’ *Epomops*. While he suggested that *Nanonycteris veldkampii* might be its closest relative, he also observed the similarities between *E. dobsonii* and *Epomophorus* species in several characters (postdental palate concavity, palatal

ridges, and the morphology of the pterygoid bone). Our molecular phylogeny is in agreement with the latter observations, recovering *E. dobsonii* within the genus *Epomophorus*. Accordingly, we remove *dobsonii* from *Epomops* and transfer the species to *Epomophorus*. A third species of *Epomops*, *E. buettikoferi*, is missing from our analysis.

The genus *Epomophorus* was revealed to be paraphyletic not only with respect to *E. dobsonii*, but also due to inclusion of *Micropteropus pusillus* within *Epomophorus* in our trees. These results are in agreement with a lack of differentiation between *Micropteropus pusillus* and *Epomophorus gambianus* at the Dloop region of the mitochondrial DNA as reported by Nesi *et al*. (2011). Our ML and MP combined trees (but not the BI) showed each of the *Epomophorus* species as monophyletic. However, we noticed that both samples of each species have come from the same localities (Appendix I), while Nesi *et al*. (2011) had samples of *M. pusillus* and *E. gambianus* from several localities across a wide geographic range. The clade formed by the typical species of *Epomophorus*, *E. dobsonii,* and *Micropteropus* apparently diversified very recently, in the Pleistocene (Fig. 4). Within this clade, *M. pusillus*, *E. gambianus,* and *E. minor* apparently split less than 0.5 Mya. Although morphological differentiation has evolved within this group, given the recent diversification of the clade, incomplete lineage sorting may blur its phylogenetic relationships. Another possible explanation for the observed results is the introgression of mitochondrial DNA from an *Epomophorus* species into *M. pusillus* following hybridization (Nesi *et al*., 2011). A better sampling of rapidly evolving nuclear loci will be necessary to distinguish between the two hypotheses and decide whether *Micropteropus* should be synonymized with *Epomophorus*.

The genus *Epomophorus* has five additional species that could not be included in the present analysis: *E. angolensis*, *E. crypturus*, *E. grandis*, *E. labiatus*, *E. minimus*, and the recently described *E. anselli* (Bergmans and van Strien, 2004; Simmons, 2005). *Epomophorous grandis* was originally described as a *Micropteropus* but was transferred to *Epomophorus* by Bergmans (1988). The other species have been linked to *E. gambianus*, although the validity of some of them (e.g., *E. crypturus*) as distinct species has been a matter of controversy (Bergmans, 1988; Boulay and Robbins, 1989; Claessen and De Vree, 1991). Also missing from our analysis is *Micropteropus intermedius*, another reason why the possible synonymization of *Micropteropus* with *Epomophorus* given their molecular affinities (see above) seems premature.

Classification

The phylogenetic trees obtained here challenge the subfamilial classification proposed by Bergmans (1997). Neither Epomophorinae nor Rousettinae sensu Bergmans (1997) are monophyletic, and Bergmans' (1997) treatment of these taxa therefore needs to be replaced by a new classification that takes into account our new understanding of phylogenetic relationships (Table 1, Fig. 5, see Appendix II — Table S2 for a species level classification of Subfamily Rousettinae). We propose retaining the name Rousettinae but revising its membership to include the entire clade composed of Scotonycterini

TABLE. 1. Bergmans' (1997) classification (left) and a new classification (right) based on the phylogenetic results presented here for the African megabats and their closely related allies

Bergmans (1997)	New classification
Harpyionycterinae	Harpyionycterinae
Dobsonia, *Aproteles*, *Harpyionycteris*	*Dobsonia*, *Aproteles*, *Boneia*, *Harpyionycteris*
Rousettinae	Rousettinae
Dobsoniini – *Dobsonia*, *Aproteles*	Rousettini – *Rousettus*
Rousettini – *Eidolon*, *Eonycteris*, *Rousettus*[1]	Eonycterini – *Eonycteris*
Epomophorinae	Scotonycterini – *Scotonycteris*, *Casinycteris*
Scotonycterini – Scotonycteris, *Casinycteris*	Epomophorini – *Epomophorus*, *Epomops*, *Hypsignathus*, *Nanonycteris*, *Micropteropus*
Epomophorini – *Epomophorus*, *Epomops*, *Hypsignathus*, *Nanonycteris*, *Micropteropus*	Stenonycterini – *Stenonycteris*
Myonycterini – *Lissonycteris*, *Myonycteris*, *Megaloglossus*	Myonycterini – *Myonycteris*[2], *Megaloglossus*
Plerotini – *Plerotes*	Plerotini – *Plerotes*
	Eidolinae
	Eidolon

[1] — inclusive of *Boneia* and *Stenonycteris*; [2] — inclusive of *Lissonycteris*

(including *Scotonycteris* and *Casinycteris*), *Eonycteris*, *Rousettus*, Myonycterini (*Megaloglossus* and *Myonycteris*), Plerotini (*Plerotes*), Stenonycterini (*Stenonycteris*), and Epomophorini (*Epomophorus*, *Epomops*, *Nanonycteris*, *Hypsignathus*, and *Micropteropus*). This clade has received high statistical support both in the analyses described in this contribution and prior family-level phylogenetic analyses published by our group (Almeida *et al*., 2011). The genera *Dobsonia* and *Aproteles* had been previously excluded from Rousettinae and placed in Harpyionycterinae together with *Boneia* and *Harpyionycteris* (Giannini *et al*., 2006, 2009). The genus *Eidolon*, also placed in Rousettinae by Bergmans (1997), is explicitly excluded from this subfamily in our new classification. Because *Eidolon* did not show close relationships to any other pteropodid genus in Almeida *et al*. (2011), we propose the erection of a new subfamily of its own, Eidolinae.

Eidolinae, New Subfamily

Type genus: *Eidolon* Rafinesque, 1815

Contents

Presently known to contain one genus and two species: *Eidolon helvum* (Kerr, 1792) and *Eidolon dupreanum* (Pollen, 1866).

Diagnosis

A large pteropodid (FA 105-135) with a dental formula of i2/2, c1/1, p3/3, m2/3 = 34. Length of rostrum much greater than width across lacrimals; anterior rim of orbit located above upper first molar; palate much broader posteriorly than between canines; gap present anteriorly between right and left premaxillae; basicranial axis moderately deflected in relation to palate; tympanic elongated to form a short, bony auditory meatus; occiput not elongated and tubular; first upper premolar much larger in cross section than upper incisors; first lower molar equal in length to length of second and third molars combined; claw present on wing digit II; short external tail present; pelage sexually dimorphic with males possessing a neck tuft and females often conspicuously larger and paler than males.

Also based on results shown herein, we propose the erection of two new exclusive tribes for the genera *Eonycteris* and *Rousettus*: Eonycterini and Rousettini respectively (Fig. 5). In this manner, the tribal classification for pteropodids will include exclusively monophyletic groups.

Biogeography

The DIVA analysis produced results in accordance with an Australasian (including the Pacific) origin of Pteropodidae as previously proposed (Butler, 1984; Aguilar *et al*., 1986; Hollar and Springer, 1997; Juste B. *et al*., 1999; Almeida *et al*., 2011) and indicated at least 4 independent colonization events of Africa, represented by the lineages comprising (1) *Eidolon*, (2) African *Rousettus*, (3) Scotonycterini, and (4) the endemic African clade including *Plerotes* (Fig. 5). This result is in accordance with and adds to Juste B. *et al*.'s (1999) hypothesis of at least three independent colonizations of Africa; the fourth colonization we inferred is due to the inclusion of scotonycterines, which were missing from their study. Our divergence time estimates suggest that these colonization events happened at different times (Fig. 4). The Scotonycterini and the endemic African clade were apparently present in Africa by the Late Miocene, when they started to diversify locally. The time of their arrival in Africa, however, is not so clear. Scotonycterini apparently split from its sister group in the Early Miocene, and the endemic African clade appeared during the Middle Miocene. Whether at the time of the split their ancestors were in Africa or elsewhere (and these clades reached Africa after cladogenesis) cannot be determined, although all their extant diversity is in Africa. Scotonycterini and members of the endemic African clade are mostly forest/woodland dwellers. If their migration route were through the Middle East, it would be more likely that it took place before increases in aridity and decreases in forest cover that occurred at end of the Miocene (Zachos *et al*., 2001; Bonnefille, 2010). A land bridge connecting Africa/Arabia and Eurasia, the Gomphotherium landbridge, was apparently present in this region since the Early Miocene (Harzhauser *et al*., 2007).

A migration route involving island-hopping through the Indian Ocean, as has been proposed in the case of Malagasy *Pteropus* (O'Brien *et al*., 2009), seems less realistic for most African pteropodids given the smaller sizes of the scotonycterines and the epomophorines and the distances involved (several hundred kilometers separate some consecutive islands), although their ancestors could have been larger and had different flight capabilities. Currently, with the exception of *Myonycteris brachycephala* from São Tomé and Príncipe, epomophorine species are not found on islands off the coast of Africa. Although an early African colonization is likely in these clades, rapid diversification during the

Pliocene and Pleistocene apparently produced most of the extant diversity. This period of rapid diversification appears to be correlated with climatic oscillations during these periods (Bonnefille, 2010), similar to those associated with the Northern Hemisphere glaciations. This relatively recent diversification had been previously noticed in Myonycterini and Scotonycterini (Nesi *et al*., 2013; Hassanin, 2014); here we extend the observation to Epomophorini. In particular, the genus *Epomophorus* + *Micropteropus* apparently diversified into 12 species in the last 2.5 million years – a remarkable radiation.

The African *Rousettus* clade originated in the late Pliocene or early Pleistocene and diversified during the Pleistocene (Fig. 4). The phylogenetic arrangement recovered herein could have resulted from an initial split between *R. leschenaultii* and the African species' ancestor somewhere in Central Asia, followed by the colonization of mainland Africa by a continental route through the Middle East, and finally the origin of the island forms by island hopping from continental Africa through Comoros and into Madagascar (Goodman *et al*., 2010). However, our topology is not totally incompatible with an oceanic, island-hopping route from South Asia. Interestingly, colonization of Africa by the *Rousettus* clade occurred during the same time period as the independent colonization of the western Indian Ocean islands from Asia by *Pteropus* species, which occurred in at least three separate events (O'Brien *et al*., 2009; Almeida *et al*., 2014). The ancestor of African *Rousettus* might have used a similar route, especially if environmental conditions at that time were favorable for island hopping, oceanic dispersal through the Indian Ocean. However, as already mentioned, *Rousettus* species are not as large as some *Pteropus* species, which makes oceanic migrations less likely in *Rousettus*.

The continental *R. aegyptiacus* has a widespread distribution from South Africa to Turkey and from Guinea to western Pakistan (Bergmans, 1994). Its current distribution in the Middle East could be recent and due to anthropogenically facilitated colonization from African stocks (Benda *et al*., 2012). Apparently, the main required condition for the presence of this generalist species is a constant, year-around fruit availability (Benda *et al*., 2012). Due to anthropogenic fruit cultivation, this condition is met in most areas of the Middle East where the species now occurs. A recent colonization scenario for the Middle East is in agreement with low levels of population genetic differentiation in the region (Benda *et al*., 2012). Besides *R. aegyptiacus*, *E. helvum* and *E. labiatus* also inhabit the Arabic peninsula, but they are restricted to its Afro-tropical region, surrounding the Mandeb strait.

The time of the arrival of the genus *Eidolon* in Africa is less clear. This genus appears to have split from its sister clade early in pteropodid history (Almeida *et al*., 2011), which would place its origin in the Miocene or Oligocene. *Eidolon* includes two species: *E. dupreanum* in Madagascar and *E. helvum* in continental Africa. *Eidolon helvum* has a widespread distribution in sub-Saharan Africa, reaching the Red Sea coast of Yemen and Saudi Arabia, and is a strong flier (Nowak, 1994). *Eidolon helvum* colonies are known to make massive seasonal migration, covering large distances (Thomas, 1983). The low level of differentiation in *E. helvum* and *R. aegyptiacus* over a large geographic range suggest ongoing gene flow between distant local populations of these species (Benda *et al*., 2012; Peel *et al*., 2013; Shi *et al*., 2014).

Implications for Zoonotic Diseases

African fruit bats have recently been in the news for their implication as most likely natural reservoir of the Ebola Zaire virus. The Ebola virus is part of the Filoviridae, which also includes the Marburg viruses; these are among the deadliest human pathogens (Wynne and Wang, 2013). Extrapolation of our phylogenetic results strongly suggest horizontal transfer of filoviruses since signs of infection have been found in species that are not closely related such as *E. helvum* and *R. aegyptiacus*. Most Ebola outbreaks in Africa have occurred in equatorial forests (one exceptional outbreak happened in South Sudan; Gatherer, 2014). Nevertheless, some of the bats implicated as Ebola Zaire reservoirs, such as *E. helvum* and *R. aegyptiacus*, are also found in areas outside the limits of African rainforests. This points to a potential risk of these bats spreading the disease out of Equatorial Africa — assuming that they are actually the reservoirs for Ebola. Moreover, several species of African fruit bats undergo annual migrations to savanna areas in the wet season when fruits are available in abundance (Thomas, 1983; Richter and Cumming, 2008). Serological studies have shown a low prevalence of viruses in bats; studies using large samples showed that less than 10% have detectable virus or antibodies (e.g., Leroy *et al*., 2005; Pourrut *et al*., 2009; Olival *et al*., 2013). It is possible that outbreaks correlate with periods of increased prevalence/virulence due to ecological factors such as food scarcity and pregnancy (Leroy

et al., 2005). Moreover, food scarcity may increase the contact between different animals species and even the human ingestion of bats (Leroy *et al*., 2005, 2014). Another important factor is the seasonal migration of fruit bats or stochastic dispersal, which also increase bat consumption by people in some areas (Leroy *et al*., 2009). These factors need to be taken into consideration in feature policies to control Ebola and other viral diseases outbreaks in Africa and elsewhere.

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Appendix I

African and related taxa samples used in this study, with their voucher numbers, source, locality, and bibliographic reference in the case of previously published sequences. Numbers after species names are references for the terminals in the illustrated phylogenies (Figs. 1–3 and Supplementary Figs. S1–S11)

Species	Voucher	Source[a]	Locality	Reference
Casinycteris argynnis	269915	AMNH	Central Africa Republic, Dzanga Sangha Forest Reserve	Almeida *et al.* (2011)
Casinycteris campomaanensis	2011-637	MNHN	Cameroon, Campo Ma'an area, Village of Nkoe´lon-Mvini	Hassanin (2014)
Eidolon helvum	102021	CMNH	Kenya, Western Province, Kakamega Dist	Giannini *et al.* (2008, 2009)
Eonycteris major	TK152179	?	Malaysia, Sarawak, Kubah National Park	unpublished
Eonycteris robusta	178362	FMNH	Philippines, Luzon, Mt Banahaw, Barangay Lalo	Almeida *et al.* (2011)
***Eonycteris spelaea* 1**	92353	CMNH	India, Andhra Pradesh, Boora Cave,	This study
***Eonycteris spelaea* 2**	176480	MVZ	China, Yunnan Prov., Yunnan Institute of Tropical Botany	This study
Eonycteris spelaea 3	176487	MVZ	China, Yunnan Prov., Yunnan Institute of Tropical Botany	Almeida *et al.* (2011)
***Epomophorus gambianus* 1**	113550	CMNH	Ghana, Northern Region	This study
***Epomophorus gambianus* 2**	113553	CMNH	Ghana, Northern Region	This study
***Epomophorus minor* 1**	102026	CMNH	Kenya, Rift Valey Prov., West Pokot Dist., Sigor	This study
***Epomophorus minor* 2**	102027	CMNH	Kenya, Rift Valey Prov., West Pokot Dist., Sigor	This study
Epomophorus wahlbergi 1	177209	FMNH	Mozambique, Zambezia, Murabue	Almeida *et al.* (2011)
***Epomophorus wahlbergi* 2**	177089	FMNH	Mozambique, Zambezia, Murabue	This study
Epomops dobsonii	115821	AMNH	Zambia, Balovale	This study
Epomops franqueti 1	269902	AMNH	Central Africa Republic, Dzanga Sangha Forest Reserve	Almeida *et al.* (2011)
***Epomops franqueti* 2**	268356	AMNH	Central Africa Republic, Dzanga Sangha Forest Reserve	This study
Hypsignathus monstrosus	116499	AMCC	?	Almeida *et al.* (2011)
Megaloglossus azagnyi 1	2011-1001	MNHN	Ivory Coast, Gboyo village	Nesi *et al* (2013)
Megaloglossus azagnyi 2	G09175	n.a.	Liberia, Gangra	Nesi *et al.* (2013)
Megaloglossus woermanni 1	268358	AMNH	Central Africa Republic, Dzanga Sangha Forest Reserve	Almeida *et al*. (2011)
***Megaloglossus woermanni* 2**	268360	AMNH	Central Africa Republic, Dzanga Sangha Forest Reserve	This study
Micropteropus pusillus	113563	CMNH	Ghana, Northern Region	Almeida *et al* (2011)
Myonycteris angolensis 1	102135	CMNH	Kenya, Western Province, Kakamega Dist	Almeida *et al* (2011)
***Myonycteris angolensis* 2**	102136	CMNH	Kenya, Western Province, Kakamega Dist	This study
Myonycteris brachycephala 1	104	ST	São Tomé, Monte Belo	Nesi *et al*. (2013)
Myonycteris brachycephala 2	105	ST	São Tomé, Monte Belo	Nesi *et al*, (2013)
Myonycteris brachycephala 2	?	?	?	Juste *et al*. (1999)
Myonycteris relicta	171299	FMNH	Tanzania, Mts Usumbara	Nesi *et al*. (2013)
Myonycteris relicta	?	?	?	Juste *et al*. (1999)
Myonycteris leptodon 1	G09196	n.a.	Cote d'Ivoire, Besso	Nesi *et al*. (2013)
Myonycteris leptodon 2	G09189	n.a.	Liberia, East Nimba	Nesi *et al*. (2013)
Myonycteris torquata 1	268362	AMNH	Central Africa Republic, Dzanga Sangha Forest Reserve	Almeida *et al*. (2011)
***Myonycteris torquata* 2**	268363	AMNH	Central Africa Republic, Dzanga Sangha Forest Reserve	This study
Nanonycteris veldkampii 1	F34376	ROM	Ivory Coast, Parc National de Mont Peko	Almeida *et al*. (2011)
***Nanonycteris veldkampii* 2**	F34377	ROM	Ivory Coast, Parc National de Mont Peko	This study
***Plerotes anchietae* 1**	85744	SMF	Malawi, 1700 m altitude	This study
***Plerotes anchietae* 2**	85745	SMF	Malawi, 1700 m altitude	This study

APPENDIX I. Continued

Species	Voucher	Source[a]	Locality	Reference
***Rousettus* sp. 1**	274231	AMNH	Vietnam, Ha Giang, Mount Tay Con Linh 2	This study
***Rousettus* sp. 2**	CMF980124	ROM	Laos, Khammouane, 2 km N of Ban Mouangkai	This study
Rousettus amplexicaudatus	23045/6	AM	Papua New Guinea	Teeling *et al*. (2002)
***Rousettus aegyptiacus* 1**	177211	FMNH	Mozambique, Zambezia, Murabu	This study
Rousettus aegyptiacus 2	?	?	?	unpublished
Rousettus leschenaultii 1	176490	MVZ	China, Yunnan Prov., 1 km E Menglung	Almeida *et al*. (2011)
Rousettus leschenaultii 2	A8	?	China, Yunnan	Li *et al*. (2007)
Rousettus leschenaultii 2	?	?	?	unpublished
***Rousettus madagascariensis* 1**	448922	NMNH	Madagascar	This study
Rousettus madagascariensis 2	449206	NMNH	Madagascar	Almeida *et al*. (2011)
Rousettus obliviosus 1	194543	FMNH	Comoros Is.	Goodman *et al*. (2010)
Rousettus obliviosus 2	194459	FMNH	Comoros Is.	Goodman *et al*. (2010)
Rousettus spinalatus 1	16	?	Malaysia, Liam Village, Sarawak	Guan *et al*. (2006)
Rousettus spinalatus 2	SNP41	?	Malaysia, Similajau N.P., Sarawak	Guan *et al*. (2006)
Scotonycteris ophiodon	256534	AMNH	Liberia, Gran Gedeh, Dugbe River	This study
Scotonycteris ophiodon	50001	ZMB	Cameroon, Bipindi	Hassanin, (2014)
Scotonycteris zenkeri 1	107997	CMNH	Cameroon, Southwest Prov., Bake River Bridge	Almeida *et al*. (2011)
***Scotonycteris zenkeri* 2**	107999	CMNH	Cameroon, Southwest Prov., Korup Natl Park	This study
Stenonycteris lanosus 1	102148	CMNH	Kenya, Western Province, Kakamega Dist	Almeida *et al*. (2011)
***Stenonycteris lanosus* 2**	102149	CMNH	Kenya, Western Province, Kakamega Dist	This study

[a] — AMNH: American Museum of Natural History, MNHN: Muséum National d'Histoire Naturelle, CMNH: Carnegie Museum of Natural History, FMNH: Field Museum of Natural History, MVZ: Museum of Vertebrate Zoology, AMCC: Ambrose Monell Cryo Collection, ST: Collection of Pr. Javier Juste Ballesta, Estación Biológica de Doñana, ROM: Royal Ontario Museum, SMF: Senckenberg Forschungsinstitut und Naturmuseum, AM: Australian Museum, NMNH: National Museum of Natural History, ZMB: Zoologisches Museum Berlin, n.a.: sample was not deposited in a museum collection

Appendix II

Supplementary material

Text. *Rousettus* specimen identification problem

A specimen collected in Vietnam that was originally identified as *R. leschenaultii* clustered with the *R. amplexicaudatus* sample (from Papua New Guinea), instead of clustering with our other *R. leschenaultii* sample from Yunnan, China. The identification was based on size and the shape of the last molar, since the two species are morphologically very similar (Lunde *et al*., 2003). When we analyzed the BRCA1 gene, we included a short sequence obtained from another specimen identified as *R. leschenaultii*, collected in Laos and archived at the Royal Ontario Museum collection (Appendix II — Fig. S4). This sequence clustered with that of the Vietnamese sample. This result prompted us to download all available 12S rRNA sequences of *Rousettus* from GenBank to try to solve this problem (Appendix II — Fig. S11). Long branches separating the different samples suggest high genetic variation within *R. amplexicaudatus* and point out to the need of further studies of what appears to be a species complex. Our analyses suggest that the specimen from Vietnam (and the one from Laos, consequently) may represent an undescribed species closely related to *R. amplexicaudatus*. We detected a clear genetic separation between *R. leschenaultii* and *R. amplexicaudatus*, despite the lack of an evident morphological distinctiveness.

Table S1. Genbank accession numbers of the samples analyzed in this study. Shaded cells represent non-available sequences

Species	Cyt*b*	12S[a]	16S	RAG1	RAG2	vWF	BRCA1
Casinycteris argynnis	JN398197	JN398168	JN398168	JN398284	JN398301	JN398268	JN398264
C. campomaanensis	KJ145797						
Eidolon dupreanum		U93058	AF293648				
E. helvum	JN398200	JN398171	JN398171	UE617950	UE617900	UE617931	JN398240
Eonycteris major	EU521600						
E. robusta	JN398201	JN398172	JN398172	JN398286	JN398303	JN398270	JN398251
E. spelaea 1	KT875801	KT875890	KT875890	KT875846	KT875829	KT875863	
E. spelaea 2	KT875794	KT875891	KT875891	KT875839	KT875822	KT875857	KT875813
E. spelaea 3	FJ218482	JN398173	JN398173	EU617951	EU617901	DQ445684	JN398254
Epomophorus gambianus 1	KT875802			KT875847	KT875830	KT875864	KT875816
E. gambianus 2	KT875803	KT875878	KT875878	KT875848	KT875831	KT875865	
E. minor 1	KT875804	KT875879	KT875879	KT875849	KT875832	KT875866	KT875817
E. minor 2	KT875805	KT875880	KT875880	KT875850	KT875833	KT875867	
E. wahlbergi 1	DQ445706	JN398174	JN398174	UE617953	EU617903	DQ445691	
E. wahlbergi 2	KT875797	KT875875	KT875875	KT875842	KT875825		JN398266
Epomops dobsonii		KT875873					
E. franqueti 1	JN398202	JN398175	JN398175	JN398287	JN398304	JN398271	JN398233
E. franqueti 2	KT875798	KT875876	KT875876	KT875843	KT875826	KT875860	
Hypsignathus monstrosus	JN398204	JN398176	JN398176	JN398289	JN398305	JN398272	JN398235
Megaloglossus azagnyi 1	JX283275						
M. azagnyi 2	JX283279						
M. woermanni 1	DQ445710	JN398180	JN398180	EU617956	EU617906	DQ445702	JN398231
M. woermanni 2	KT875799	KT875877	KT875877	KT875844	KT875827	KT875861	
Micropteropus pusillus	JN398208	JN398183	JN398183	JN398292	JN398308	JN398275	JN398241
Myonycteris angolensis 1	JN398205	JN398177	JN398177	JN398290	JN398306	JN398273	JN398243
M. angolensis 2	KT875806	KT875881	KT875881	KT875851	KT875834	KT875868	
M. brachycephala 1	JX283222						
M. brachycephala 2	JX283221		AF044613				
M. relicta	JX283220		AF044618				
M. leptodon 1	JX283217						
M. leptodon 2	JX283215						
M. torquata 1	FJ218483	JN398184	JN398184	EU617958	EU617908	DQ445700	JN398232
M. torquata 2	KT875795	KT875874	KT875874	KT875840	KT875823	KT875858	
Nanonycteris veldkampii 1	JN398209	JN398185	JN398185	JN398293	JN398309	JN398276	JN398253
N. veldkampii 2	KT875809	KT875883	KT875883	KT875854	KT875837	KT875871	KT875818

Appendix II. Continued

Table 1. Continued

Species	Cyt*b*	12S[a]	16S	RAG1	RAG2	vWF	BRCA1
Plerotes anchietae 1	KT875811	KT875884					
P. anchietae 2	KT875812	KT875885	KT875885	KT875856			
Rousettus sp. 1	KT875796	KT875887	KT875887	KT875841	KT875824	KT875859	KT875814
R. sp. 2							
R. amplexicaudatus			AF203742	AF447512	AF447529	AF447547	AF447500
R. aegyptiacus 1	KT875800	KT875886	KT875886	KT875845	KT875828	KT875862	KT875815
R. aegyptiacus 2	AB205183	AB205183	AB205183				
R. leschenaultii 1	JN398218	JN398190	JN398190	JN398300	JN398313	JN398283	JN398219
R. leschenaultii 2	DQ888669	AB293940					
R. madagascariensis 1	KT875810			KT875855	KT875838	KT875872	
R. madagascariensis 2	JN398214	JN398191	JN398191	JN398296	JN398311	JN398279	JN398224
R. obliviosus 1	GU228752						
R. obliviosus 2	GU228766						
R. spinalatus 1	EF105522	EF139882					
R. spinalatus 2	EF105524	EF139884					
Scotonycteris ophiodon		KT875889					
S. ophiodon	KJ145798						
S. zenkeri 1	JN398216	JN398192	JN398192	JN398297	JN398312	JN398280	
S. zenkeri 2	KT875808	KT875882	KT875882	KT875853	KT875836	KT875870	JN398265
Stenonycteris lanosus 1	JN398215	JN398193	JN398193	JN398298	JN400925	JN398281	
S. lanosus 2	KT875807	KT875888	KT875888	KT875852	KT875835	KT875869	JN398244
Desmalopex leucopterus	JN398198	JN398169	JN398169	UE617966	UE617915	UE617929	JN398252
Pteropus medius	JN398211	JN398314	AY011170d	EU617964	EU617913	EU617935	JN398242
Nyctimene albiventer	DQ314264	U61077	AF293640	AF447514	AF447531	AF447549	AF447502
Cynopterus sphinx	DQ445703	GQ410302	GQ410336	UE617947	UE617897	DQ445697	KT875821
Haplonycteris fischeri	GQ410225	GQ410319	GQ410342	GQ410270	GQ410247	GQ410293	JN398245
Syconycteris australis	FJ218479	JN398195	JN398195	FJ218466	FJ218462	FJ218470	JN398247
Harpyionycteris whiteheadi	DQ445708	FJ218474	FJ218474	EU617954	EU617904	DQ445690	JN398246
Dobsonia moluccensis	FJ218484	FJ218472	JN398196	EU617949	EU617899	EU617930	JN398220
Hipposideros vittatus		AY395856	AY395856	AF203760	AF203770	AF203778	AF203752
Megaderma lyra	DQ888678	AF069538	AF069538	AF203757	AF203767	U31616	AF203749

[a] — For most individual samples the sequences of 12S rRNA, tRNA-val, and 16S rRNA were deposited in the GenBank in a single string, under the same accession number. When otherwise, tRNA-val (~70 bp) may or may not be available and was deposited together with either ribosomal RNA gene sequences

Appendix II. Continued

Table S2. New classification of Subfamily Rousettinae at species level. Changes since the last mammal list (Simmons, 2005) are underlined

Subfamily Rousettinae Andersen, 1912
- **Tribe Rousettini** Andersen, 1912
  - Genus *Rousettus* Gray, 1921
    - *R. amplexicaudatus* Geoffroy, 1810
    - *R. leschenaultii* Desmarest, 1820
    - *R. spinalatus* Bergmans and Hill, 1980
    - *R. madagascariensis* Grandidier, 1928
    - *R. obliviosus* Kock, 1928
    - *R. aegyptiacus* Geoffroy, 1810
    - *R. linduensis*[a] Maryanto and Yani, 2003
    - *R. celebensis*[a] Andersen, 1907
    - *Rousettus* sp. (?)[b]
- **Tribe Eonycterini** Almeida, Giannini and Simmons, 2015 new tribe
  - Genus *Eonycteris* Dobson, 1873
    - *E. spelaea* Dobson, 1871
    - *E. major* Andersen, 1910
    - *E. robusta* Miller, 1913
- **Tribe Scotonycterini** Bergmans, 1997
  - Genus *Scotonycteris* Matschie, 1894
    - *S. zenkeri* Matschie, 1894
    - *S. occidentalis* Hassanin *et al*., 2015
    - *S. bergmansi* Hassanin *et al*., 2015
  - Genus *Casinycteris* Thomas, 1910
    - *C. argynnis* Thomas, 1910
    - *C. ophiodon*[c] (Pohle, 1943)
    - *C. campomaanensis* Hassanin, 2014
- **Tribe Epomophorini** Gray 1866
  - Genus *Nanonycteris* Matschie, 1899
    - *N. veldkampii* Jentink, 1888
  - Genus *Hypsignathus* Allen, 1861
    - *H. monstrosus* Allen, 1861
  - Genus *Epomops* Gray, 1870
    - *E. franqueti* Tomes, 1860
    - *E. buettikoferi*[a] Matschie, 1899
  - Genus *Epomophorus* Bennett, 1835
    - *E. gambianus* Ogilby, 1835
    - *E. minor* Dobson, 1879
    - *E. wahlbergi* Sandevall, 1846
    - *E. dobsonii* (Bocage, 1889)[d]
    - *E. pusillus* (Peters, 1867)[e]
    - *E. angolensis*[a] Gray, 1870
    - *E. crypturus*[a] Peters, 1852
    - *E. grandis*[a] Sanborn, 1950
    - *E. labiatus*[a] Temminck, 1837
    - *E. minimus*[a] Claessen and De Vree, 1991
    - *E. anselli*[a] Bergmans and van Strien, 2004
  - Genus *Micropteropus*[f] Matschie, 1899
    - *M. intermedius*[a] Hayman, 1963
- **Tribe Stenonycterini** Nesi *et al.*, 2012
  - Genus *Stenonycteris* Thomas, 1906
    - *S. lanosus* Thomas, 1906
- **Tribe Myonycterini** Lawrence and Novick, 1963
  - Genus *Myonycteris* Matschie, 1899
    - *M. torquata* Dobspn, 1878
    - *M. leptodon*[g] Andersen, 1908
    - *M. brachycephala* Bocage, 1889
    - *M. relicta* Bergmans, 1980
    - *M. angolensis* Bocage, 1889
  - Genus *Megaloglossus* Pagenstecher, 1885
    - *M. woermanni* Pagenstecher, 1885
    - *M. azagnyi* Nesi, Kadjo, and Hassanin, 2012
- **Tribe Plerotini** Bergmans, 1997
  - Genus *Plerotes* Andersen, 1910
    - *P. anchietae* Seabra, 1900

[a] — No molecular data is available for these species and their tentative classifications are based on results of previous work using morphological traits for interspecies comparisons;

[b] — Phylogenetic results shown herein suggest there might a cryptic species of *Rousettus* in Southeast Asia, related to *R. amplexicaudatus*. Further work is necessary to confirm this hypothesis;

[c] — Described as *Scotonycteris ophiodon*, transferred t Casinycteris by Hassanin (2014);

[d] — Described as *Epomops dobsonii*, new combination;

[e] — Described as *Micropteropus pusillus*, new combination;

[f] — Our analyses recovered *M. pusillus* deeply nested within *Epomophorus* (sister to *E. gambianus* and *E. minor*), contesting the validity of the genus *Micropteropus*. Rearrangements involving *Micropteropus* and *Epomophorus* species are pending due to missing data for *M. intermedius* and 6 *Epomophorus* species;

[g] — Considered synonymous to *M. torquata* by Bergmans (1997), revalidate by Nesi *et al*. (2013)

Appendix II. Continued

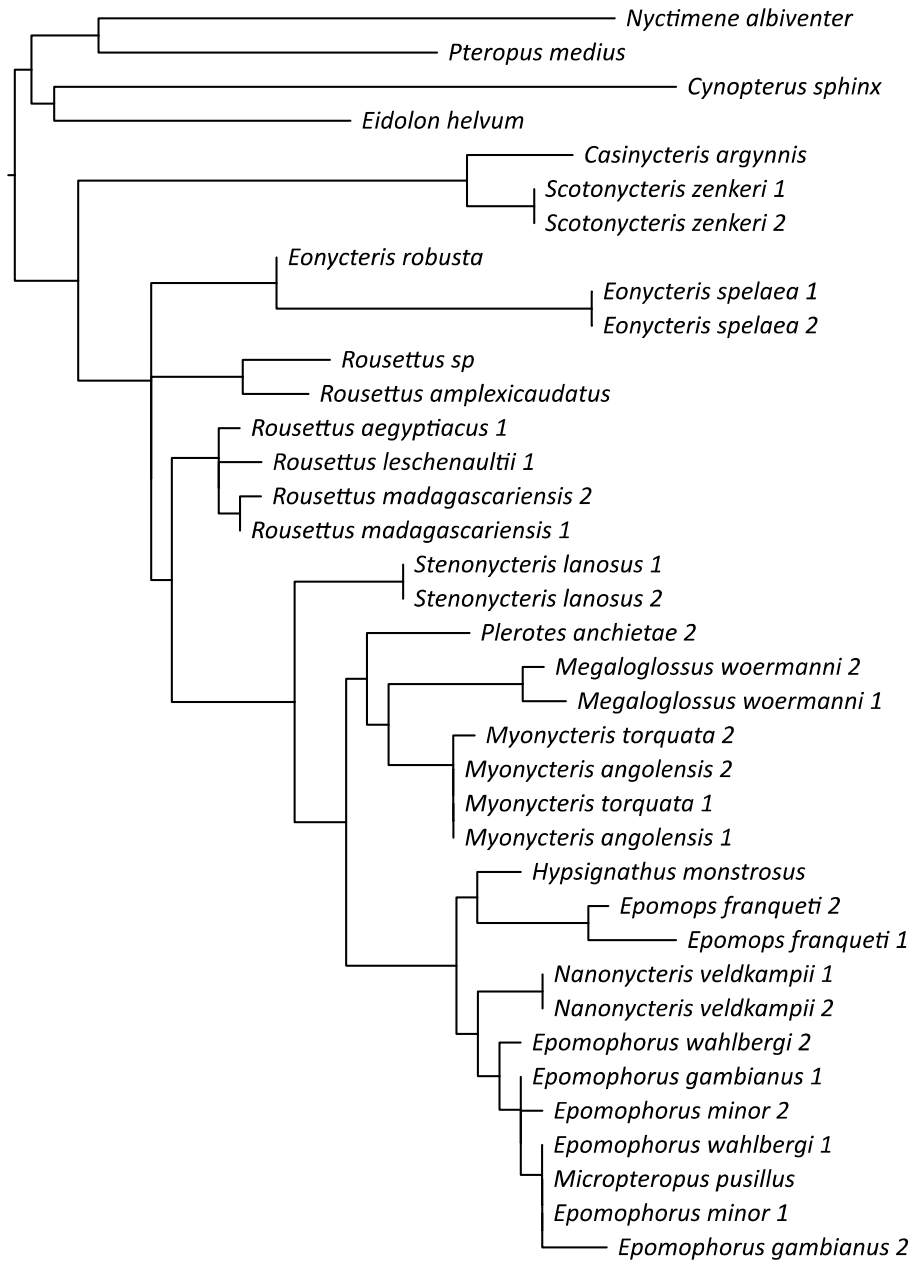

Fig. S1. Maximum likelihood tree obtained with the RAG1 gene

Appendix II. Continued

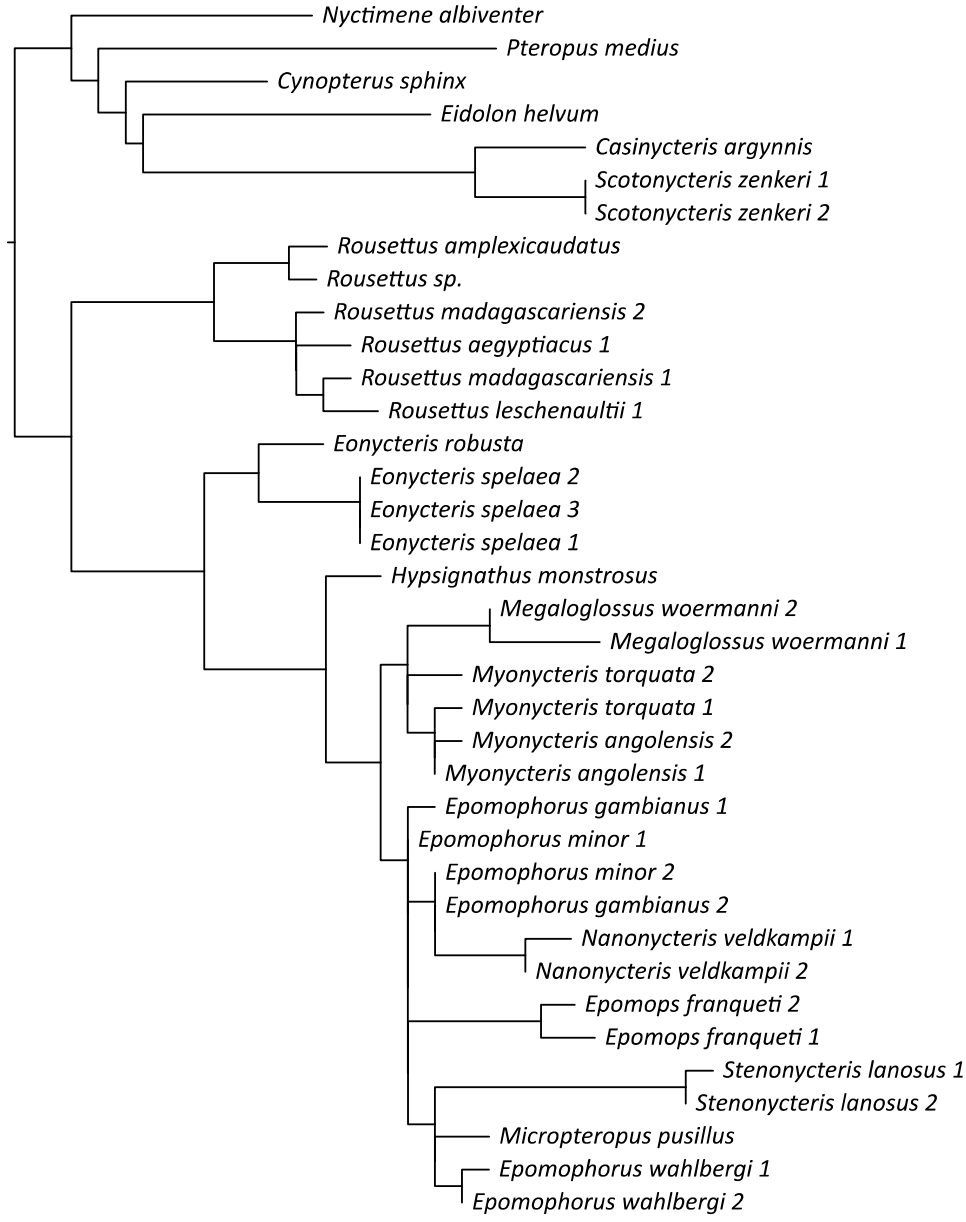

Fig. S2. Maximum likelihood tree obtained with the RAG2 gene

Appendix II. Continued

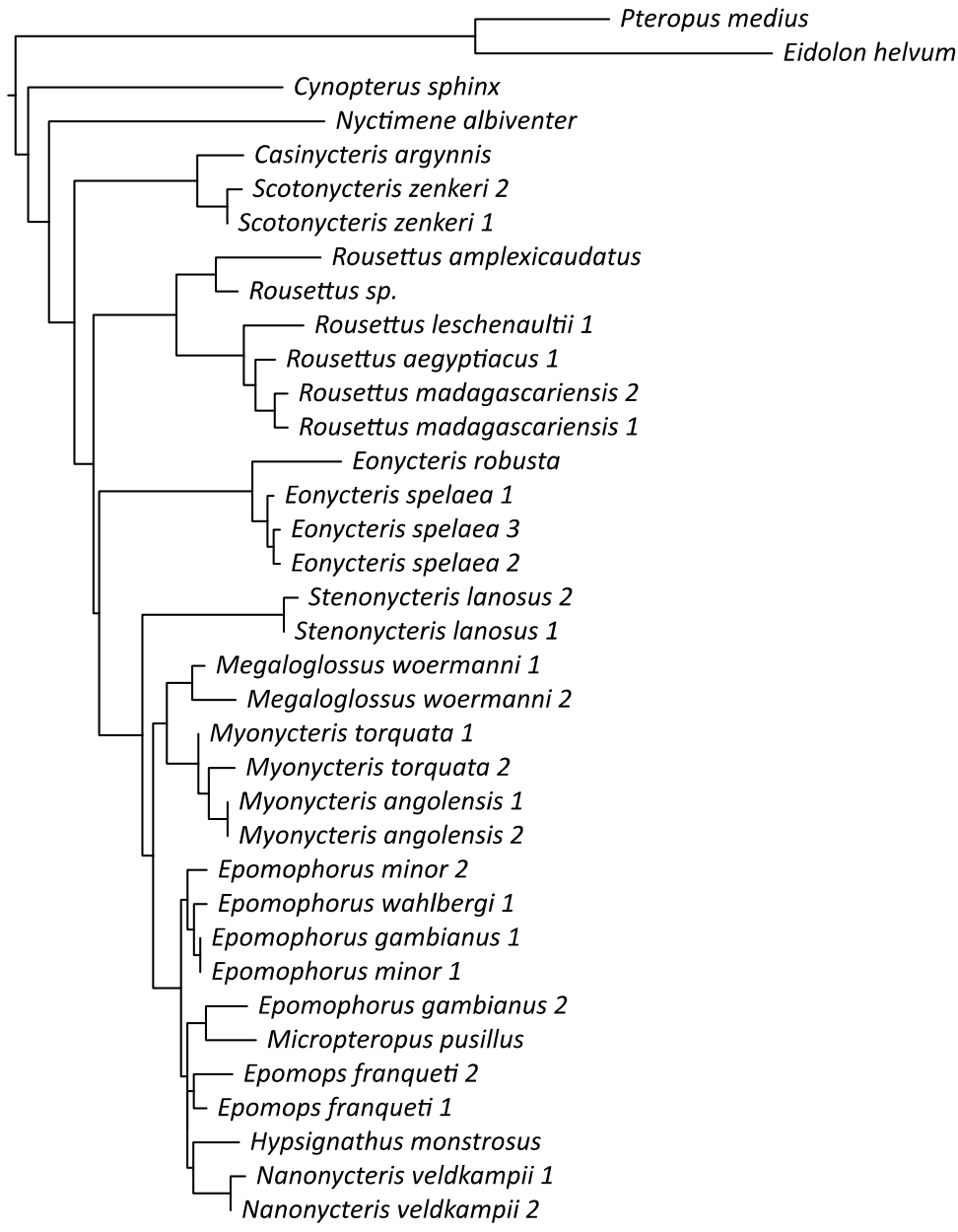

Fig. S3. Maximum likelihood tree obtained with the vWF gene

Appendix II. Continued

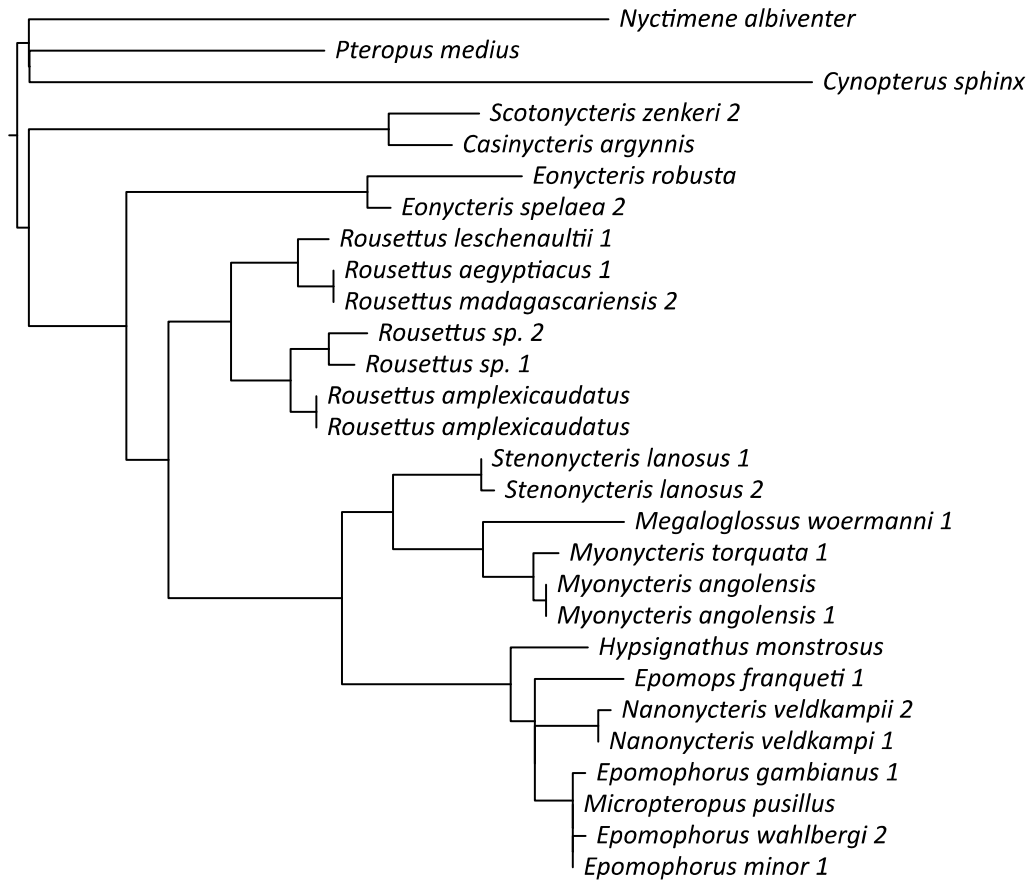

Fig. S4. Maximum likelihood tree obtained with the BRCA1 gene. A *Rousettus amplexicaudatus* sequence from Genbank (accession number AY057829) and a *Myonycteris angolensis* unpublished sequence (voucher number 177097, housed at the Field Museum of Natural History) were included in this analysis, but not in the combined analyses

Appendix II. Continued

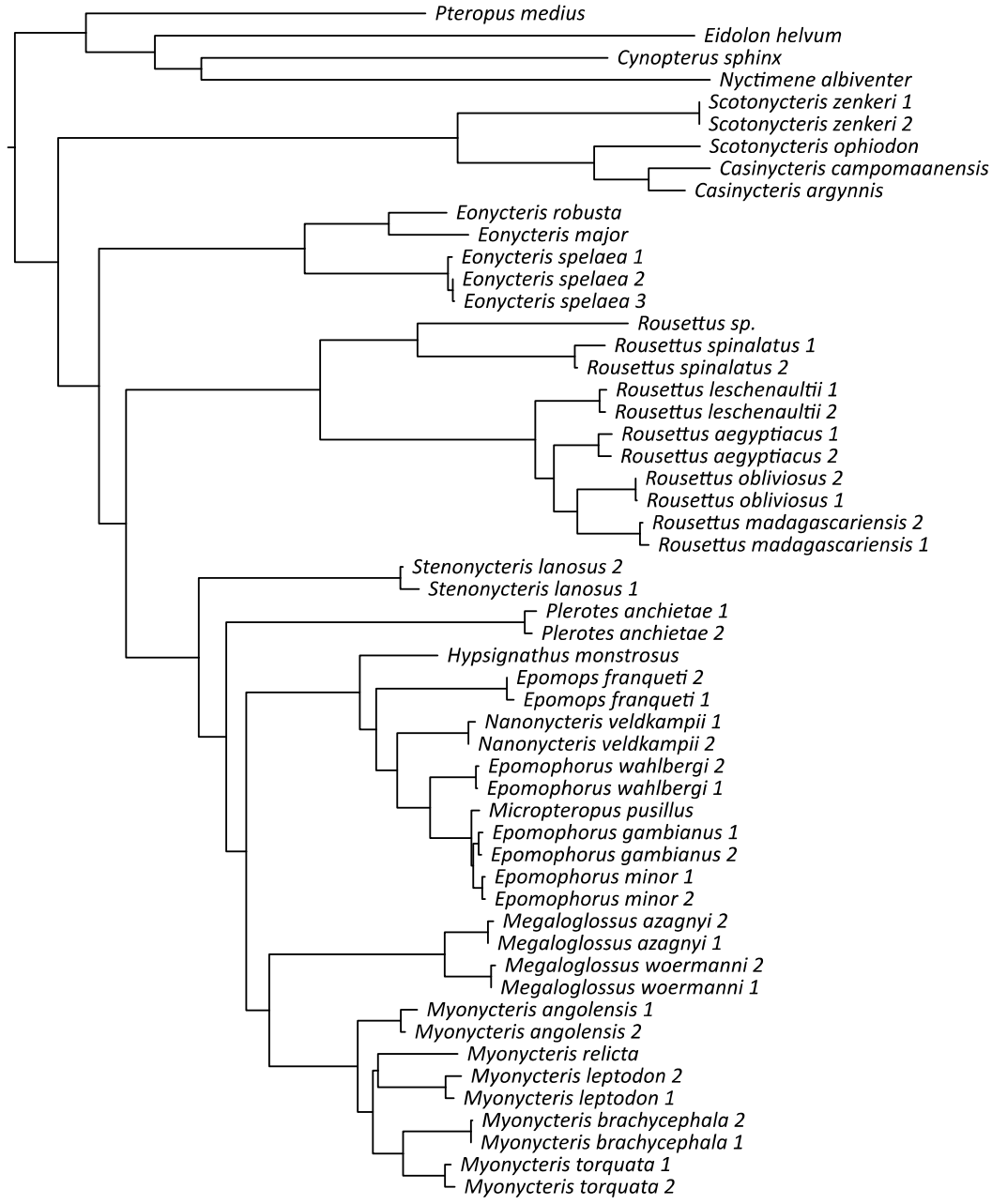

Fig. S5. Maximum likelihood tree obtained with the Cyt*b* gene

Appendix II. Continued

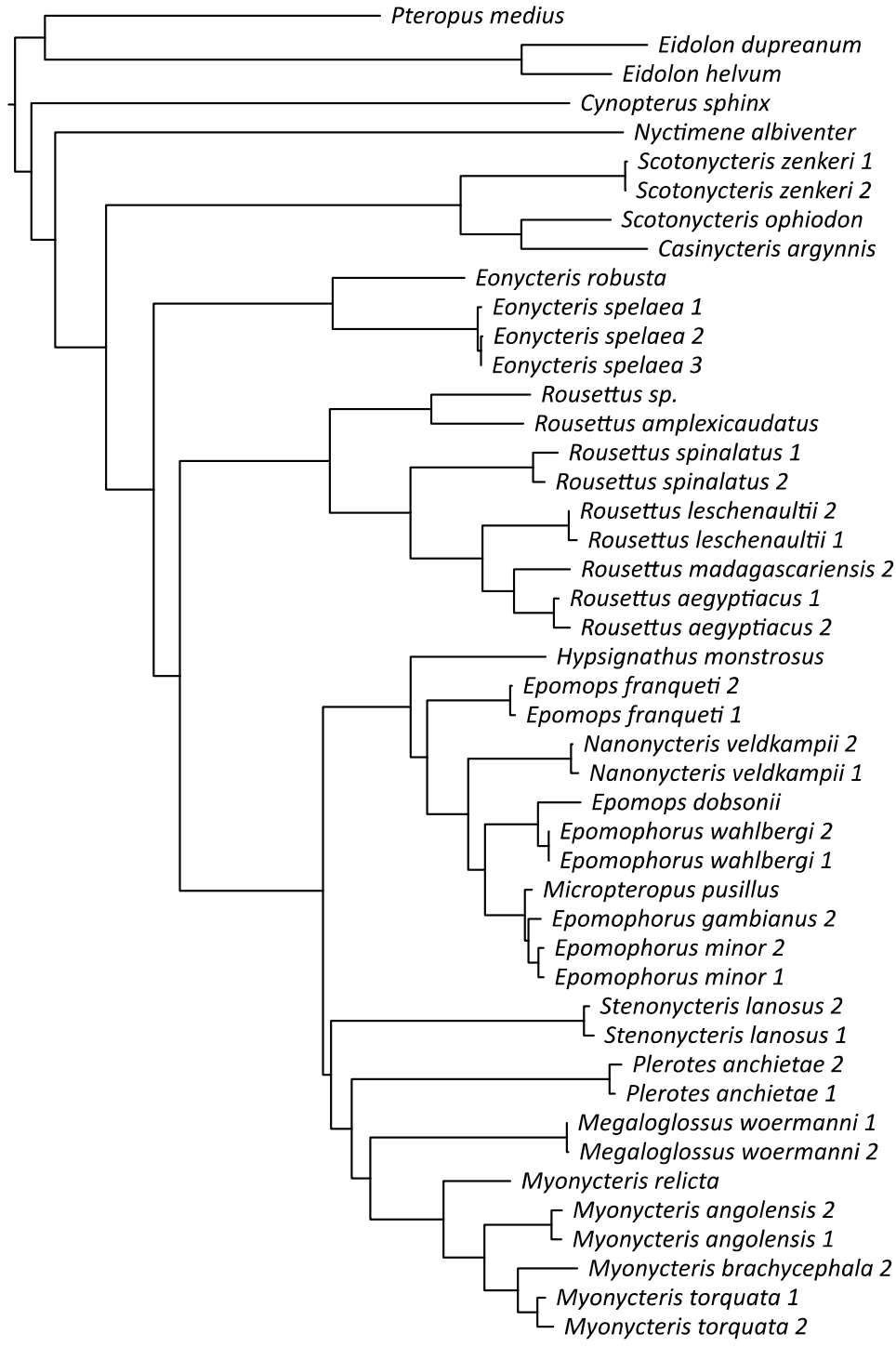

Fig. S6. Maximum likelihood tree obtained with the mitochondrial DNA fragment containing the 12S rRNA and 16S rRNA genes

Appendix II. Continued

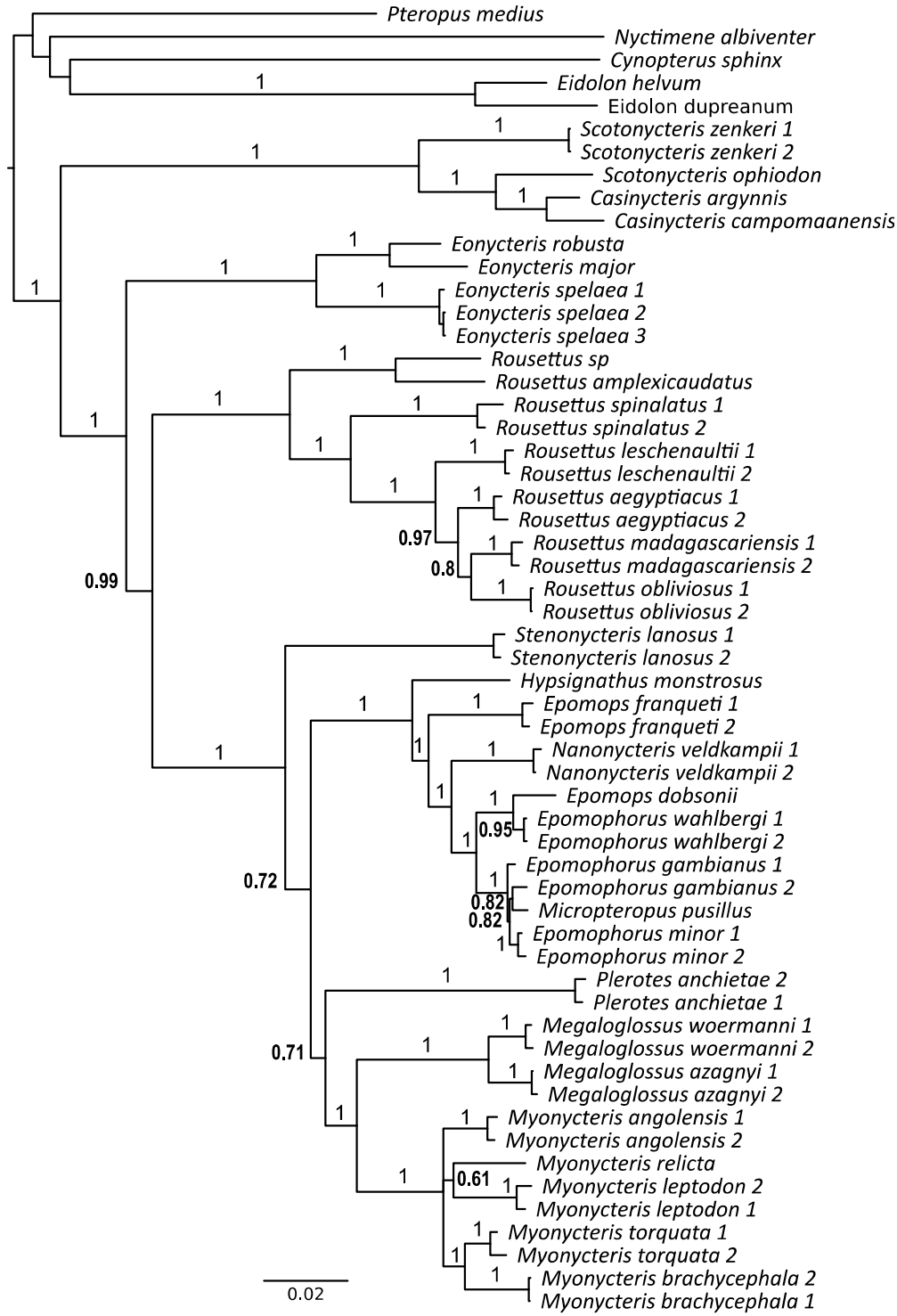

Fig. S7. Bayesian Inference tree based on the combined dataset. Numbers on nodes are posterior probabilities

Appendix II. Continued

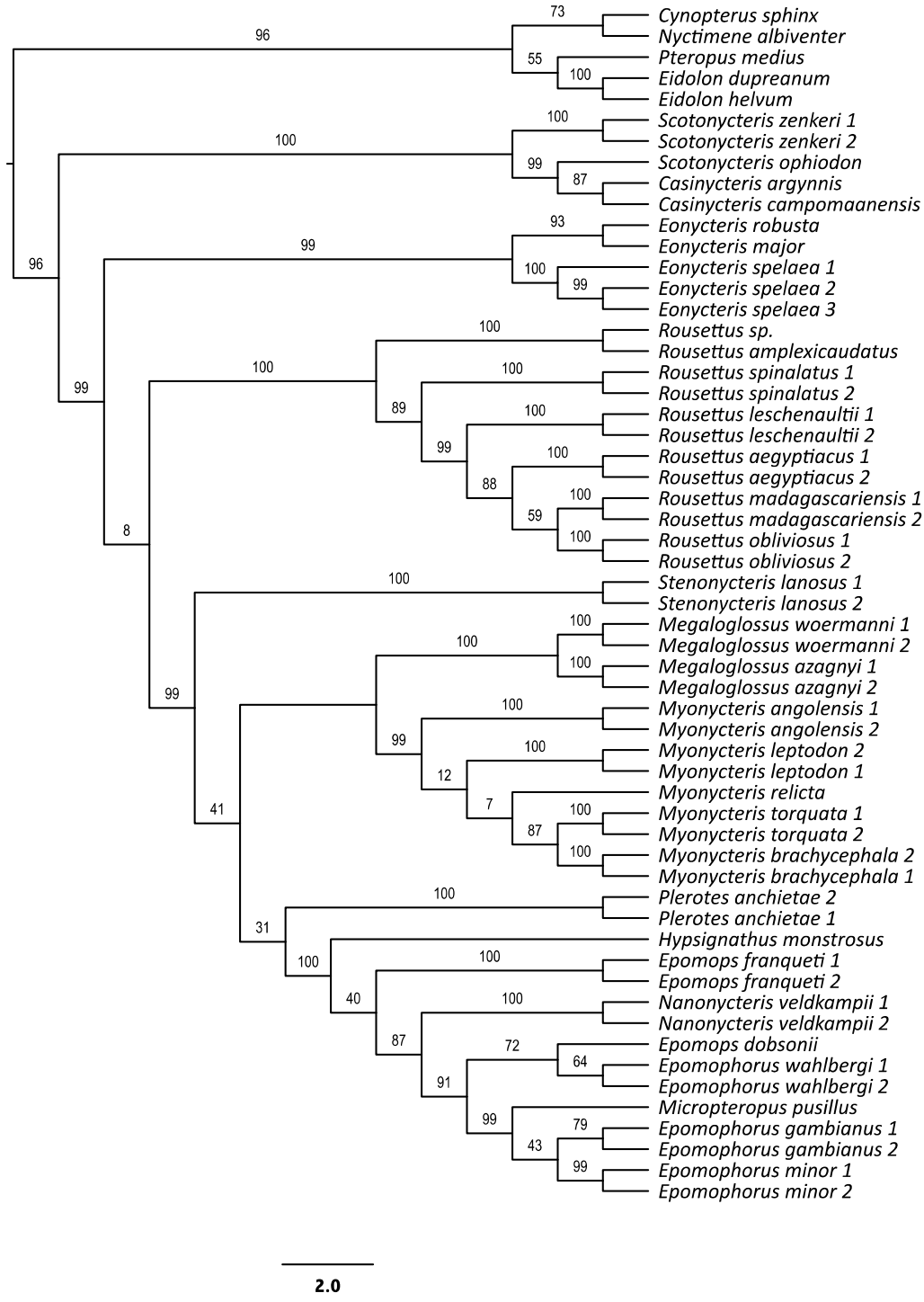

Fig. S8. Maximum parsimony tree based on the combined dataset. Numbers on nodes are bootstrap values

Appendix II. Continued

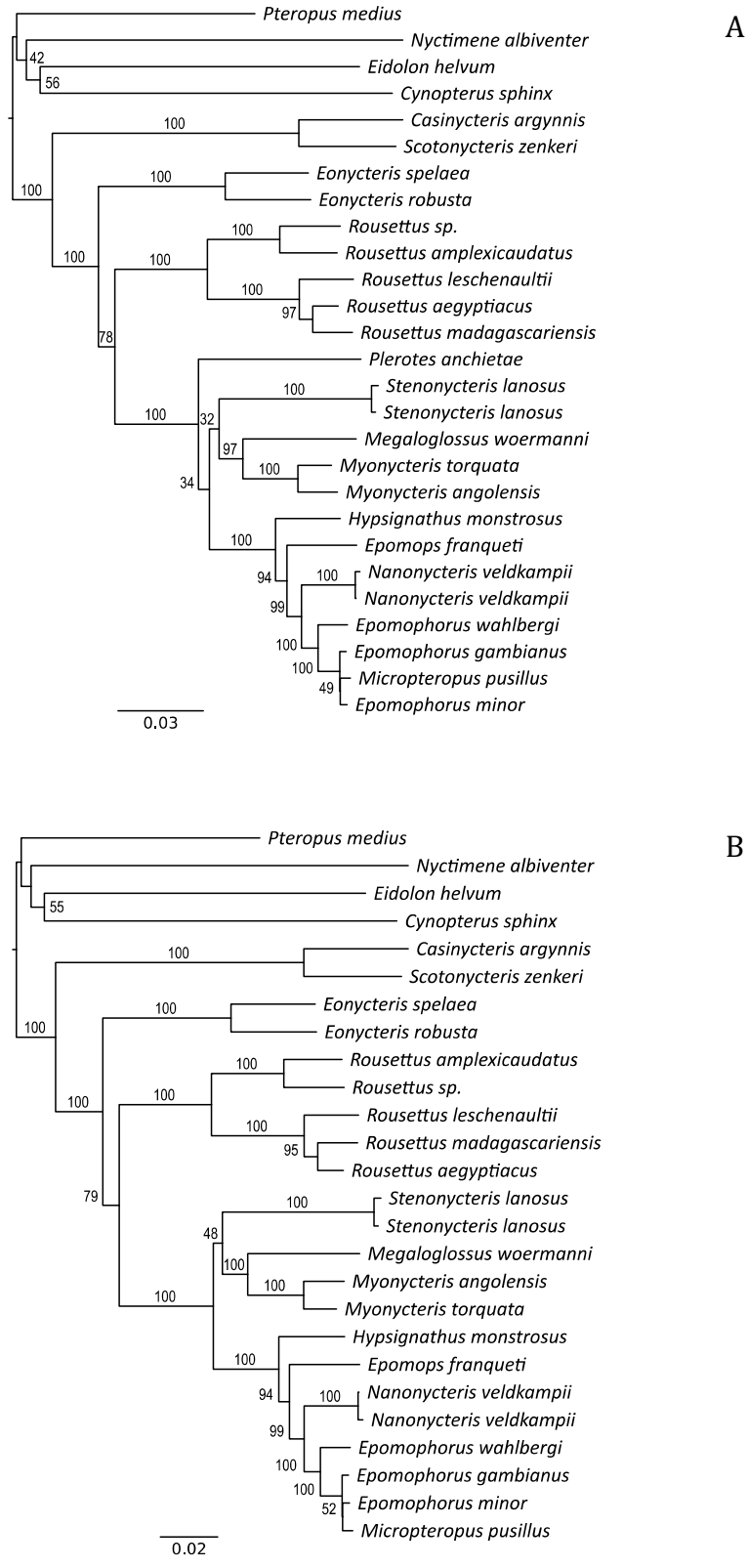

Fig. S9. Maximum likelihood trees based on reduced dataset to minimize missing data, with A — inclusions and B — exclusion of *Plerotes anchietae*. Numbers on nodes are bootstrap values

Appendix II. Continued

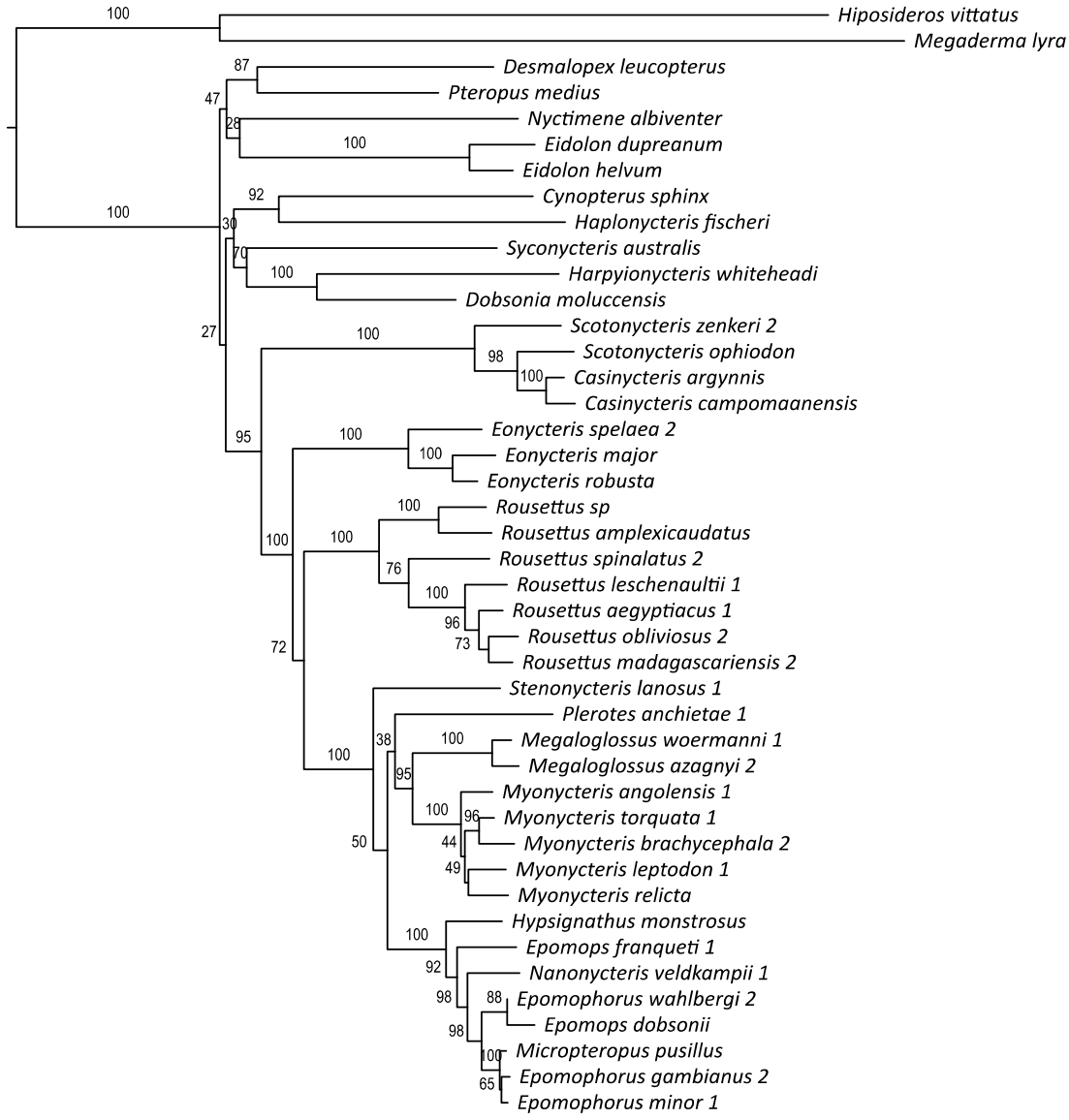

Fig. S10. Maximum likelihood tree obtained for the Biogeographic analysis (DIVA) with bootstrap values

Appendix II. Continued

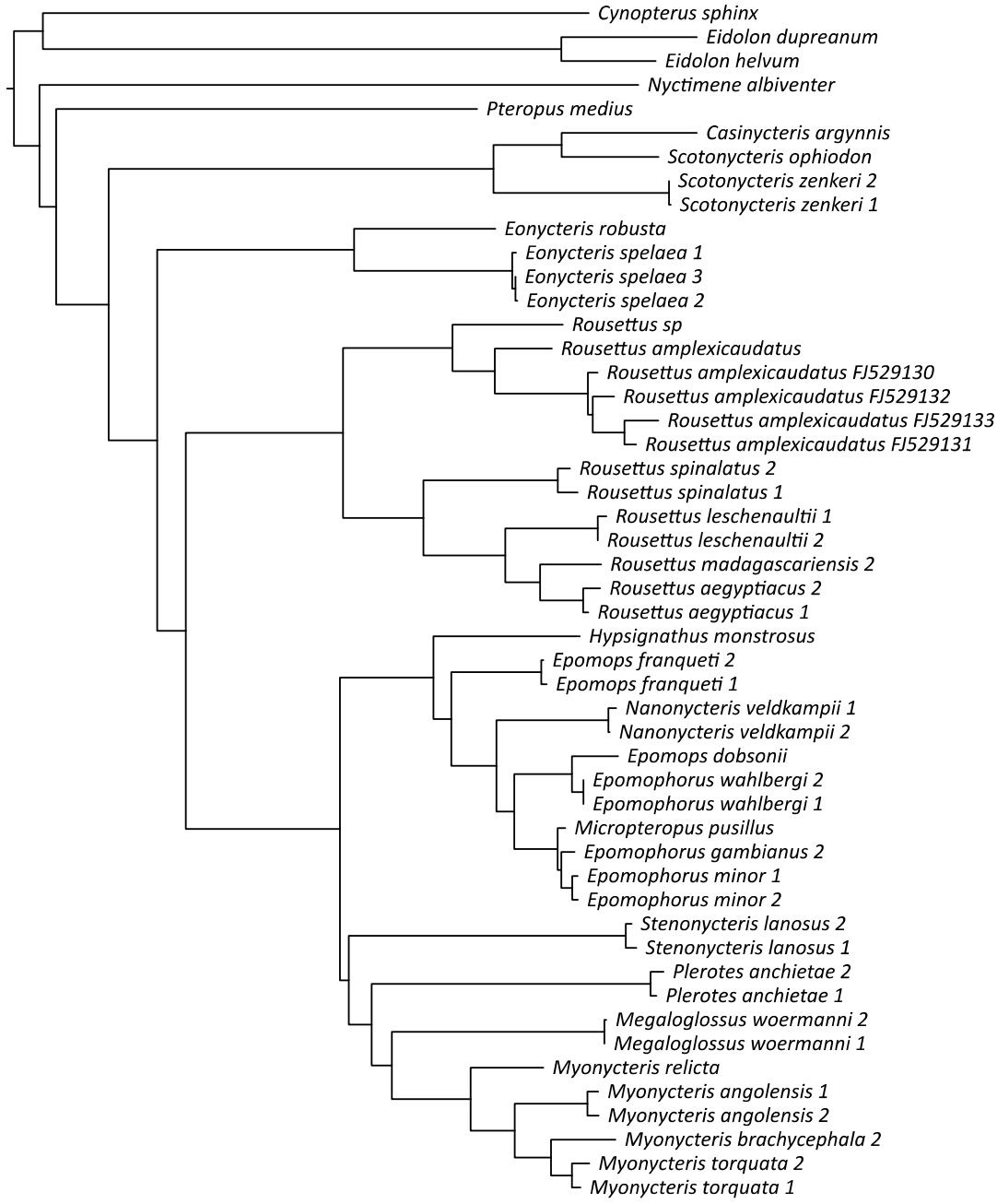

Fig. S11. Maximum likelihood tree obtained with a fragment containing the 12S rRNA and 16S rRNA genes and additional *Rousettus amplexicaudatus* samples from Malaysia (FJ529130–FJ529133), obtained from the GenBank (Guan *et al*., 2006). The other *Rousettus amplexicaudatus* sample is from Papua New Guinea (Teeling *et al*., 2002)