PHYLOGENETIC RELATIONSHIPS OF FIVE MEMBERS OF THE FAMILY VESPERTILIONIDAE (CHIROPTERA) FROM MALAYSIAN BORNEO

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ABSTRACT

Several studies have been conducted to refine the historically unclear phylogeny of chiropterans within the family Vespertilionidae. However, the phylogenetic affinities of some taxa remain poorly resolved. My objective was to clarify the classification and phylogenetic affinities of five species (*Pipistrellus petersi, Glischropus tylopus, Hesperoptenus tomesi, Philetor brachypterus,* and *Arielulus cuprosus*) using DNA sequence data from the 12S rRNA mitochondrial gene and RAG2 nuclear gene. A total of 587 nucleotides of the 12S rRNA gene were aligned for 35 taxa, and for nuclear marker RAG2, 1231 nucleotides were aligned for 40 taxa. I performed maximum likelihood and Bayesian inference phylogenetic analyses on these taxa. Although resolution was poor overall, *A. cuprosus* and *H. tomesi* clustered with tribe Nycticeiini/Eptesicini, with *Philetor brachypterus* clustering with *Hesperoptenus*. Furthermore, *Pipistrellus petersi* clustered within the Hypsugine group instead of the predicted tribe Pipistrellini. Lastly, *G. tylopus* formed a polytomy with members of various tribes. There has been a uniform lack of resolution for this family in recent literature and the results presented here similarly provide unresolved relationships.

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INTRODUCTION

Historically, the phylogenetics of Vespertilionidae, particularly at its supergeneric level, has been of great debate and discussion since testable hypotheses for classification of bats within the family were first introduced by Tate (1942). Recent studies have attempted to elucidate many of the issues, focusing on identifying genetic and karyotypic composition (Volleth *et al*., 2001) and suggesting specific tribal and generic phylogenies based on morphological (Hoofer and Van Den Bussche, 2003) and, very recently, molecular data (Hoofer and Van Den Bussche, 2003; Lack and Van Den Bussche, 2010; Lack *et al.,* 2010; Roehrs *et al*., 2010). However, many of the characters utilized in previous studies have been unable to fully clarify phylogenetic relationships, primarily due to the ambiguous nature of some of these characters. Specifically, convergent or phylogenetically uninformative characters have hindered researchers from constructing a fully acceptable phylogeny. In addition, presumably very rapid diversification of these species has led to problems recovering deep branching patterns (Lack and Van Den Bussche, 2010; Roehrs *et al*., 2010).

Although the higher-level relationships have resisted resolution, based on karyotypic (Volleth and Heller, 1994) and molecular data (Hoofer and Van Den Bussche, 2003; Lack and Van Den Bussche, 2010; Lack *et al*., 2010; Roehrs *et al*., 2010) there is good statistical support for the monophyly of specific tribes and subfamilies. These studies have examined both mitochondrial and nuclear DNA sequence data to provide a strong foundation to the currently accepted phylogenies of Vespertilionidae. Hoofer and Van Den Bussche (2003) used molecular data to recognize seven tribes (Plecotini, Lasiurini, Scotophilini, Nycticeiini, Pipistrellini, Vespertilionini, Antrozoini) in the subfamily Vespertilioninae. Roehrs *et al*. (2010) proposed five traditional tribes (Plecotini, Lasiurini, Scotophilini, Vespertilionini,

Antrozoini), two new unnamed clades (Hypsugine group and Perimyotine group), and Nycticeiini/Eptesicini, the latter requiring further elucidation (Roehrs *et al*., 2011). For this study, I followed the taxonomy proposed by Simmons (2005) with regard to tribe Pipistrellini and Roehrs *et al*. (2010; 2011) for the remaining clades.

Roehrs *et al*. (2010) conducted an extensive phylogenetic study, comparing traditional morphological phylogenies (Tate, 1942; Simpson, 1945; Hill and Harrison, 1987) to recent karyotypic (Volleth *et al*., 2001) and molecular vespertilionid phylogenies (Hoofer and Van Den Bussche, 2003; Simmons, 2005). However, Roehrs *et al.* (2010) did not include any of the species from Malaysian Borneo that were analyzed and sequenced in this study (TABLE 1, FIG. 1). Hoofer and Van Den Bussche (2003) and Lack *et al.* (2010) also did not study the five species sequenced in this investigation. These exemptions were due to the rarity of these specimens, as they had not been widely collected in the field and tissue samples were previously limited, if available.

The five taxa (TABLE 1, FIG. 1) that were analyzed in this study currently belong to the subfamily Vespertilioninae within family Vespertilionidae based on morphological (Koopman, 1994; Simmons, 1998; Simmons and Geisler, 1998; Simmons, 2005) and karyotypic data (Volleth and Heller, 1994; Volleth *et al*., 2001). However, tribal and supergeneric relationships remain unclear and lack full acceptable resolution, particularly because of the absence of molecular data.

The genus *Pipistellus* has undergone extensive revision as new research emerges (Hoofer and Van Den Bussche, 2003; Lack and Van Den Bussche, 2010; Lack *et al*., 2010; Roehrs *et al*., 2010; Roehrs *et al*., 2011) with a large number of publications placing more than 30 species in the genus (Koopman, 1994; Volleth and Heller, 1994; Simmons, 1998;

Simmons and Geisler, 1998; Volleth *et al*., 2001, Simmons, 2005). The genus *Pipistrellus* cannot be diagnosed by any universal morphological characters, though it is diagnostically separated from other vespertilionids by their bacular morphology (Hill and Harrison, 1987; Bates and Harrison, 1997). The species in this genus occur in the Old World, occupying a variety of areas in Asia, Europe, and Africa. *Pipistrellus petersi* has been placed in the genus *Falsistrellus* (Simmons, 2005), along with 4 other species by some authorities (Kitchener *et al.,* 1986; Simmons, 2005), though Payne and Francis (2007) retained the genus *Pipistrellus petersi* in their field guide. Payne and Francis (2007) reported its projected distribution as Sulawesi and the Moluccas, whereas Simmons (2005) described its distribution as Indonesia and the Minahassa peninsula.

The genus *Glischropus* was synonymized with *Pipistrellus* by Menu (1987) but was transferred to its own generic status by Corbet and Hill (1992). The genus contains only two members, with *G. javanus* distributed in western Java in Indonesia and *G. tylopus* found in both Peninsular Malaysia and Malaysian Borneo (Anwarali Khan *et al*., 2008) as well as Burma, Thailand, Philippines and Sumatra (Payne and Francis, 2007). *Glischropus tylopus* was considered a member of the tribe Pipistrellini based on karyotypic data (Volleth *et al*., 2001) and morphological data (Simmons, 2005).

Philetor brachypterus is a monotypic species classified in the tribe Vespertilionini by Simmons (2005) and Volleth *et al*. (2001), with its distribution reported to include Peninsular Malaysia, Philippines, Sumatra, Java, and New Guinea (Payne and Francis, 2007). Though *Philetor brachypterus* is the currently accepted name, this bat was initially described as *Vespertilio brachypterus* and two other species have been synonymized under *Philetor brachypterus* – *Philetor rohui* and *Eptesicus verecundus* (Bates and Harrison, 1997).

The genus *Arielulus* was originally named as a subgenus of *Pipistrellus* by Hill and Harrison (1987) and members of this genus were subsequently transferred to *Eptesicus* by Heller and Volleth (1984) and Volleth and Heller (1994), but it was not recognized as a distinct genus until Csorba and Lee (1999). The genus has been placed in the tribe Nycticeiini/Eptesicini most recently (Simmons, 2005; Roehrs *et al*., 2010; Roehrs *et al*., 2011) and currently contains five species (Simmons, 2005) that are distributed across Thailand, Cambodia, Vietnam, Borneo, Burma, Nepal, China, India and Taiwan. *Arielulus cuprosus* occupies areas in Sabah and Sarawak (Anwarali Khan *et al*., 2008).

Lastly, the genus *Hesperoptenus*, revised by Hill (1976), contains five species occupying mostly Indomalayan areas, including Malaysia, Sulawesi, Indonesia, Burma, Cambodia, Thailand, Laos, India, Bihar and Chaibassa. *Hesperoptenus tomesi* was placed in the tribe Nycticeiini/Eptesicini by Simmons (2005) and it has been reported to occur in both Peninsular Malaysia and Malaysian Borneo (Anwarali Khan *et al*., 2008).

Lack *et al*. (2010) and Roehrs *et al*. (2010; 2011) specifically targeted genes highly conserved in mammalian taxa; these included both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) genes. Previous studies (Matthee *et al*., 2001; Eick *et al*., 2005) have discussed the importance of including both mtDNA and nDNA in phylogenetic studies. For this research, the inclusion of both types of DNA potentially allows for a more robust phylogenetic tree. It would take into account the high evolutionary rate of certain species by utilizing the high mutation rates in the mitochondrial genome to better describe phylogenetic relationships at the generic level. Conversely, nDNA analyses should recover phylogenetic relationships at deeper nodes and help to resolve relationships at the supergeneric level. Therefore, I focused on two genes: a mitochondrial ribosomal gene (12S rRNA) and a

nuclear gene, recombination activating gene II (RAG2), in order to examine the systematics of select vespertilionid taxa.

The 12S mitochondrial gene contains the ribosomal RNA that partly makes up the small subunit of the mitochondrial ribosome (Hillis and Dixon, 1991). RAG2 genes encode one of two essential enzymes that assist in the initiation of V(D)J recombination during B and T cell development, accounting for the variability found in immunoglobulins and T cell receptors generated by lymphocytes (Oettinger *et al*., 1990).

The specimens in my study were collected in Malaysian Borneo, also known as East Malaysia or Sabah and Sarawak, the eastern part of Malaysia located in the northern portion of the island of Borneo (Payne and Francis, 2007). The island's tropical locality results in high and almost constant rainfall (Payne and Francis, 2007). Most of the country's landscape is primarily lowland rain forests with some areas of mountainous rain forests (Payne and Francis, 2007). East Malaysia is less populated than its western counterpart, allowing for greater conservation and greater biodiversity, which includes a large number of primates, chiropterans and rodents (Payne and Francis, 2007).

These specimens offered a terrific opportunity to conduct studies on very rare species previously unexamined molecularly. To date, the genetic makeup of these specimens has not been published, nor has their current phylogeny and classification been tested with a molecular approach. Therefore, my objectives were to clarify their classification within their respective tribes (Pipistrellini, Vespertilionini, and Nycticeiini/Eptesicini) and to better define the relationships of tribes within Vespertilioninae. To do this, I amplified and analyzed DNA sequences from two genes used by Roehrs *et al*. (2010; 2011) in order to test the following hypotheses: *Pipistrellus petersi* and *Glischropus tylopus* are members of tribe

Pipistrellini (*sensu* Simmons, 2005), *Philetor brachypterus* is a member of tribe Vespertilionini, and lastly, *Arielulus cuprosus* and *Hesperoptenus tomesi* are members of the Nycticeiini/Eptesicini clade.

MATERIALS AND METHODS

Molecular Methods

In this study, I focused on five species (*Pipistrellus petersi, Glischropus tylopus, Philetor brachypterus, Arielulus cuprosus, Hesperoptenus tomesi*), using tissues previously collected from Malaysian Borneo in 2006 and 2010. I isolated whole genomic DNA from frozen liver, kidney or heart tissues or from samples stored in lysis buffer using a DNeasy Extraction Kit (QIAGEN Inc., Valencia, California) following manufacturer's protocol. Polymerase chain reaction amplifications for the 12S mitochondrial ribosomal gene were carried out using 200-500ng of DNA, 1 unit of *Taq* polymerase, 2.5 mM of each dinucleoside triphosphate, 1X *Taq* buffer, 1.5-2.0 mM of $MgCl_2$, and 0.16 µM of forward and reverse primers in a 25-µL total volume reaction. The general polymerase chain reaction thermal profile used for these reactions began with an initial 2-min denaturing at 94ºC, followed by 35 cycles at 94ºC for 40 s, 50ºC for 2 min, and 72ºC for 3 min, with amplification ending after a final elongation at 72ºC for 30 min.

Polymerase chain reactions for exons of the RAG2 gene were carried out using 200-500 ng of DNA, 1 unit of *Taq* polymerase, 2.5 mM of each dinucleoside triphosphate, 1X *Taq* buffer, 4 mM of $MgCl_2$, and 0.16 µM of each primer in a 25-µL total volume reaction. The general polymerase chain reaction thermal profile used for these reactions began with an initial 2-min denaturing of 94ºC, followed by 33 cycles of 94ºC for 30 s, touchdown 60-58ºC (decreasing in 0.5ºC increments), then 33 cycles of 57ºC for 60 s, and 72ºC for 90 s, followed by a final elongation period of 72º for 10 min. For both genes, amplification was carried out utilizing AmpliTaq 360 DNA polymerase and its corresponding buffer (5U/µl; Applied Biosystems, Foster City, California).

Primers utilized in this study were previously outlined by Roehrs *et al*. (2010), Van Den Bussche and Hoofer (2000), and Hoofer and Van Den Bussche (2003) and targeted approximately 2000 nucleotides (TABLE 2). Following DNA amplification, products were quantified using a Qubit fluorometer (Invitrogen, Carlsbad, California) and purified using ExoSAP-IT (USB-Affymetrix, Cleveland, Ohio). Samples were sequenced using a Genome DTCS-Quick Start Kit in a Beckman Coulter CEQ 8000 automated sequencer following manufacturer's protocol with reactions quartered rather than the suggested amounts listed in the protocol (Beckman Coulter Inc., Fullerton, California). 12S reactions were sequenced utilizing primarily 12a, 12b, 12c and 12g primers, whereas RAG2 sequencing was carried out using the same primers utilized for PCR as well as additional internal primers to allow for overlapping areas to increase the accuracy of the sequence acquired (TABLE 2).

Phylogenetic Analyses

Initially, analyses in this study were carried out with over a hundred species within subfamily Vespertilioninae. In addition, the sequence alignment utilized in Lack and Van Den Bussche (2010) was used in this study together with my newly sequenced data, and was acquired directly from TreeBASE (www.treebase.org). However, with the inclusion of such a large number of taxa, no resolution was achieved and most of the phylogenies presented were unsupported with many polytomies extensively present throughout. To improve resolution, I truncated a large number of taxa and chose individuals whose phylogenetic affinity was well supported in previous studies (Volleth *et al*. 1994; Hoofer and Van Ben Bussche, 2003; Simmons, 2005; Roehrs *et al.*, 2010). These individuals were selected from each of the tribes recognized by Roehrs *et al.* (2010) in the subfamily Vespertilioninae. The subfamily Myotinae (Vespertilionidae) was utilized as the outgroup following previous

molecular studies that ascertained its phylogenetic affinity (Lack *et al.*, 2010; Roehrs *et al.*, 2010).

DNA sequences from other vespertilionid species from closely related tribes (33 taxa for 12S and 32 taxa for RAG2) were acquired from GenBank (www.ncbi.nlm.nih.gov/genbank/) and aligned by eye in MEGA version 5 (Tamura *et al*. 2011) and Sequencher version 5.0 (Gene Codes Corporation, Ann Arbor, Michigan) with my newly-generated sequences (7 taxa for 12S and 3 for RAG2; Appendix). For the 12S phylogenetic analyses, a total of 109 nucleotides were eliminated to account for regions that appeared to violate the assumption of positional homology (Roehrs *et al.*, 2010). Alignment parameters for 12S in MEGA 5 were carried out utilizing MUSCLE, using Unweighted Pair-Group Method with Arithmetic Mean for its clustering method, and open gap penalties of -400. Parameters of RAG2 in MEGA 5 were carried out using ClustalW, with a DNA Weight Matrix set at IUB and gap opening penalties for both pairwise and multiple alignments set at 15 bases.

The mtDNA and nDNA gene alignments were analyzed independently using maximum likelihood (ML) analyses in MEGA (Felsenstein, 1981; Felsenstein, 1985). The best-fit evolutionary models for both the 12s and RAG2 data sets were calculated using the Model Selection analysis included in MEGA5. Nodes in resulting trees containing ≥70% maximum-likelihood bootstrap support were considered statistically significant (Hillis and Bull, 1993).

Bayesian Inference (BI) analyses were conducted using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The BI analyses consisted of 2 simultaneous runs each with four Markov Chain Monte Carlo chains (one heated, three cold) run for 1 million

generations on each of the data sets. Nodes in resulting trees containing ≥0.95 Bayesian posterior probabilities were considered statistically significant (Ronquist and Huelsenbeck, 2003). Lastly, pairwise distance analyses for tribe Nycticeiini/Eptesicini for both genes (12S and RAG2) were conducted to explain genetic divergence between species.

RESULTS

Both ML analyses recovered significant support for the outgroup Myotinae, but only the RAG2 ML recovered support for two additional clades, tribe Lasiurini and the Hypsugine group (FIG. 2, 4). Furthermore, both BI analyses carried out for both genes recovered statistical support for four different tribes: Hypsugine group, tribe Lasiurini, tribe Antrozoini, and the outgroup subfamily Myotinae (FIG. 3, 5). The specific outcomes and degree of resolution for each of the target species varied.

12S Phylogenetic Analyses

Maximum Likelihood- A total of 587 nucleotides were aligned from 40 taxa, with 33 individuals included from GenBank and 7 novel sequences (Appendix), resulting in 161 phylogenetically informative characters. A total of 109 nucleotides were eliminated to account for regions that appeared to violate the assumption of positional homology (Roehrs *et al.*, 2010) The best-fit evolutionary model for this data set was determined as the Tamura-Nei with invariable gamma distribution (T92+G+I) with 1000 bootstrap replicates carried out.

Significant support was recovered using ML for the placement of *H. tomesi* and *Philetor brachypterus* in the same clade within the Nycticeiini/Eptesicini tribe. However, *H. tomesi* conspecifics are in a polytomy with *Philetor brachypterus.* Furthermore, *A. cuprosus* clustered sister to *A. aureocollaris* though that relationship does not bear statistical support. Interestingly, *G. tylopus* forms a polytomy with tribes Scotophilini, Nycticeiini/Eptesicini, Vespertilionini, and members of both the Perimyotine and Hypsugine groups [*sensu* Roehrs *et al*., (2010)]. Lastly, *Pipistrellus petersi* falls within the Hypsugine group and as a sister to *Chalinolobus gouldii* (FIG. 2).

Bayesian Analysis- A total of 587 nucleotides from 40 taxa were included in the Bayesian analysis for the 12S mitochondrial gene, with 33 taxa included from GenBank and 7 novel sequences. The same evolutionary model (T92+G+I) utilized in ML analysis was used in the BI analysis.

Similar relationships previously illustrated in the ML analysis were also represented here. Four of the seven taxa grouped in polytomy within Nycticeiini/Eptesicini, with *A. cuprosus* falling sister to its congener and *H. tomesi* this time clustering sister to *Philetor brachypterus* within the same clade. *Pipistrellus petersi* clustered within the Hypsugine group and both *G. tylopus* samples clustered sister to each other but formed a polytomy with other members of Vespertilionini (FIG. 3). Furthermore, none of these relationships were statistically significant based on Bayesian posterior probability scores.

RAG2 Phylogenetic Analyses

Maximum Likelihood- A total of 1234 nucleotides were aligned from 35 taxa, with 32 individuals included from GenBank and 3 novel sequences (Appendix), resulting in 251 phylogenetically informative characters. The best-fit evolutionary model for this data set was determined as the Kimura with Gamma distribution (K2+G).

Maximum likelihood analysis accounted for the placement of *A. cuprosus* and *H. tomesi* within the Nycticeiini/Eptesicini tribe. Furthermore, *A. cuprosus* clustered sister to *A. aureocollaris*, while the two *Hesperoptenus* species clustered sister to each other (FIG. 4). These relationships, however, were not statistically supported.

Bayesian Analysis- A total of 1234 nucleotides from 35 taxa were included in the Bayesian analysis for the RAG2 nuclear gene, with 32 individuals acquired from GenBank

and 3 novel sequences included in the analysis. Similarly, the same evolutionary model (K2+G) and utilized in ML analysis was used in this BI analysis.

Relationships recovered in the BI analysis were also very similar to those produced by the ML analysis. Significant support was recovered for *A. cuprosus* clustering sister to its congener, *A. aureocollaris*, within the Nycticeiini/Eptesicini tribe. *Hesperoptenus tomesi* similarly grouped together, though its sister relationship to *Glauconycteris egeria* was not supported (FIG. 5).

The 12S pairwise distance analysis was calculated using a Tamura-Nei model with Gamma distributed rates among sites, accounting for transitions occurring at two different rates and transversions occurring at a different rate. In addition, under the Tamura-Nei model base frequencies occur unequally. RAG2 analysis followed a Kimura-2 parameter model with Gamma distributed rates among sites, with transitions and transversions being treated at different rates, and base frequencies occurring at equal rates. Pairwise distance analyses for tribe Nycticeiini/Eptesicini for both genes resulted in an 11% (12S) and 7% (RAG2) distance between the *Arielulus* species (TABLE 3, 4). Furthermore, in the 12S pairwise distances analysis for the Nycticeiini/Eptesicini clade, *Hesperoptenus* and *Philetor* distances ranged from 0.6% to 2% (TABLE 3).

DISCUSSION

Phylogenetic Relationships

Based on morphological and molecular evidence (Hoofer and Van Den Bussche, 2003; Lack *et al*., 2010; Roehrs *et al*., 2010), *Arielulus cuprosus* was expected to cluster sister to its congener, *A. aureocollaris*, and fall within tribe Nycticeiini/Eptesicini (*sensu* Roehrs *et al*., 2010). My molecular analyses of the 12S mitochondrial gene illustrated this expected relationship, although it does not bear statistical support (FIG. 2, 3). Similarly, analyses of the RAG2 nuclear gene depicted this relationship, and in both cases, bootstrap values and posterior probabilities were statistically significant (FIG. 4, 5). However, in these latter analyses, the monophyly of tribe Nycticeiini/Eptesicini remained in question, with several members of Nycticeiini/Eptesicini, such as *Eptesicus* and *Nycticeius*, clustering with members of several tribes. The same trend was observed in the ML analysis of the 12S mitochondrial gene (FIG. 2).

Pairwise distance analyses provided a measurement of genetic divergence amongst some members of tribe Nycticeiini/Eptesicini (TABLE 3, 4). In the instance of the *Arielulus* species, the 11% divergence illustrated by the 12S fragment is consistent with proposed divergence values in Baker and Bradley (2006) observed at the intrageneric level. In addition, both values (7% for RAG2 and 11% for 12S) were typical values observed amongst sister taxa. However, Baker and Bradley (2006) mainly depicted values for another mitochondrial gene, cytochrome b, and as such, direct conclusions comparing their values to other chiropteran divergence values must be considered with caution. *Arielulus cuprosus* contained approximately 130 sites of missing data for the 12S analyses and 580 sites of

missing data for the RAG2 analyses, which could be a possible reason why there is a long branch present at the node between the two *Arielulus* species (FIG. 2, 4).

Prior to this study, *Glischropus tylopus* was only grouped with other members of tribe Pipistrellini based on karyotypic data (Volleth *et al*., 2001), and molecular analyses for this species were absent from the literature. In this study, molecular analyses of the 12S mitochondrial gene placed this species in a polytomy within Vespertilionini (*sensu* Roehrs *et al*., 2010) (FIG. 2, 3). Unfortunately, obtaining sequences for RAG2 for *G. tylopus* proved unsuccessful. Future approaches should adjust PCR conditions and try higher DNA concentrations, higher Mg concentrations, as well as redesign primers for the RAG2 gene in hopes of attaining sequence data. As suggested by Matthee *et al*. (2001) and Eick *et al*. (2005), the inclusion of nuclear data for *G. tylopus* would provide additional necessary robustness to the depicted relationships, perhaps providing statistical support for relationships at deeper nodes due to the slower evolutionary rate of nuclear genes as opposed to mitochondrial genes.

In accordance with the taxonomic arrangement presented in Simmons (2005), *Hesperoptenus tomesi* clustered within tribe Nycticeiini/Eptesicini. In both ML and BI analyses for RAG2, *H. tomesi* clustered sister to *Glauconycteris egeria*, though that relationship bore no statistical support (FIG. 4, 5). Interestingly, in both ML and BI analyses for 12S, *Philetor brachypterus* clustered either sister to one of the two *Hesperoptenus* specimens included in this study or in a polytomy with the *Hesperoptenus* conspecifics, and this relationship was statistically supported. Furthermore, divergence values between *Hesperoptenus* and *Philetor* members varied from 0.6 to 2%; these values are typical of intraspecific relationships (Baker and Bradley, 2006). Due to previous karyotypic and

morphological studies (Volleth et al. 2001; Simmons 2005) grouping *Philetor brachypterus* with other members of tribe Vespertilionini (and not Nycticeiini/Eptesicini), it is highly probable that in this situation contamination could have occurred within the laboratory and led to these results. I evaluated the skins and skulls from each of the voucher specimens in this analysis and identification in the field was carried out accurately. Contamination could have also taken place in the field, such as improper sterilization of the equipment utilized for the preparation of the voucher specimens, or mislabeling the tubes containing tissue samples. However, this relationship could also be real, and as such, additional evidence is necessary to elucidate the phylogenetic affinity of *Philetor brachypterus.*

Lastly, *Pipistrellus petersi* was placed within the Hypsugine group (*sensu* Roehrs *et al*., 2010) for both ML and BI analyses for the 12S mitochondrial gene, rather than the proposed tribe Pipistrellini. Because it grouped apart from other pipistrelles (*Pipistrellus*), the name proposed by Kitchener *et al.* (1986), *Falsistrellus petersi,* is more applicable to the species (Simmons, 2005). According to Kitchener *et al.* (1986), *Falsistrellus* species differ from *Pipistrellus* by being considerably larger in all morphological measurements.
Furthermore, Simmons (2005) included *Falsistrellus* in tribe Vespertilionini, along with *Chalinolobus, Hypsugo, Laephotis, Neoromicia, Tylonycteris,* and *Vespadelus.* Roehrs *et al.* (2010) included these genera, except *Falsistrellus*, within a redefined Hypsugine group based on molecular data. However, similarly to *G. tylopus,* having additional nuclear data for this species would help to elucidate its placement and allow for a more robust depiction of its phylogenetic relationship with other members of this family.

Tribal Relationships

Members of the traditional tribe Pipistrellini, a name utilized by both Volleth *et al*. (2001) and Simmons (2005) were combined into tribe Vespertilionini by Roehrs *et al*. (2010). Initially, my hypothesis still separated these two tribes, with *Pipistrellus* paced in Pipistrellini and *Vespertilio* placed in Vespertilionini. I predicted that *Pipistrellus petersi* and *Glischropus tylopus* would cluster within what Simmons (2005) considered Pipistrellini, but results placed members of the genus *Pipistrellus* with *Vespertilio.* In this case, the name Vespertilionini appears to be more applicable to this grouping. However, this tribal relationship is not statistically supported.

Roehrs *et al*. (2010) also proposed a Hypsugine group, and in this study, the monophyly of this group is supported in both ML and BI for the RAG2 nuclear gene and the BI analysis for the 12S mitochondrial gene (FIG. 3, 4, 5). Furthermore, the Hypsugine grouping was sister to Vespertilionini in both ML and BI analyses for the RAG2 gene, though this relationship lacks statistical support. The monophyly of Lasiurini was also supported in all analyses except for the ML analysis of the 12S mitochondrial gene. The status of tribe Lasiurini has been supported since Tate (1942) proposed the grouping, and in accordance to results presented previously, herein it is presented as a natural tribal grouping bearing statistical support.

Tribe Antrozoini, a traditionally unstable grouping, is supported herein only in BI analyses for both 12S and RAG2 genes, with ML analyses including members of other tribes clustering with *Antrozous* or *Rhogeesa.* Other tribes suggested by Roehrs *et al*. (2010), such as Nycticeiini/Eptesicini, follow a similar course of unresolved status, with members of the tribe scattered in an unresolved polytomy throughout the phylogenetic tree. The other proposed grouping, the North-American Perimyotine, by Roehrs *et al*. (2010) also contains a

member of tribe Plecotini (*Barbastella barbastella)* in the ML analyses for both genes. The only instance where the Perimyotine group forms a natural grouping is in the BI analysis for the nuclear RAG2 gene, though that relationship is also unsupported statistically (FIG. 5). The last tribe mentioned by Roehrs *et al*. (2010), Scotophilini, was also unsupported by this study, although it is supported in the literature (Tate, 1942; Koopman, 1994; Volleth *et al*., 2001; Hoofer and Van Den Bussche, 2003; Simmons, 2005).

Conclusions

Initially, analyses for this study were carried out with over a hundred species of the subfamily Vespertilioninae since Roehrs *et al*. (2010) warned that conducting phylogenetic analyses with too few taxa would lead to biased relationships or an untrue phylogeny. However, other recent molecular studies containing many taxa still showed a uniform lack of resolution for this family (Hoofer and Van Den Bussche, 2003), a trend that was observed herein as well, even when utilizing a large number of taxa. Therefore, I truncated all analyses in this study to contain fewer than 50 taxa in order to focus on specific groups and attempt to elucidate relationships. Unfortunately, similar results of unresolved relationships were gathered in this study and elucidation was problematic.

Previous studies have also shown that the inclusion of additional genetic markers, both nuclear and mitochondrial, tend to provide more robust results and in many cases, resolved relationships at various phylogenetic levels (Matthee *et al*., 2001; Eick *et al*., 2005; Lack *et al*., 2010). Additional approaches to solving poor resolution could include mitochondrial markers such as 16S (Hillis and Dixon, 1991), the ribosomal counterpart to 12S. In addition, other coding nuclear markers, similar to RAG2 that may provide clarity would include apolipoprotein B (APOB) and dentin matrix acidic phosphoprotein 1 (DMP1)

(Roehrs *et al*., 2010; Roehrs *et al*., 2011). For higher variability amongst nuclear markers, the inclusion of protein kinase C, iota (PRKCι), signal transducer and activator of transcription 5A (STAT5A), and thyrotropin (THY) may prove to be beneficial in providing some resolution (Roehrs *et al*., 2010; Roehrs *et al*., 2011).

Two of the three main hypotheses in this study were rejected. *Pipistrellus petersi* and *G. tylopus* did not cluster with other members of tribe Pipistrellini. Instead, *P. petersi* grouped within the Hypsugine group and *G. tylopus* was found in a polytomy with a variety of taxa from different tribes. *Philetor brachypterus* also did not group as predicted, instead grouping sister to *H. tomesi* in the Nycticeiini/Eptesicini clade. The only hypothesis supported was the predicted grouping of *A. cuprosus* and *H. tomesi* within the Nycticeiini/Eptesicini clade. Of the eight clades proposed by Roehrs *et al.* (2010), three were statistically supported (Hypsugine, Lasiurini and Antrozoini) in this study, with two others grouping naturally but unsupported (Scotophilini and Perimyotine). The remaining three clades (Vespertilionini, Nycticeiini/Eptesicini and Plecotini) were unsupported and individuals of these groupings were scattered throughout the phylogenetic trees.

In recent morphological, karyotypic and molecular studies, there has been a uniform lack of resolution for the phylogenetics of this family, particularly at the generic and tribal levels (Volleth *et al*., 2001; Hoofer and Van Den Bussche, 2003; Simmons, 2005; Lack *et al*., 2010; Roehrs *et al*., 2010; Roehrs *et al*., 2011). Many tribes that are supported in one study are disputed in other studies as additional data become available. That same trend is observed here and a lack of resolution is found throughout these results. Previous literature has stated that rapid radiation and diversification has been the main source of problems leading to deep branching patterns and subsequent lack of resolution amongst members of

this family (Roehrs *et al.*, 2010). Therefore, it is important for studies to continue within this family. The acquisition of additional taxa, especially those that are particularly rare such as the ones presented herein, can only benefit researchers and allow elucidation of relationships among these taxa. Furthermore, a larger number of genetic markers should be utilized in further studies, as well as the inclusion of large sample size of taxa in order to provide more robust answers and elucidate this family's evolutionary history.

TABLE 1. Taxa and tissue collection numbers utilized in this study. Tissues were acquired on loan from the Museum of Texas Tech University (TTU). GenBank Accession numbers are provided for both mitochondrial (12S) and nuclear (RAG2) data.

Taxon	Tissue Number	GenBank Accession No.		Collection Locality
		12S	RAG2	
Arielulus cuprosus	TK168438	KC887906	KC887913	Mount Penrissen, Borneo Heights Resort, Sarawak, Malaysia
Pipistrellus petersi	TK168445	KC887912	--	Mount Penrissen, Borneo Heights Resort, Sarawak, Malaysia
Glischropus tylopus	TK152090	KC887907	--	Kinabalu National Park- Mongis Substation, Sabah, Malaysia
Glischropus tylopus	TK152163	KC887908	--	Kabah National Park, Sabah, Malaysia
Philetor brachypterus	TK168444	KC887911	--	Mount Penrissen, Borneo Heights Resort, Sarawak, Malaysia
Hesperoptenus tomesi	TK168442	KC887909	KC887915	Mount Penrissen, Borneo Heights Resort, Sarawak, Malaysia
Hesperoptenus tomesi	TK168464	KC887910	--	Mount Penrissen, Borneo Heights Resort, Sarawak, Malaysia
Hesperoptenus tomesi	TK168441	--	KC887914	Mount Penrissen, Borneo Heights Resort, Sarawak, Malaysia

TABLE 2. Primers utilized in PCR and DNA sequencing for RAG2 and 12S rRNA. Primers were acquired from Lack *et al*., (2010)

Gene	Name	Sequence (5'-3')
12S	12a	AAAAAGCCTTCAAACTGGGATTAGATCCCCACTAT
	12b	TGACTGCAGAGGGTGACGGGCGGTGTGT
	12c	AAAGCAAARCACTGAAAATG
	12g	TTTCATCTTTTCCTTGCGGTAC
RAG2	R1	AACYTGYTTATTGTCTCCTGGTATGC
	R2	GRAAGGATTTCTTGGCAGGAGT
	F1	GGCYGGCCCAARAGATCCTG
	F2	TTTGTTATTGTTGGTGGCTATCAG

TABLE 3. Average Tamura-Nei 12S distances between members of tribe Nycticeiini/Eptesicini based on 577 base pairs of the 12S rRNA gene for 8 taxa.

	1	2	3	4	5	6	7	8
1 *Arielulus cuprosus*	—							
2 *Arielulus aureocollaris*	0.113	—						
3 *Eptesicus furinalis*	0.091	0.111	—					
4 *Eptesicus fuscus*	0.076	0.117	0.044	—				
5 *Philetor brachypterus*	0.097	0.134	0.099	0.095	—			
6 *Hesperoptenus tomesi* (442)	0.126	0.165	0.128	0.124	0.022	—		
7 *Hesperoptenus tomesi* (464)	0.106	0.143	0.108	0.103	0.006	0.029	—	
8 *Nycticeius humeralis*	0.100	0.124	0.087	0.085	0.085	0.112	0.092	—

TABLE 4. Average Kimura-2 parameter RAG2 distances between members of tribe Nycticeiini/Eptesicini based on 1234 base pairs of the RAG2 gene for 7 taxa.

	1	2	3	4	5	6	7
1 *Arielulus cuprosus*	—						
2 *Arielulus aureocollaris*	0.076	—					
3 *Eptesicus furinalis*	0.105	0.044	—				
4 *Eptesicus fuscus*	0.108	0.044	0.007	—			
5 *Hesperoptenus tomesi* (441)	0.103	0.042	0.053	0.049	—		
6 *Hesperoptenus tomesi* (442)	0.101	0.040	0.051	0.047	0.002	—	
7 *Nycticeius humeralis*	0.120	0.049	0.054	0.054	0.070	0.068	—

FIG. 1. Two of the five species utilized for this study. A: Coppery pipistrelle, *Arielulus cuprosus.* B: Tomes' false serotine, *Hesperoptenus tomesi.* Photographs by Robert J. Baker.

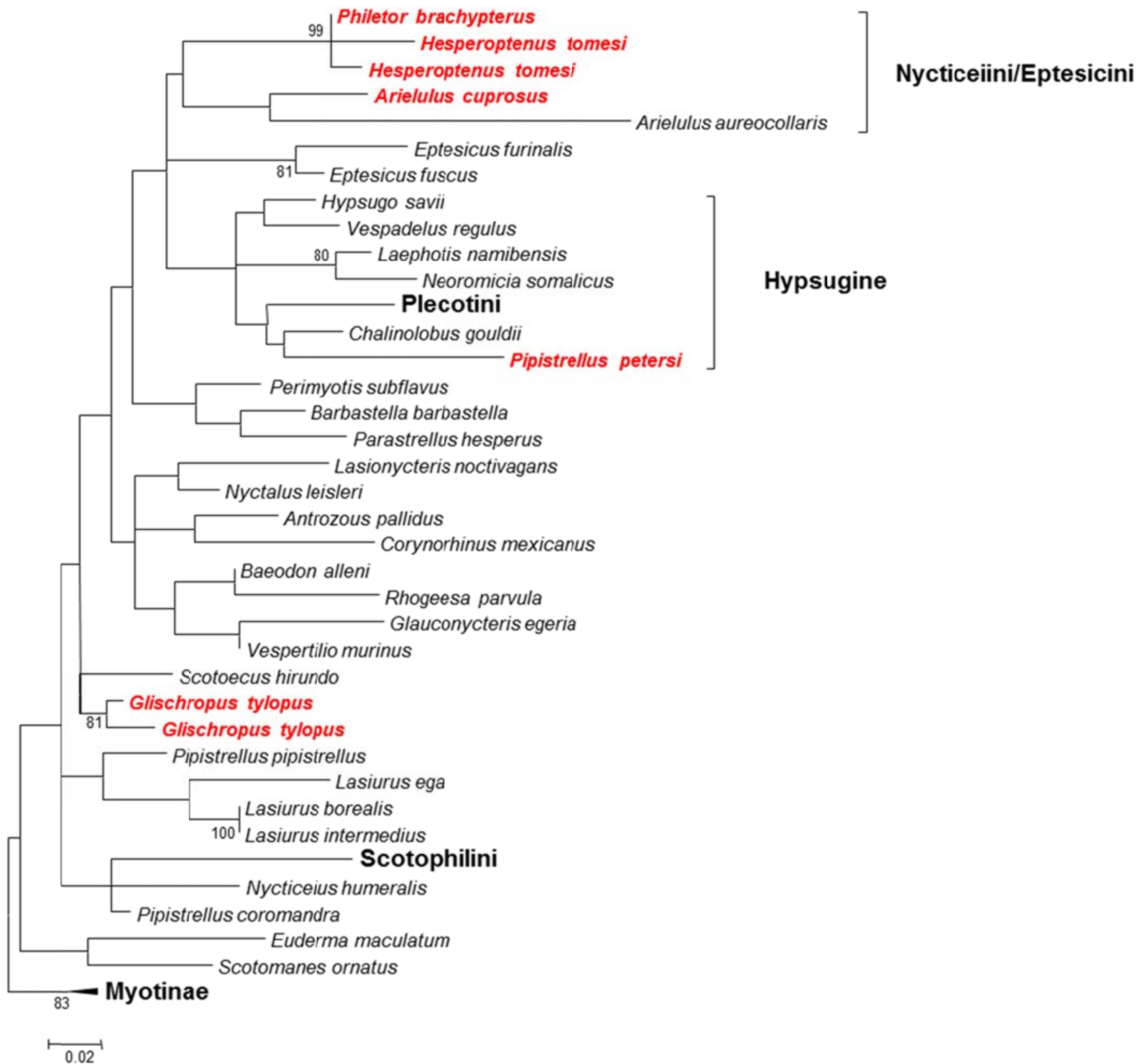

FIG. 2. Phylogram resulting from ML analysis of the mitochondrial 12S ribosomal gene (40 taxa, 587 nucleotides, T92+G+I, 1000 pseudoreplicates). Specimens highlighted in red indicate organisms sequenced for this study. Nodes with ≥70% bootstrap values are shown. Scale is in number of substitutions per site.

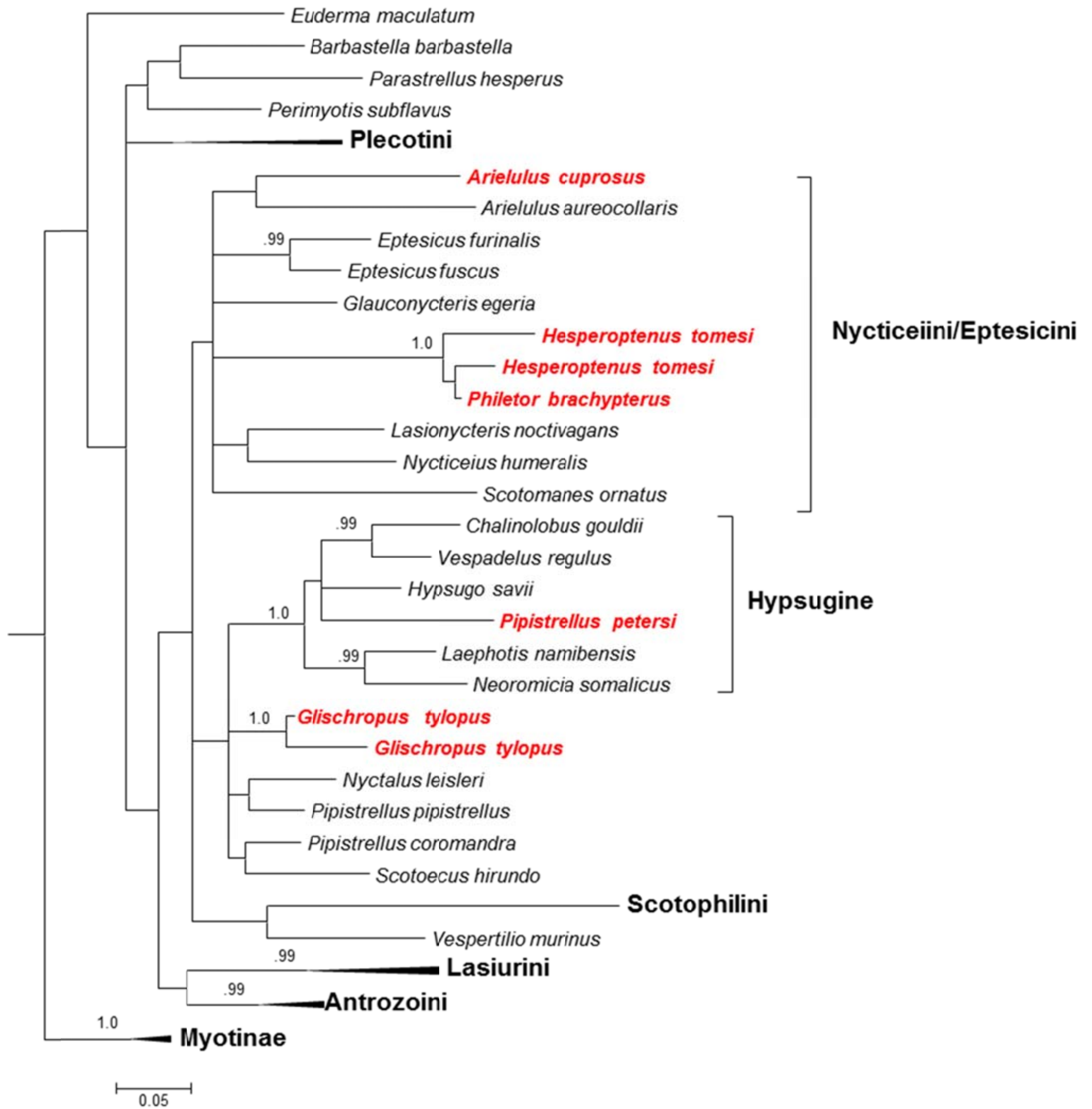

FIG. 3. Phylogram resulting from BI analysis of the mitochondrial 12S ribosomal gene (40 taxa, 1231 nucleotides, T92+G+I, 1×10^6 generations). Specimens highlighted in red indicate organisms sequenced for this study. Nodes with ≥0.95 posterior probabilities are shown. Scale is in number of substitutions per site.

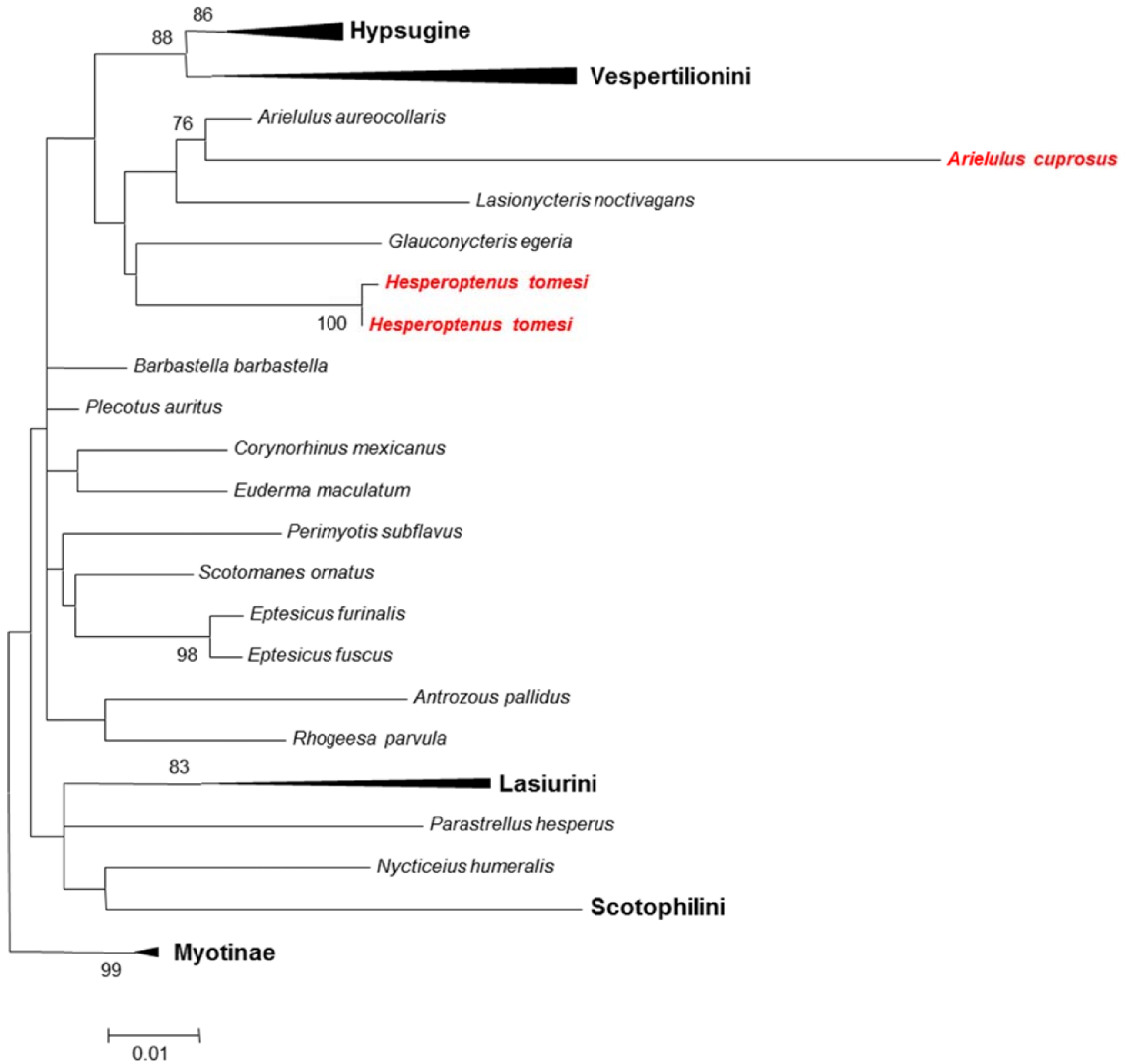

FIG. 4. Phylogram resulting from ML analysis of the RAG2 nuclear gene (35 taxa, 1231 nucleotides, K2+G, 1000 pseudoreplicates). Specimens highlighted in red indicate organisms sequenced for this study. Nodes with ≥70% bootstrap values are shown. Scale is in number of substitutions per site.

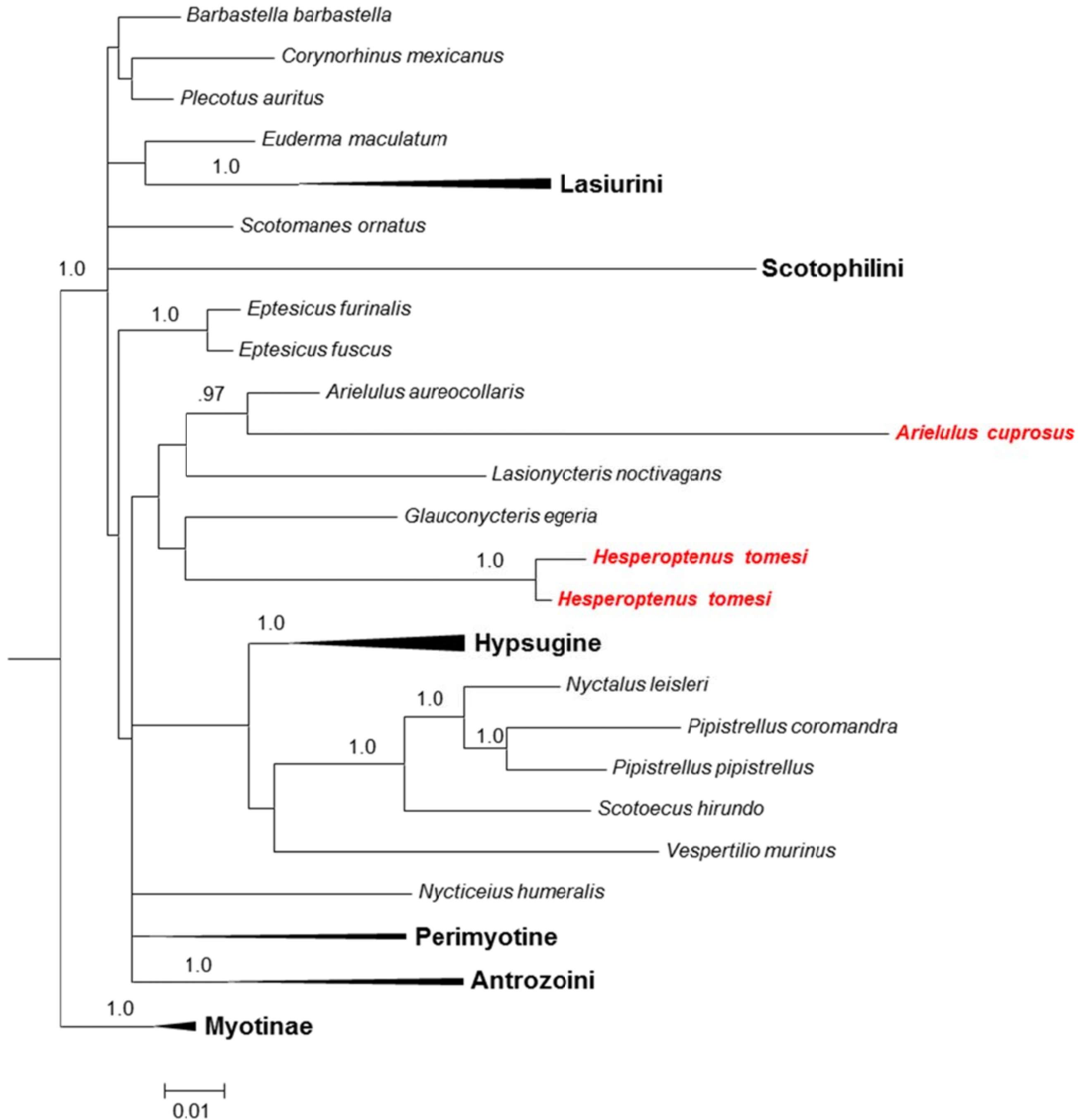

FIG. 5. Phylogram resulting from BI analysis of the RAG2 nuclear gene (35 taxa, 1231 nucleotides, K2+G, 1×10^6 generations). Specimens highlighted in red indicate organisms sequenced for this study. Nodes with ≥0.95 posterior probabilities are shown. Scale is in number of substitutions per site.

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APPENDIX

Species examined- Specimens utilized in this study acquired from GenBank and their accession numbers. mtDNA sequences include both 12S and 16S sequences. All specimens were originally published by Lack and Van Den Bussche (2010).

Taxon	mtDNA	RAG2
Antrozous pallidus	AF326088	GU328047
Arielulus aureocollaris	HM561624	HM561643
Barbastella barbastella	AF326089	GU328049
Baeodon alleni	AF326108	--
Chalinolobus gouldii	AY495461	HM561665
Corynorhinus mexicanus	AF326090	GU328053
Eptesicus furinalis	AF263234	AY141030
Eptesicus fuscus	AF326092	GU328058
Euderma maculatum	AF326093	GU328060
Glauconycteris egeria	AY495470	HM561654
Hypsugo savii	AY495475	HM561667
Laephotis namibensis	AY495477	HM561668
Lasionycteris noctivagans	AF326095	GU328065
Lasiurus borealis	AY495480	HM561637
Lasiurus ega	AY495483	HM561639
Lasiurus intermedius	HM561627	HM561640
Myotis californicus	AY495495	GU328078
Myotis velifer	AF263237	AY141033
Myotis volans	AY495510	GU328092
Neoromicia somalicus	AY495516	HM561671
Nyctalus leisleri	AY495517	HM561567
Nycticeius humeralis	AF326102	GU328096
Parastrellus hesperus	AY495522	GU328099
Perimyotis subflavus	AY495523	GU328103
Pipistrellus coromandra	AY495524	GU328102
Pipistrellus pipistrellus	AY495529	HM561662
Plecotus auritus	AF326106	GU328100
Rhogeessa parvula	AF326109	GU328108
Scotoecus hirundo	AY495536	HM561664
Scotomanes ornatus	AY495537	HM561656
Scotophilus leucogaster	AY395867	GU328114
Vespadelus regulus	AY495539	GU328119
Vespadelus murinus	AY395866	HM561676