Molecular phylogeny of hipposiderid bats from Southeast Asia and evidence of cryptic diversity

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ARTICLE INFO

ABSTRACT

Old World leaf-nosed bats (Hipposideridae) are among the most widespread and ecologically diverse groups of insectivorous bats in the Old World tropics. However, phylogenetic relationships in Hipposideridae are poorly resolved at both the generic and species levels, and deep genetic divergence within several Southeast Asian species suggests that current taxonomy underestimates hipposiderid diversity in this region. We used mitochondrial and nuclear sequence data to conduct the first extensive molecular phylogenetic analysis of Southeast Asian hipposiderid bats. Inclusion of multiple samples per taxon allowed testing for evidence of evolutionarily distinct lineages within taxa currently defined as single species. In contrast to earlier phylogenies based on morphometrics, molecular data support monophyly of *Hipposideros*, but are ambiguous regarding the monophyly of Hipposideridae. With a few exceptions, molecular data also support currently recognized species groups classified by qualitative morphological characters. Widespread paraphyly and polyphyly within many currently recognized species of *Hipposideros* indicates that evolutionary diversity in the genus is underrepresented by current nomenclature. Comparison of available morphological and echolocation data suggest that both geographic isolation and ecological selection have contributed to the diversification of Southeast Asian hipposiderid bats.

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1. Introduction

As we face increasing levels of extinctions due to human impacts on the environment, there is a new urgency to document and understand biodiversity (Ehrlich and Pringle, 2008; Myers and Knoll, 2001; Wake and Vredenburg, 2008; Willis et al., 2008). Although biodiversity is the sum of biological processes, ecosystems, organisms, and genetic diversity, species are most often the primary unit of study for assessing biodiversity (Wilson, 1999). Species traditionally have been defined based on morphological criteria; however, many animals live and communicate in a world dominated by tactile, auditory, and/or chemical cues, and may exhibit little differentiation in morphological characters (e.g. Hebert et al., 2004; Irwin et al., 2001; Kingston et al., 2001; Knowlton, 1993; Smith et al., 2007). Measures of range size, niche width, and intraspecific variation may be overestimated if morphologically cryptic species are not recognized, which can result in

incorrect conclusions about habitat specificity, levels of biodiversity, and species response to habitat alteration and climate change. To accurately assess the number of species both locally and globally, and to set conservation priorities, it is essential to identify and describe cryptic diversity.

Cryptic diversity is predicted to be relatively common in bats, especially in Hipposideridae and Rhinolophidae, which have highly specialized echolocation systems that potentially constrain social communication (Jones, 1997). Hipposideridae, also known as roundleaf or Old World leaf-nosed bats, is one of the most widespread and abundant groups of insectivorous bats in the Old World tropics, consisting of species inhabiting tropical and subtropical regions of Africa and the Middle East, through Asia and Australia (Simmons, 2005). Including three recently described species, *Hipposideros boeadii*, *Hipposideros khaokhouayensis* and *Hipposideros khasiana* (Bates et al., 2007; Guillen and Francis, 2006; Thabah et al., 2006), there are 70 species currently recognized within *Hipposideros* and 14 species spread across the remaining eight genera (*Anthops*, *Asellia*, *Aselliscus*, *Coelops*, *Cloeotis*, *Paracoelops*, *Rhinonicteris*, and *Triaenops*; Simmons, 2005). Species within *Hipposideros* historically have been grouped based on morphology. Tate (1941)

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described 11 species groups in his revision of the genus, whereas Hill (1963) only recognized seven distinct groups. Koopman (1994) and Simmons (2005) subsequently modified the classification scheme into the nine currently recognized species groups (Table 1). However, it is not known whether these morphological groups reflect evolutionary relationships within the genus.

The first quantitative analyses of phylogenetic relationships within Hipposideridae were based solely on morphology (Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998). There was little agreement among the morphological phylogenies, but they shared three main conclusions: Hipposideridae formed a monophyletic group, Hipposideros was paraphyletic, and the morphological characters used were plagued with homoplasy. A more recent study utilized all available systematic data to infer a bat supertree and found little resolution within Hipposideridae, highlighting the lack of congruence among morphological phylogenies (Jones et al., 2002). Several recent studies have used molecular markers to examine population level divergence within species (Armstrong, 2006; Chinnasamy et al., 2011; Russell et al., 2007, 2008), assess evolutionary relationships among a few hipposiderid species (Guillen and Francis, 2006; Kanagaraj et al., 2010; Li et al., 2007; Sun et al., 2009; Vallo et al., 2008; Wang et al., 2003), and examine the feasibility of DNA barcoding in Southeast Asian bats (Francis et al., 2010). The later study included 21 hipposiderid species and demonstrated previously unrecognized diversity in the family, but the basal nodes of the mitochondrial COI gene tree lacked resolution and phylogenetic relationships were not discussed (Francis et al., 2010). Currently, there are no published molecular phylogenetic studies that include a large number of hipposiderid species. Moreover, it has been over 40 years since the last major revision of Hipposideros (Hill, 1963). In that time, approximately 30 new species of Old World leaf-nosed bats have been described (e.g., Bates et al., 2007; Flannery and Colgan, 1993; Francis et al., 1999; Guillen and Francis, 2006; Hill and Yenbutra, 1984; Hill et al., 1986; Huihua et al., 2003; Kitchener and Maryanto, 1993; Kock and Bhat, 1994; Smith and Hill, 1981; Topal, 1975, 1993). Deep genetic divergence within several lineages recently considered conspecific suggests that additional cryptic diversity within Hipposideros remains to be described (e.g. Francis et al., 2010; Kingston et al., 2001; Thabah et al., 2006).

In this study we use mitochondrial and nuclear sequence data to infer phylogenetic relationships among leaf-nosed bats from Southeast Asia. We compare our results to those of morphology-based phylogenies and ask whether Hipposideridae and Hipposideros are monophyletic groups. We examine the currently recognized species groups within Hipposideros (Simmons, 2005), and determine whether divergent genetic lineages exist within species defined by morphology. Because many species of uncertain taxonomic status are morphologically cryptic but differ in echolocation call frequency, we include available data on body size and call frequency for comparative purposes.

2. Materials and methods

2.1. Sampling

Bats were identified to species using morphological characters and echolocation call frequency following Medway (1982), Payne and Francis (1985), and Kingston et al. (2006), supplemented by recent revisions or descriptions of individual taxa (Bates et al., 2007; Thabah et al., 2006). Some individuals were captured in the same area and fit the same published species description, but differed in other morphological characters and/or echolocation call frequency (e.g., Hipposideros larvatus, Hipposideros cineraceus, and Hipposideros pomona). We distinguish among these taxa in our analyses using numbers or letters for the different lineages within each recognized species.

Field research for this study was focused throughout peninsular Malaysia at cave roosts and in forested areas, and supplemented by museum specimens and vouchers collected by other researchers working in Southeast Asia. Three of the four hipposiderid genera (Aselliscus, Coelops, and Hipposideros) and representatives of all five species groups of Hipposideros (bicolor, pratti, diadema, armiger, and larvatus groups; Table 1) that occur in Southeast (SE) Asia were included in our study. Following current taxonomy (Bates et al., 2007; Guillen and Francis, 2006; Simmons, 2005; Thabah et al., 2006), 25 of the approximately 40 species of Old World leaf-nosed bats described from mainland SE Asia east to Sulawesi were analyzed (Fig. 1; Appendix A). Rhinonicteris aurantia from Australia, Hipposideros fulvus from Sri Lanka and an individual of H. pomona from China were also included in our analyses. Fifteen recognized SE Asian species were not included in the study because samples were not available. Of these, 10 are considered rare or limited in range: two are known only from the type specimens (Hipposideros nequam and Paracoelops megalotis), seven have very limited ranges and/or are extremely rare (Hipposideros breviceps, Hipposideros coxi, Hipposideros inexpectatus, Hipposideros madurae, Hipposideros macrobullatus, Hipposideros orbiculus, and Hipposideros sorenseni), and at least one is of questionable taxonomic status (Hipposideros crumeniferus; Simmons, 2005). When possible, we sequenced multiple individuals (2–20) within species, including representatives from different localities to identify possible divergent lineages in widely distributed species. One individual from each unique lineage or location was included in the final analyses.

Two Rhinolophus species, Rhinolophus creaghi and Rhinolophus stheno, were used as the outgroup in this study. Both

Table 1
List of currently recognized Hipposideros species, classified into morphological species groups (in bold) as recognized by Simmons (2005) and Koopman (1994). These classifications largely follow Hill (1963), except that Hill considered the commersoni group to be part of the diadema group, and the larvatus group to be part of the speoris group. The recently described species, H. khaokhouayensis and H. khasiana were grouped based on their morphological similarities to other species in their groups (Guillen and Francis, 2006; Thabah et al., 2006). Bates et al. (2007) noted that H. boeadii could not be readily matched with any group.

Armiger group	Bicolor group (cont.)	Cyclops group	Larvatus group
H. armiger[a]	H. hypophyllus	H. camerunensis	H. grandis
H. turpis	H. jonesi	H. corynophyllus	H. khasiana
	H. khaokhouayensis[a]	H. cyclops	H. larvatus[a]
Bicolor group	H. lamottei	H. edwardshilli	H. madurae
H. ater[a]	H. macrobullatus	H. muscinus	H. sorensenii
H. beatus[b]	H. maggietaylorae	H. semoni	H. sumbae
H. bicolor[a]	H. marisae	H. stenotis	
H. breviceps[b]	H. nequam	H. wollastoni	**Megalotis group**
H. caffer[b]	H. obscurus[a]		H. megalotis
H. calcaratus	H. orbiculus	**Diadema group**	
H. cervinus[a,b]	H. papua[b]	H. demissus	**Pratti group**
H. cineraceus[a]	H. pomona[a]	H. diadema[a]	H. lylei[a]
H. coronatus	H. pygmaeus[b]	H. dinops	H. pratti
H. coxi[b]	H. ridleyi[a]	H. inexpectatus	H. scutinares[a]
H. cruminiferus	H. rotalis[a]	H. inornatus	
H. curtus[b]	H. ruber	H. lankadiva	**Speoris group**
H. doriae[a]		H. lekaguli	H. abae
H. durgadasi	**Commersoni group**	H. pelingensis[a]	H. speoris
H. dyacorum[a]	H. commersoni		
H. fuliginosus[b]	H. gigas		**Unclassified**
H. fulvus[a]	H. thomensis		H. boeadii[a]
H. galeritus[a,b]	H. vittatus		
H. halophyllus[a]			

[a] Species included in the current study.
[b] Hill (1963) placed these species in the galeritus subgroup within the bicolor group, while the remaining species were in the bicolor subgroup.
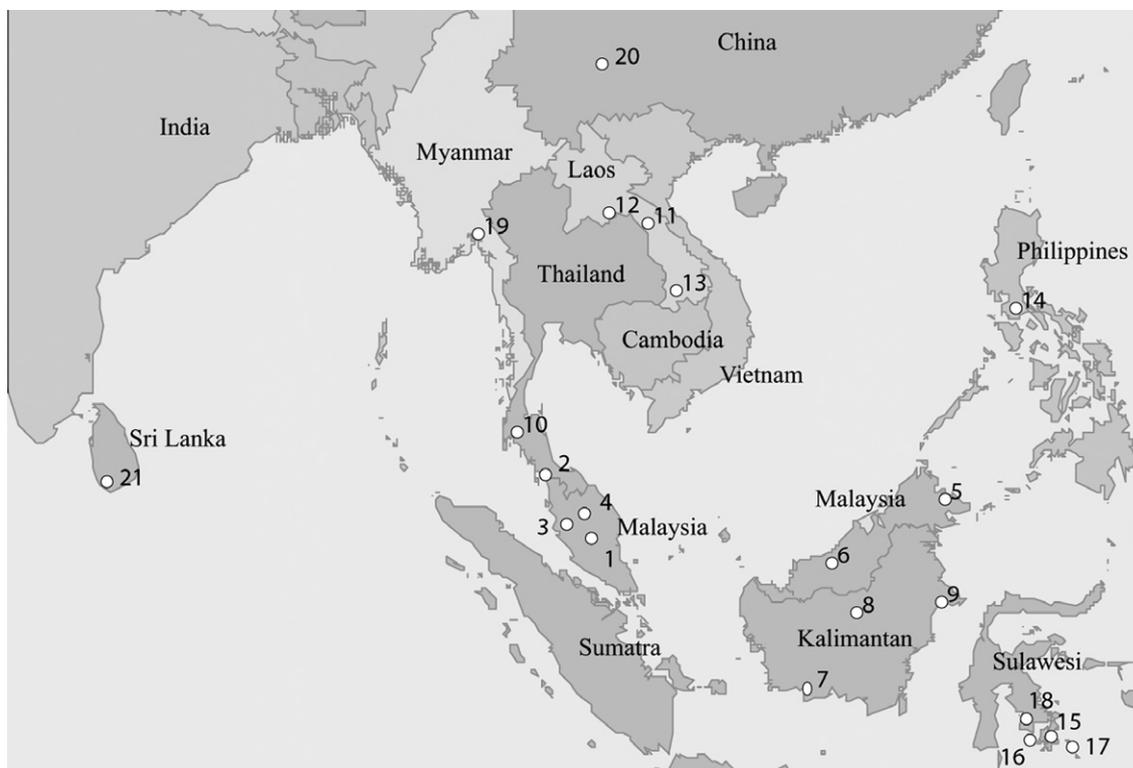
Fig. 1. Map of sample localities: 1. Pahang, Malaysia; 2. Perlis, Malaysia; 3. Perak, Malaysia; 4. Kelantan, Malaysia; 5. Sabah, Malaysia; 6. Sarawak, Malaysia; 7. Central Kalimantan-a, Indonesia; 8. Central Kalimantan-b, Indonesia; 9. East Kalimantan, Indonesia; 10. Krabi, Thailand; 11. Khammouan, Laos; 12. Vientiane, Laos; 13. Champassak, Laos; 14. Luzon, Philippines; 15. Buton Island, Sulawesi; 16. Kabaena Island, Sulawesi; 17. Lintea Island, Sulawesi; 18. Kendari, Southeast Sulawesi; 19. Mon State, Myanmar; 20. Yunnan Province, China; 21. Wavulpane, Sri Lanka. Sequence data for *Rhinonicteris aurantia* from Australia were included, but locality data are not shown. The island of Borneo includes the Malaysian states of Sabah and Sarawak and the Indonesian states of East, West, and Central Kalimantan. The map was created using www.planiglobe.com.

morphological and molecular evidence strongly support the sister relationship and reciprocal monophyly between Hipposideridae and Rhinolophidae (Baker et al., 1991; Eick et al., 2005; Hutcheon and Kirsch, 2004; Hutcheon et al., 1998; Simmons, 1998; Simmons and Geisler, 1998; Springer et al., 2001; Teeling et al., 2002, 2005). Although many researchers have considered Hipposideridae as a subfamily of Rhinolophidae (Koopman, 1993, 1994; McKenna and Bell, 1997; Novacek, 1991; Simmons, 1998; Simmons and Geisler, 1998; Teeling et al., 2002; Van Valen, 1979), we recognize them as separate families based on the differences in molecular, immunological, and morphological characters (Bates and Harrison, 1997; Bogdanowicz and Owen, 1998; Corbet and Hill, 1992; Hand and Kirsch, 1998; Pierson, 1986; Simmons, 2005).

2.2. DNA extraction, PCR and sequencing

Total genomic DNA was isolated from ethanol preserved tissue using DNeasy Tissue Kits (Qiagen) and associated protocols. We amplified 509 base pairs (bp) of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) with primers L5758.M (5'-GGH TGA GGN GGM CTN AAY CAR AC-3') and H6305.M (5'-GGC TTT GAA GGC YCT TGG TC-3'; M. Sorenson, pers. com.). The likelihood of amplifying nuclear copies of ND2 was reduced by using degenerate primers (Sorenson et al., 1999), and there were no insertions, deletions or stop codons in the translated sequences, suggesting that the sequences were indeed of mitochondrial origin. For all but nine individuals (Appendix A), we amplified 761 bp of the nuclear recombination activating gene 1 (RAG1) with primers F1705 (5'-GCT TTG ATG GAC ATG GAA GAA GAC AT-3') and R2864

(5'-GAG CCA TCC CTC TCA ATA ATT TCA GG-3') from Teeling et al. (2000).

Partial sequences for ND2 and RAG1 were amplified in 25 µl reaction volumes containing 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.2 mM of each primer, 0.5 units of Ampli*Taq* gold DNA Polymerase with appropriate 10X buffer (Applied Biosystems). All double stranded amplifications started with an initial denaturation at 95 °C (9 min), followed by strand denaturation at 94 °C (30 s), annealing at 59–48 °C (45 s), and primer extension at 72 °C (1 min) repeated for 35 cycles followed by a final extension at 72 °C for 7 min. The touchdown PCR protocol was used, wherein the first cycle had an annealing temperature of 59 °C and each successive cycle had an annealing temperature one degree lower than the previous until the annealing temperature attained and remained at 48 °C. Amplified DNA fragments were purified with EXO-SAPIt (USB Corporation) or run on a 1.5% agarose (Promega) gel, excised using a sterile razor blade, and purified using QIAquick Gel Extraction Kit (Qiagen). Cycle sequencing reactions were labeled with Big Dye terminator (ver. 3.1, Applied Biosystems), purified using Sephadex columns, and run on an ABI 3100 automated Genetic Analyzer (Applied Biosystems).

Three additional taxa were added to the study using ND2 sequences from GenBank: *R. aurantia* from Australia and two hipposiderids from Sulawesi (*Hipposideros diadema* and *Hipposideros galeritus*). RAG1 amplifications for outgroup taxa were unsuccessful, so we concatenated the RAG1 sequence of *R. creaghi* from GenBank with the ND2 sequence of *R. stheno* produced in this study. Both morphometric and molecular phylogenies for Rhinolophidae place *R. creaghi* and *R. stheno* in the same clade (Bogdanowicz, 1992; Guillen et al., 2003).

2.3. Phylogenetic reconstruction

Both ND2 and RAG1 are coding genes so the sequences were edited and aligned by eye using CodonCode Aligner (ver. 1.5.2, CodonCode Corporation). Modeltest version 3.7 (Posada and Crandall, 1998) was used to estimate the best-fit evolutionary model of nucleotide substitution for the mitochondrial, nuclear, and concatenated datasets. For both the ND2 and concatenated datasets the general time reversible (GTR) model of sequence evolution with a gamma (Γ) distribution of rates and a proportion of invariant sites (I) had the best Akaike information criterion (AIC) and likelihood ratio test (LRT) scores. Using the AIC, the best-fit model for the RAG1 dataset was the transitional model with equal base frequencies (TIMef) + Γ + I, whereas the best model based on the LRT was the Kimura two parameter + Γ (K80; Kimura, 1980). We chose to use the TIMef + Γ + I model for the RAG1 dataset because the AIC penalizes for extraneous parameters and tests for goodness of fit (Posada and Buckley, 2004). Corrected and uncorrected (observed) genetic distances were calculated in PAUP* version 4.0b10 (Swofford, 2000); corrected genetic distances were estimated using maximum likelihood settings and parameters from Modeltest.

Congruence between the nuclear and mitochondrial data was tested using the partition homogeneity test in PAUP*, which is equivalent to the incongruence length difference (ILD) test of Farris (Farris et al., 1994, 1995; Yoder et al., 2001). All uninformative characters were excluded and 100 replicates were run using a heuristic search. There was no significant difference between data partitions ($p = 1.0$), so all remaining analyses were conducted with the concatenated dataset.

Phylogenetic analyses using maximum parsimony (MP) and maximum likelihood (ML) criteria were inferred in PAUP*. The MP analysis used equal character weights, an heuristic search, and 5000 random addition-sequence replicates with tree-bisection-reconnection (TBR). Nodal support was estimated with 1000 bootstrap replicates, each with 100 addition-sequence replicates and TBR. The ML analysis used an heuristic search with 200 addition-sequences, TBR, and the GTR + Γ + I model fit to the concatenated data set (base frequencies: A = 0.327, C = 0.307, G = 0.167, T = 0.1986; rate matrix: A–C = 0.881, A–G = 5.678, A–T = 0.669, C–G = 0.193, C–T = 10.890, G–T = 1.000; gamma distribution shape parameter: 0.573: proportion of invariant sites: 0.559). Nodal support for the ML analysis was assessed in PAUP* with 100 bootstrap replicates, each with one addition-sequence replicate.

Bayesian inference (BI) was implemented in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) for the concatenated dataset using the GTR + Γ + I model with parameters estimated in the analysis. Two sets of four chains (three hot and one cold) were run for 1,000,000 generations, sampling trees every 100 generations. The two sets of chains converged, and we excluded the first 2500 samples as the burnin time and calculated a consensus tree and posterior probabilities with the remaining samples. We repeated the Bayesian analysis three additional times to control for the possibility of finding a local but not global best solution due to the randomly selected initial tree for each run.

3. Results

3.1. Comparison of inference methods

The concatenated data matrix comprised 1270 bp with 319 parsimony informative sites: 254 out of 509 bp for ND2 and 65 out of 761 bp for RAG1. For the MP analysis, there were two most parsimonious trees, each with 1754 steps. Among the four separate BI runs there were no differences in tree topology, so mean posterior probabilities were calculated across the four runs. There were some differences in topology among the BI, MP and ML trees (Figs. 2–4), but all analyses recovered the same four basal clades, as labeled in the BI tree (Fig. 4: clades A–D). With all of the poorly supported nodes collapsed, there were no differences in topology between the ML and BI trees (Figs. 3 and 4). However, a few species demonstrated affinities to different clades in the MP analysis compared to the ML/BI trees. In clade B, for example, *H. galeritus* from Sulawesi was sister to all *Hipposideros cervinus* in both the ML and BI analyses (Fig. 3, ML bootstrap <50; Fig. 4, posterior probability (pp) = 84), but in the MP tree, *H. galeritus* from Sulawesi fell within all other *H. galeritus* (Fig. 2, bootstrap <50). Although the relationship between *H. galeritus* from Sulawesi and the remainder of clade B remains equivocal, the MP analysis probably was affected by long-branch attraction (see relative branch lengths in Fig. 4; Felsenstein, 1978). A second difference between the MP and ML/BI analyses was the placement of *H. fulvus* from Sri Lanka. This taxon was in a poorly supported clade with *Hipposideros ater* from Borneo and *Hipposideros bicolor* from the Philippines in the MP analysis (Fig. 2, bootstrap <50), whereas in the ML and BI trees, *H. fulvus* was sister to a clade containing *Hipposideros ridleyi* and an unnamed species from Sulawesi (Fig. 3, ML bootstrap = 77; Fig. 4, pp = 99). It is likely that the inclusion of more taxa from the Indian subcontinent would help solidify the position of *H. fulvus* within the *bicolor* group.

Both *H. boeadii* and *Hipposideros obscurus* proved troublesome in the current analyses and accounted for the final difference in tree topology. There was little support for the placement of these taxa in the MP analysis (bootstrap <50), with *H. obscurus* basal to *Hipposideros*, and *H. boeadii* sister to clade D (Fig. 2). In both the ML and BI trees, *H. obscurus* and *H. boeadii* were sister taxa (ML bootstrap = 57; pp = 100), and formed a basal polytomy with clades B–D (Figs. 3 and 4). The lack of stability of the positions of *H. boeadii* and *H. obscurus* did not affect relationships among other species in the analyses. However, removing one of these taxa at a time in the Bayesian analysis affected the position of the other (data not shown): *H. boeadii* demonstrated an affinity to clade D in the BI tree when *H. obscurus* was excluded (pp = 76), and when *H. boeadii* was excluded *H. obscurus* was sister to clade B (pp = 56). Clearly, more taxa and molecular markers are needed to clarify the positions of *H. boeadii* and *H. obscurus* within Hipposideridae.

3.2. Genetic distances

Genetic divergence between *Rhinolophus* and each ingroup taxon was 16.3–21.8% for ND2 and 5.8–9.1% for RAG1. Pairwise sequence divergence among the genera *Aselliscus*, *Coelops* and *Hipposideros* ranged from 13.2% to 21.8% for ND2, and 2.0–5.9% for RAG1. *Rhinonicteris* sequence data were only available for ND2; there was 17.7–22.6% observed divergence between *Rhinonicteris* and the other three genera of the ingroup, and only 20% divergence between *Rhinonicteris* and *Rhinolophus*. Genetic divergence within the genus *Hipposideros* ranged from 0.6% to 19.6% for ND2 and from 0% to 5.5% for RAG1.

3.3. Relationships within Hipposideridae

Monophyly of Hipposideridae was equivocal; the position of *R. aurantia* was uncertain but basal to the remainder of the family (Fig. 4). As noted in Section 3.2, genetic distances for this species were among the highest for ND2, and pairwise distance between *Rhinonicteris* and the remaining ingroup genera was generally higher than the distance between *Rhinonicteris* and the outgroup. Resolution of the question of monophyly for Hipposideridae will require inclusion of the hipposiderid genera not represented in this
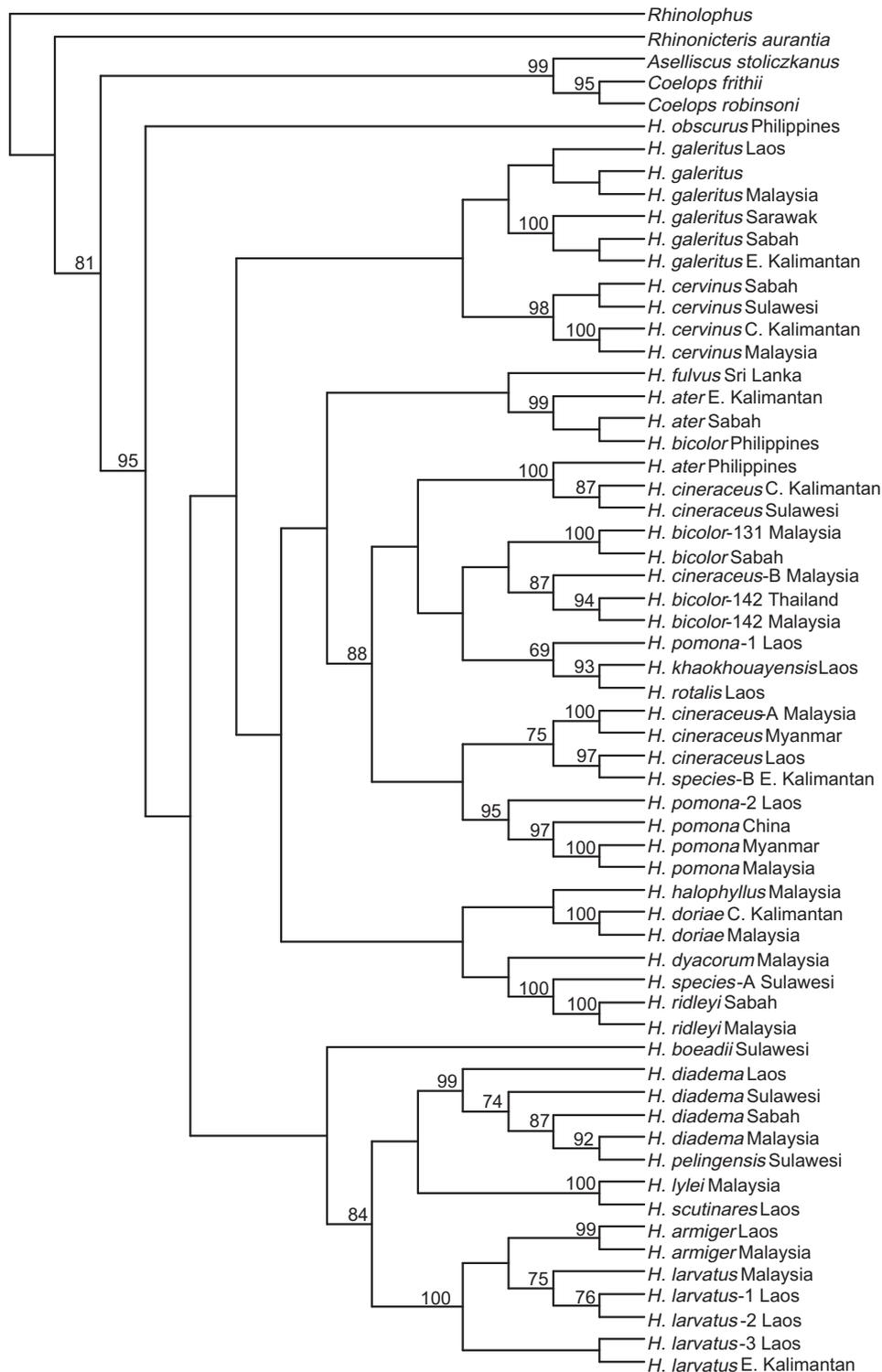
Fig. 2. Majority rule consensus tree of two most parsimonious trees, each with 1754 steps. The trees were inferred using maximum parsimony criterion in PAUP* for the concatenated data set (ND2 and RAG1 genes). Bootstrap values greater than 50 are shown to the left of each node and were estimated with 1000 bootstrap replicates.

analysis (i.e. *Anthops*, *Asellia*, *Cloeotis*, *Paracoelops* and *Triaenops*), together with nuclear sequence data for *Rhinonicteris*. Monophyly of the genus *Hipposideros* was, however, well-supported in all analyses (Figs. 2–4).

All three phylogenetic analyses recovered four main clades (Figs. 2–4). Clade A, which included *Aselliscus stoliczkanus*, *Coelops frithii*, and *Coelops robinsoni,* was the most basal and sister to all of *Hipposideros*. Within *Hipposideros*, clades B and C were sister to each other in all of the analyses, but there was little support

for this relationship (MP and ML bootstraps <50, pp = 50). If the branch supporting clades B and C is collapsed, clades B, C and D form a polytomy with *H. obscurus* and *H. boeadii* at the base of *Hipposideros* (Fig. 4).

Clade B included all individuals of *H. galeritus* and *H. cervinus*, which are in the *galeritus* subgroup (Hill, 1963; Table 1) of the *bicolor* species group (Simmons, 2005). In the ML and BI trees, *H. galeritus* was paraphyletic due to the placement of *H. cervinus* within *H. galeritus*, whereas *H. galeritus* and *H. cervinus* were
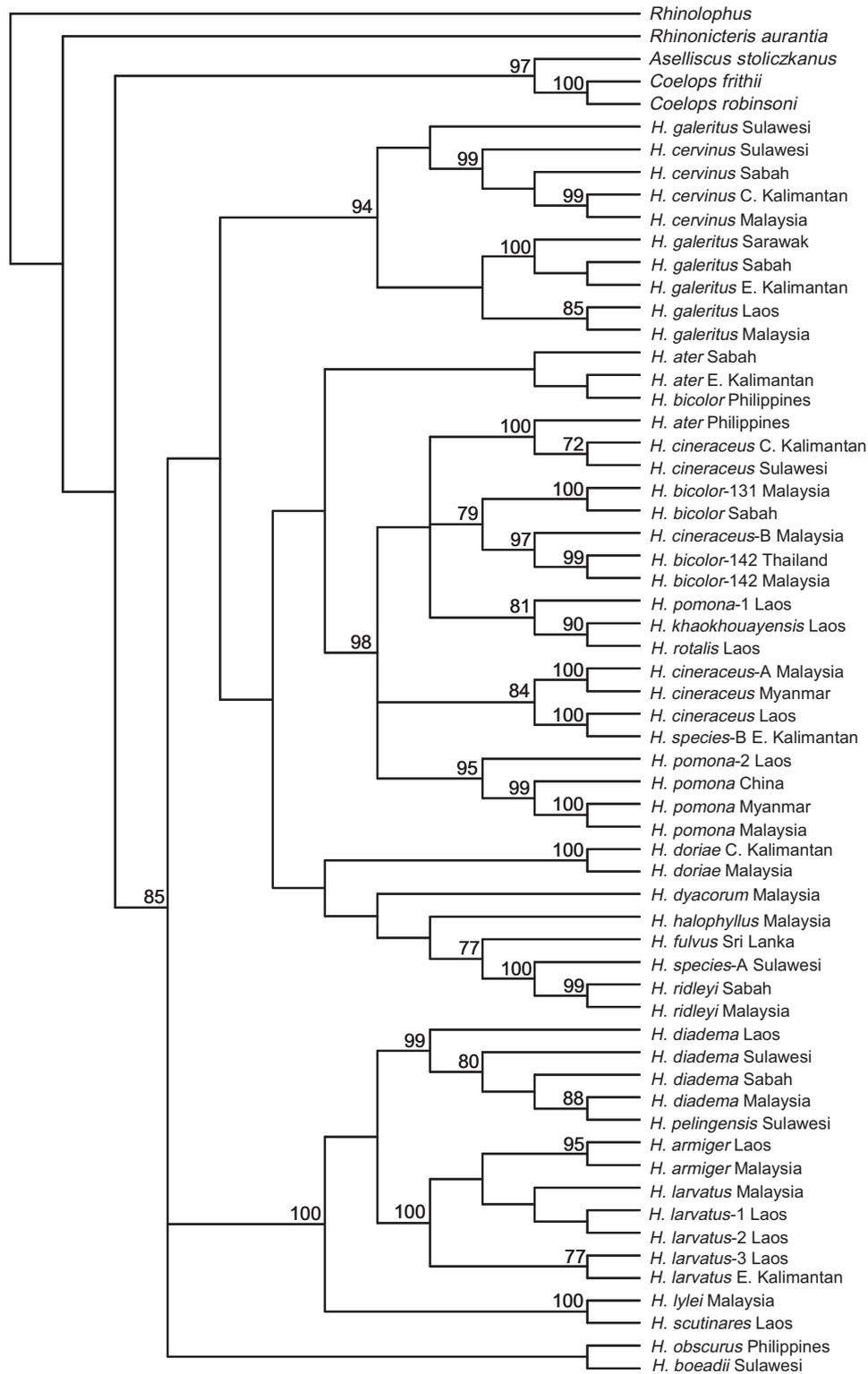
Fig. 3. Phylogenetic tree inferred in PAUP* using maximum likelihood criterion for the concatenated data set (ND2 and RAG1 genes). Bootstrap values were calculated from 100 bootstrap replicates; values greater than 50 are shown to the left of each node.

reciprocally monophyletic in the MP analysis (Figs. 2–4). However, the position of *H. galeritus* Sulawesi was poorly supported in the MP and ML trees (bootstraps <50) and only moderately supported in the BI tree (pp = 84). Sequence divergence between *H. galeritus* and *H. cervinus* (10.6–13.9%) was similar to the divergence between these groups and *H. galeritus* from Sulawesi (11.8–15.3%; Table 2), but there was higher sequence divergence within

H. galeritus (3.9–14.4%) compared to *H. cervinus* (2.8–7.5%; excluding *H. galeritus* Sulawesi).

Clade C comprised species assigned to the *bicolor* subgroup of the *bicolor* species group (Hill, 1963; Table 1). Although there were several well-supported groups within this clade (phylogroups 1–7, Fig. 4), the relationships among many of them were equivocal. Phylogroups 2–6 formed a well supported clade (pp = 99, MP
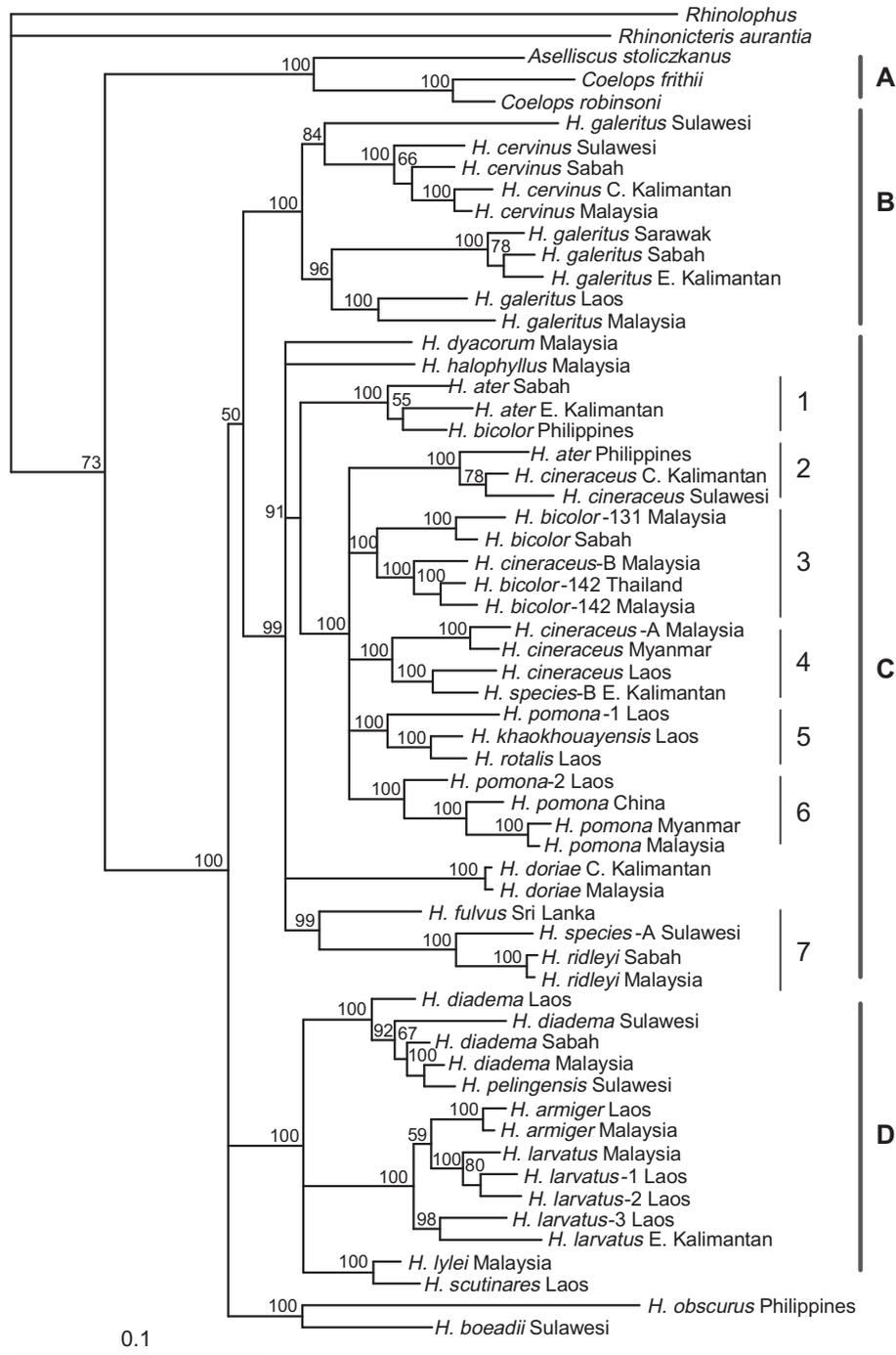
Fig. 4. Tree inferred using bayesian inference employed in MrBayes 3.1.2 for the concatenated dataset (ND2 and RAG1 genes) using the GTR + Γ + I model with parameters estimated in the analysis. Posterior probabilities, shown to the left of each node, were averaged across the four runs.

bootstrap = 88, ML bootstrap = 98), which was sister to group 1 (pp = 91, ML bootstrap = 69, MP bootstrap <50). Together, phylogroups 1–6 joined the polytomy at the base of clade C. Within phylogroups 1–6, four species were polyphyletic: *H. cineraceus*, *H. bicolor*, *H. ater*, and *H. pomona*. Maximum ND2 sequence divergence within these species was high, ranging from 13.6% to 15.1%.

Phylogroup 7 consisted of three morphologically divergent taxa from different geographical regions, with uncorrected sequence divergence for ND2 ranging from 7.3% to 13.4% (Table 3). *H. fulvus* is endemic to the Indian subcontinent (Bates and Harrison, 1997), *H. species*-A has only been documented from Sulawesi, and

H. ridleyi is only found in peninsular Malaysia, Singapore, and northern Borneo (Simmons, 2005). Although the relationships among *H. fulvus*, *H. ridleyi* and the undescribed species from Sulawesi were well-supported in the BI and ML analyses (pp = 99, ML bootstrap = 77), only the relationship between *H. ridleyi* and *H. species*-A had high bootstrap support in the MP analysis (bootstrap = 100). The individual representing *H. species*-A was very small (FA = 36.8 mm; Table 4) and resembled *H. cineraceus*-A in nose morphology (Fig. S1, Supplementary online), whereas *H. ridleyi* is a medium sized hipposiderid (mean FA = 48.3; Table 4) with a highly derived noseleaf (Fig. S1). *Hipposideros fulvus* is small

Table 2
Percent uncorrected (below the diagonal) and corrected (above the diagonal) genetic distances among individuals of *H. galeritus* (H.ga) and *H. cervinus* (H.ce) for ND2. The labels for individuals include the capture site: Sulawesi (Sul), Sabah (Sabah), Sarawak (Sar), East Kalimantan (E.Kal), Central Kalimantan (C.Kal), Malaysia (Mal), and Laos (Lao).

	H.ga Sul	*H.ce* Sab	*H.ce* C.Kal	*H.ce* Sul	*H.ce* Mal	*H.ga* Sab	*H.ga* Sar	*H.ga* E.Kal	*H.ga* Lao	*H.ga* Mal
H.ga Sul	–	24.5	29.5	27.1	28.9	44.1	44.8	49.0	24.7	27.6
H.ce Sab	11.8	–	7.4	7.8	6.1	30.3	28.5	27.4	23.3	21.5
H.ce C.Kal	13.6	6.3	–	10.0	2.6	38.9	37.4	37.0	24.6	20.1
H.ce Sul	12.8	6.3	7.5	–	8.9	32.0	29.7	28.7	25.9	25.2
H.ce Mal	13.4	5.5	2.8	7.1	–	34.9	35.4	34.9	22.9	18.6
H.ga Sab	14.5	11.8	13.8	12.8	13.0	–	5.0	4.2	24.6	37.1
H.ga Sar	14.7	12.0	13.9	12.2	13.6	4.5	–	5.0	25.6	35.3
H.ga E.Kal	15.3	11.0	13.4	12.0	13.0	3.9	4.5	–	29.4	35.3
H.ga Lao	11.8	11.2	11.6	12.2	11.2	10.8	11.2	12.0	–	17.3
H.ga Mal	13.6	11.4	11.0	12.8	10.6	14.1	13.8	13.8	11.0	–

Table 3
Percent uncorrected (below the diagonal) and corrected (above the diagonal) genetic distances for ND2 among the different phylogroups in the *bicolor* species group (Fig. 4 clade C). Percentages along the diagonal are within group uncorrected genetic distance.

	Group-1	Group-2	Group-3	Group-4	Group-5	Group-6	Group-7	*H. doriae*	*H. dyacorum*	*H. halophyllus*
Group-1	5.9–7.1	25.3–32.8	21.3–29.7	24.1–30.9	21.2–32.8	19.3–33.4	17.7–37.2	27.2–35.9	20.0–31.2	22.4–26.8
Group-2	13.2–16.5	5.1–8.4	16.4–24.7	18.4–26.0	16.5–21.7	16.3–26.3	23.9–39.1	28.6–35.8	27.2–29.1	24.4–30.6
Group-3	11.6–13.9	11.0–14.1	3.5–9.8	16.7–21.7	14.0–20.2	13.6–23.4	20.5–40.5	27.9–34.1	21.4–31.4	23.3–30.7
Group-4	12.6–14.7	12.2–14.5	10.2–12.2	3.7–10.6	16.0–24.6	13.7–25.4	24.2–38.7	35.7–39.2	30.2–34.7	24.4–34.8
Group-5	12.6–15.9	10.6–13.6	9.0–12.0	10.2–13.6	3.9–10.4	13.4–23.1	21.3–35.9	29.5–36.9	17.9–26.9	22.3–32.3
Group-6	12.0–15.9	11.6–14.9	9.4–12.6	9.6–13.8	9.6–14.3	1.8–8.4	19.8–37.2	26.3–43.5	20.1–28.0	23.9–34.3
Group-7	10.2–15.7	13.4–17.3	11.0–16.5	12.2–17.1	11.0–16.5	11.0–16.5	7.3–13.4[a]	25.0–35.9	17.6–23.8	17.0–28.0
H. doriae	13.0–14.9	14.3–15.9	13.6–15.3	14.7–16.1	13.8–16.3	13.4–17.3	12.4–16.1	0.6	23.4–24.7	22.0–22.3
H. dyacroum	11.4–12.8	12.6–13.4	11.6–13.4	12.0–14.7	11.0–14.3	12.0–14.5	9.6–13.2	11.8–12.0	–	18.5
H. halophyllus	11.0–14.1	13.0–14.1	11.2–13.4	13.4–14.1	10.0–13.8	11.0–13.2	10.2–13.4	11.6–11.8	10.4	–

[a] There was only 1.0% sequence divergence between *H. ridleyi* from Sabah and peninsular Malaysia.

(FA = 40.3 mm), and was formerly thought to be a subspecies of *H. bicolor* (Bates and Harrison, 1997; Hill et al., 1986).

Hipposideros dyacorum, *Hipposideros halophyllus*, and *Hipposideros doriae* formed a polytomy at the base of Clade C. Until recently, *H. doriae* from peninsular Malaysia was referred to *Hipposideros sabanus* (Kingston et al., 2006; Simmons, 2005). However, both *H. doriae* and *H. sabanus* were originally described from Sarawak (Borneo), and a recent morphological study comparing the lectotype of *H. doriae* (as designated by Benda (2000)) with individuals of *H. sabanus* from peninsular Malaysia found them to be synonymous (Benda, 2000). Consistent with this result, uncorrected ND2 sequence divergence between *H. doriae* from peninsular Malaysia and Central Kalimantan (Borneo) was the lowest in this study (0.6%). Following Benda (2000), *H. doriae* is accepted as the senior synonym.

Three subclades in clade D, comprising species in the *diadema*, *armiger*, *larvatus*, and *pratti* species groups, formed a basal polytomy each with posterior probabilities of 100 and bootstrap values >99 (Fig. 4; Table 1). *H. diadema* was paraphyletic with respect to *Hipposideros pelingensis* from Sulawesi, and sequence divergence between *H. pelingensis* and *H. diadema* from peninsular Malaysia was lower than divergence within *H. diadema* (2.8% vs. 2.9–8.1%; Table 5). Similarly, *H. larvatus* was paraphyletic with respect to *Hipposideros armiger*. *H. armiger* from Laos and peninsular Malaysia exhibited low levels of sequence divergence (2% for uncorrected ND2; Table 5) and formed a well-supported group (pp = 100, ML and MP bootstraps >95) within *H. larvatus*. However, the position of *H. armiger* relative to the *larvatus* group was unclear (pp = 59, ML and MP bootstraps <50). Within *H. larvatus*, two genetically distinct lineages had high support indices: *H. larvatus*-1 from Laos, *H. larvatus*-2 from Laos and *H. larvatus* from peninsular Malaysia formed one group, whereas *H. larvatus*-3 from Laos and *H. larvatus* from East Kalimantan (Borneo) formed the second group (Figs. 2–4). Finally, the two representatives of the *pratti* species group, *H. lylei* and *H. scutinares*, formed a monophyletic group (pp and bootstraps = 100).

4. Discussion

Ideally, taxonomic classifications should provide an index of biodiversity at the species level, and nomenclature should reflect ancestor–descendent relationships in deeper evolutionary time. Molecular phylogenies have provided tremendous insight into the position of bats in the mammalian radiation (Springer et al., 2004), and relationships at the base of Chiroptera (Teeling et al., 2005). However, relationships below the familial level remain poorly resolved for many lineages, particularly in the tropics where species diversity is highest. We conducted the first extensive molecular phylogenetic analysis of Hipposideridae, one of the most speciose and ecologically diverse families of Paleotropical insectivorous bats. Our results are in poor agreement with earlier morphological phylogenies, which supported monophyly for Hipposideridae but not *Hipposideros*. Within *Hipposideros*, molecular data generally support species groups as first defined by Hill (1963) and later Koopman (1994) and Simmons (2005), and conflict with some of the phylogenetic relationships inferred from morphology (Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998, 2003). Importantly, we find evidence for extensive undescribed diversity within *Hipposideros* species. Below, we briefly discuss the question of monophyly for Hipposideridae and *Hipposideros*, and then focus in more detail on relationships among and within species groups.

4.1. Monophyly of Hipposideridae

Phylogenies based on morphological characters have consistently supported monophyly of Hipposideridae (Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998, 2003). However, the equivocal position of *R. aurantia* at the base of Hipposideridae (Figs. 2–4) suggests that the hypothesis of monophyly for the family warrants re-evaluation. Historically, *Rhinonicteris* was closely allied with *Cloeotis* and *Triaenops* (not included in this study), based on the "cellular" posterior noseleaf found in all three genera (Hill, 1982). This taxonomy links species with disparate distributions: *R. aurantia*

Table 4
Length of forearm, echolocation call frequency and nose morphology (also see Fig. S1) of bats in clade C (Fig. 4). For species captured in peninsular Malaysia, the mean from multiple individuals are presented (n = sample size). For all other species, measurements are from the same individual listed in Appendix A, unless otherwise noted. All measurements are from adults.

Species	Subgroup (Fig. 5)	Locality	Forearm (mm) (n)	Echol. (kHz) (n)	Internarial septum
H. ater	1	Sabah	41.6	–	–
H. ater	1	E. Kalimantan	41.8[a]	–	–
H. bicolor	1	Philippines	43.1[b]	136[b]	Triangular[c]
H. ater	2	Philippines	41.1[b]	–	Straight and wide
H. cineraceus	2	Sulawesi	39.5[d]	–	Straight and wide
H. cineraceus	2	C. Kalimantan	40.1[a]	–	–
H. bicolor	3	Sabah	46.7		
H. bicolor-131	3	Malaysia	45.4 (146)	132.8 (31)	Straight
H. bicolor-142	3	Malaysia	42.9 (278)	142.3 (106)	Straight
H. cineraceus-B	3	Malaysia	39.3 (1)	144.0 (5)[e]	Small swelling
H. cineraceus-A	4	Malaysia	35.1 (44)	152.6 (16)	Straight and wide
H. cineraceus	4	Myanmar	34.5[f]	–	Straight and wide
H. cineraceus	4	Laos	35.4	–	–
H. species-B	4	E. Kalimantan	35.6[a]	–	–
H. pomona-1	5	Laos	41.3	–	–
H. khaokhouayensis	5	Laos	47.1 (8)[g]	88.6 (6)[g]	Small disk
H. rotalis	5	Laos	47.2 (11)[g]	70.3 (16)[g]	Large disk
H. pomona-2	6	Laos	42.1	–	–
H. pomona	6	China	43.8[f]	~131[h]	–
H. pomona	6	Malaysia	43.4 (3)	138.1 (3)	Straight
H. pomona	6	Myanmar	42.2[f]	–	Straight
H. ridleyi	7	Malaysia	48.3 (10)	62.4 (3)	Large disk
H. species	7	Sulawesi	36.8	–	Straight and wide
H. fulvus	7	Sri Lanka	40.3	–	Damaged
H. doriae	–	Malaysia	36.3[i]	–	Straight
H. doriae	–	C. Kalimantan	35.6[j]	–	–
H. halophyllus	–	Malaysia	38.1 (30)	185.4 (11)	Small disk
H. dyacorum	–	Malaysia	44.0 (8)	138.1 (7)	Very narrow

[a] M. Struebig (pers. comm.).
[b] J. Sedlock (pers. comm.).
[c] Internarial septum narrow in the middle and wider at the base.
[d] These measurements are from a specimen that was identified as the same species as the H. cineraceus used in the genetic analysis and differs by 7 base pairs for ND2.
[e] Kingston et al. (2000).
[f] Measurements from alcohol preserved specimens.
[g] Guillen and Francis (2006).
[h] G. Jones (pers. comm.).
[i] Forearm measurement from an individual captured at the same site as the one in the genetic analyses.
[j] Struebig et al. (2006).

is an Australian endemic while *Cloeotis* and *Triaenops* are mainly restricted to Africa (Simmons, 2005). While morphological phylogenies generally supported Hill's (1982) assessment (Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998, 2003), the relationship of *Rhinonicteris* to other hipposiderid genera was not stable across analyses. For example, Bogdanowicz and Owen (1998) found that *Rhinonicteris* was most closely related to *Triaenops* using mensural characters, whereas discrete-state characters placed *Rhinonicteris* in a clade with *Coelops*, *Cloeotis*, *Hipposideros pygmaeus*, and *Aselliscus*. Although molecular data recovered a well-supported sister relationship between *Coelops* and *Aselliscus* (Figs. 2–4; see also Li et al., 2007; Sun et al., 2009), *Rhinonicteris* was consistently basal to this clade, and fell outside of Hipposideridae in the Bayesian analysis (Fig. 4). Notably, corrected ND2 distances between *Rhinonicteris* and *Hipposideros* were, on average, higher than the pairwise distances between all other ingroup taxa and the outgroup. These results, together with the uncertain affinities of *Rhinonicteris* inferred from morphological and immunological data (Bogdanowicz and Owen, 1998; Pierson, 1986), suggest that Hipposideridae including *Rhinonicteris* may not be a monophyletic group.

While inclusion of representatives from all hipposiderid genera and nuclear data for *Rhinonicteris* will help resolve this issue, placement of *Rhinonicteris* in relation to extant hipposiderids will likely pose a challenge. Phylogenetic analysis of discrete-state morphological characters for extant and fossil hipposiderids placed *Rhinonicteris* in a basal clade with extinct taxa from Australia, suggesting that it is the sole extant representative of a once more diverse group (Hand

and Kirsch, 1998, 2003). Thus, deep divergence between *Rhinonicteris* and other extant hipposiderid genera may reflect extinction of closer relatives, rather than a unique evolutionary origin.

4.2. Monophyly of Hipposideros

The results of the current study support *Hipposideros* monophyly with respect to *Rhinonicteris*, *Aselliscus*, and *Coelops* (Figs. 2–4). In contrast, morphology-based phylogenies have consistently found *Hipposideros* to be paraphyletic (Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998, 2003). The morphological studies included eight of the nine extant genera of Old World leaf-nosed bats (excluding *Paracoelops*, which is known only from a damaged type specimen), whereas our study only included four. Thus, inclusion of *Anthops*, *Asellia*, *Cloeotis* and *Triaenops* could alter our inference of monophyly for *Hipposideros*. However, given that mensural data placed both *Coelops* and *A. stoliczkanus* within *Hipposideros* (Bogdanowicz and Owen, 1998), whereas molecular data place these taxa as basal to *Hipposideros*, we suspect that more complete taxon sampling will not resolve this conflict between morphological and molecular phylogenies.

4.3. Species groups of Hipposideros

Currently, there are nine recognized species groups within *Hipposideros* (Table 1; Koopman, 1994; Simmons, 2005), including all seven of Hill's (1963) groups, plus Tate's (1941) *commersoni* group

and a *larvatus* group, which was previously part of the *speoris* species group. While morphological phylogenies have provided little support for the hipposiderid species groups (Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998, 2003), molecular data are broadly in agreement with Hill's classification scheme. The *diadema* and *pratti* species groups are monophyletic, and the *bicolor* group is monophyletic if *H. obscurus* is removed (as recommended below). This suggests that the general features of cranial and external morphology studied by Hill (1963) are conserved within hipposiderid subclades.

Based on morphological characters, Hill (1963) found it difficult to determine the evolutionary relationships among what he considered the three main lineages (*bicolor* and *megalotis* groups; *cyclops* group; and the *armiger*, *diadema/commersoni*, *pratti*, and *speoris/larvatus* groups), suggesting that there was a very early split among them. Phylogenetic analysis recovered three main clades within *Hipposideros* (Fig. 4): clade B consisted of species in what Hill (1963) labeled as the *galeritus* subgroup of the *bicolor* group; clade C included species in the *bicolor* subgroup of the *bicolor* group; clade D included larger-bodied species in the *armiger*, *diadema*, *larvatus*, and *pratti* groups. The relationships between the three clades in this study are equivocal, possibly supporting an old but relatively rapid split among lineages as proposed by Hill (1963). The addition of more molecular markers and more taxa, including species from the *cyclops* group, could help resolve the polytomy at the base of *Hipposideros*.

The *galeritus* and *bicolor* subgroups were previously recognized as separate species groups by Tate (1941), but Hill (1963) merged them into the *bicolor* group based on unifying morphological characters. Specifically, *H. dyacorum* and *H. doriae* have shorter, more robust skulls that resemble members of the *galeritus* subgroup, while their external appearance is that of bats in the *bicolor* subgroup (Fig. S1; Benda, 2000; Hill, 1963; Tate, 1941). Tate (1941) placed both of these species in his *galeritus* group, whereas Hill (1963) assigned them to his *bicolor* subgroup. In support of Hill's position, molecular data suggest that *H. doriae* and *H. dyacorum* are more closely related to other species in the *bicolor* subgroup than to species in the *galeritus* subgroup (Fig. 4). Given that both the *galeritus* and *bicolor* subgroups formed well-supported exclusive clades (Fig. 4: clades B and C, respectively), we propose that the *galeritus* subgroup should be resurrected to group status. We also recommend the removal of *H. obscurus* from the *bicolor* group, as it fell outside of the main *Hipposideros* clades in this study (Fig. 4).

We found strong support for monophyly of Hill's large-bodied species lineage (Fig. 4, clade D). However, within this lineage Hill (1963) hypothesized a close relationship between the *armiger* and *pratti* groups, and between the *diadema* and *speoris* (including *larvatus*) groups. In contrast, our results support a closer relationship between members of the *armiger* and *larvatus* groups, while representatives of the *diadema* and *pratti* groups form distinct subclades. We suggest that the *armiger* and *larvatus* groups should be merged into one, for which *larvatus* is the earliest name. Following current taxonomy, the only other species in the *armiger* group is *H. turpis*, which was not included in this study. *Hipposideros t. alongensis* from Vietnam was originally identified as a subspecies of *H. larvatus* (Topal, 1993), suggesting that *H. turpis* is morphologically similar to both *H. armiger* and *H. larvatus*, and thus may fall within the proposed *larvatus* group.

4.4. Polyphyly, paraphyly and cryptic diversity

Morphologically cryptic species are predicted to be relatively common in bats, especially horseshoe bats (Rhinolophidae) and Old World leaf-nosed bats (Jones and Barlow, 2004). Thus, one of our primary objectives was to determine if species defined by

morphology contained genetically distinct lineages. Sampling for twelve species in *Hipposideros* was sufficient to address this question (see Appendix A for sample and locality data). We found no evidence for cryptic diversity in five of these species. *H. armiger* lineages from peninsular Malaysia and Laos differed by only 2% at ND2 (Table 5) and shared a RAG1 genotype (data not shown). Likewise, *H. doriae*, *H. dyacorum*, and *H. ridleyi* sampled from peninsular Malaysia and Borneo all exhibited less than 1% intraspecific sequence divergence at ND2 (Table 3; *H. dyacorum* Borneo not shown) and did not differ at RAG1. Moderate levels of divergence among *H. cervinus* lineages from peninsular Malaysia, Borneo and Sulawesi (2.8–7.5% at ND2; Table 2) may be explained by the complex geological histories of Sulawesi and the islands of the Sunda shelf (e.g. Moss and Wilson, 1998; Voris, 2000; Hall, 2002). However, more divergent lineages may exist within *H. cervinus*; we did not have samples from the entire species range, including the nominate form from Australia.

Of the remaining seven species, four were polyphyletic (*H. ater*, *H. bicolor*, *H. cineraceus*, and *H. pomona*; Fig. 4) and three were paraphyletic (*H. galeritus*, *H. diadema* and *H. larvatus*; Fig. 4). The following discussion of these taxa is organized by the species groups defined in Table 1. While our results strongly suggest that species delimited by morphology comprise multiple evolutionary lineages, there are limitations to the data presented here that are important to keep in mind. First, this study does not include extensive sampling for any one species and, therefore, has not sampled all potential populations and/or diversity. Just as additional evolutionarily distinct lineages may exist within species in our phylogeny, population level studies may reveal gene flow between lineages that appear distinct in the current analysis. While many of these species are in need of taxonomic revision, assignment of appropriate nomenclature will require additional sampling and examination of type specimens. Second, because there was little divergence in RAG1 within distal hipposiderid groups, inferences of evolutionary relationships are based mainly on a single mitochondrial gene. Thus, the ND2 gene tree represents an incomplete estimate of the true genealogies of taxa in this study (e.g., Avise et al., 1990; Hughes and Vogler, 2004; Morando et al., 2004).

4.4.1. Galeritus group

H. galeritus is a widespread taxon, occurring from Sri Lanka and India, throughout mainland Southeast Asia, Java and Borneo, and possibly to the Moluccas Islands east of Sulawesi (Bonaccorso, 1998; Simmons, 2005). In this study, *H. galeritus* samples from Borneo formed a well-supported monophyletic group (Fig. 4, Clade B) and exhibited relatively low sequence divergence (<5% uncorrected, Table 2). In contrast, uncorrected divergence among individuals from Borneo, peninsular Malaysia and Laos was 10.8–14.1%, a level consistent with species level divergence in bats (Baker and Bradley, 2006; Bradley and Baker, 2001; Jones, 1997). Length of forearm did not differ among individuals from Borneo (East Kalimantan: 46.5 mm; M. Struebig, pers. com.), Laos (46.5 mm) and peninsular Malaysia (*n* = 6, mean FA = 46.0 mm, standard deviation (SD) = 1.3 mm), although the individual from Sabah, Borneo was larger (FA = 49.3 mm). There were, however, substantial differences in the echolocation call frequency from each region: peninsular Malaysia (*n* = 3, mean call frequency = 89.1, SD = 1.5 kHz), Laos (~108 kHz), and Borneo (Sabah: ~115 kHz). Together, relatively large genetic distances and differences in echolocation call frequency suggest that *H. galeritus* lineages from Borneo, peninsular Malaysia and Laos represent three evolutionarily and ecologically distinct species.

H. galeritus was paraphyletic with respect to *H. cervinus* in the ML and BI trees due to the placement of the lineage from Sulawesi at the base of the *H. cervinus* subclade (Figs. 3 and 4). While echolocation data were not available for *H. galeritus* from Sulawesi, the individual

included in this study was larger than other *H. galeritus* or *H. cervinus* (FA = 52.3 mm). Thus, molecular and morphological data indicate that additional sampling of *H. galeritus* from Sulawesi and the Moluccas may uncover another undescribed species in this group.

4.4.2. Bicolor group

Based on data from RAG1 and ND2 genes, four species in the *bicolor* group were polyphyletic: *H. ater*, *H. bicolor*, *H. cineraceus*, and *H. pomona* (Fig. 4). *H. ater* fell into two separate clades in the analysis (Fig. 4). Phylogroup one consisted of *H. ater* from Borneo (Sabah and E. Kalimantan) and an individual from the Philippines identified as *H. bicolor*. Phylogroup two comprised *H. cineraceus* from Sulawesi and Borneo (C. Kalimantan) and *H. ater* from the Philippines. While all three individuals of *H. ater* were similar in size (Table 4), uncorrected sequence divergence between *H. ater* from Borneo and the Philippines was 13.2–14.1%. Deep divergence between polyphyletic lineages suggests that there are at least two distinct species within *H. ater*.

The Philippine *H. bicolor* included in this study was identified based on morphological similarity to *H. bicolor*-142 and an echolocation call frequency intermediate between the *H. bicolor* lineages from mainland SE Asia and Borneo (Table 4; J. Sedlock, pers. comm.). While additional sampling will be required to determine whether this lineage represents an undescribed species or is conspecific with a previously named form, (e.g. *Hipposideros erigens* Lawrence, 1939), molecular data strongly suggest that morphological similarity to other Southeast Asian *H. bicolor* is due to convergence, not recent common ancestry.

The two other lineages of *H. bicolor* are similar in external appearance (Fig. S1) and overlap in size, but are genetically distinct and differ in echolocation call frequency (Fig. 4; Table 4). Kingston et al. (2001) demonstrated that peninsular Malaysian *H. bicolor* comprised two divergent lineages, designated *H. bicolor*-142 and -131 according to echolocation call frequency. The same two phonic types are co-distributed on the southern portion of the Thai peninsula (Douangboubpha et al., 2010). While we recovered distinct lineages corresponding to *H. bicolor*-142 and -131, the presumed sister relationship between the two phonic types (i.e. Kingston et al., 2001) was not supported. All individuals of *H. bicolor* from Borneo grouped in the clade with *H. bicolor*-131 from peninsular Malaysia, whereas *H. bicolor*-142 was sister to *H. cineraceus*-B (Fig. 4).

Like *H. bicolor*, *H. cineraceus* included in this study comprise a minimum of three evolutionary lineages (Fig. 4, phylogroups 2–4), some of which exhibit differences in morphology and echolocation call frequency (Fig. S1; Table 4). For example, *H. cineraceus* from phylogroup 4 are smaller than individuals from phylogroups 2 and 3 (mean FA = 35 vs. 40 mm), and *H. cineraceus*-A and -B echolocate at 152 kHz and 144 kHz, respectively (Table 4). Given the sister relationship between *H. cineraceus*-B and *H. bicolor*-142, it is notable that the two taxa overlap in echolocation frequency (Table 4; Kingston et al., 2000). However, differences in morphology suggest that they are not conspecific: *H. cineraceus*-B is smaller (FA = 39.3 vs. 42.9 mm), and has a small swelling in the septum that separates the nostrils (internarial septum; Table 4, Fig. S1) that is absent in *H. bicolor*-142, and in other *H. cineraceus* lineages.

In addition to differences in morphology and echolocation, deep genetic divergences indicate that there may be four distinct species within *H. cineraceus*. Individuals from Central Kalimantan and Sulawesi (phylogroup 2, Fig. 4) only differed by 5.1%, but had genetic divergences of 11.0 – 15.1% with all other *H. cineraceus*. Likewise, the two groups within phylogroup 4 (*H. cineraceus*-A from Malaysia/*H. cineraceus* from Myanmar, and *H. cineraceus* from Laos/*H. species*-B from East Kalimantan, Borneo) demonstrated relatively low within-group genetic divergence (3.7–5.9%), but high genetic divergence relative to each other, and to other *H. cineraceus* (9.2–15.1%). Finally, *H. cineraceus*-B from peninsular Malaysia

differed from all other *H. cineraceus* in this study by 10.4–12.2% sequence divergence for the ND2 gene.

H. pomona fell into two distinct clades and likely consists of two species: *H. pomona*-1 from Laos formed phylogroup five with *Hipposideros rotalis* and *H. khaokhouayensis*, while a second lineage from Laos (*H. pomona*-2) grouped with other individuals identified as *H. pomona* from China, Myanmar, and peninsular Malaysia in phylogroup six (Fig. 4; Table 3). Together, *H. pomona*-1, *H. rotalis* and *H. khaokhouayensis* represent a small radiation that may be endemic to Laos, a country with several endemic or semi-endemic bat species (Guillen and Francis, 2006). Interestingly, two of these taxa are morphologically convergent with another species in the *bicolor* group. *H. pomona*-1 and *H. pomona*-2 from Laos are similar in appearance (C.M. Francis, pers. obs.) but are genetically divergent (12% uncorrected). *H. rotalis* has an expanded noseleaf and large internarial disk, which was thought to be a synapomorphy uniting *H. rotalis*, *H. ridleyi*, and *H. orbiculus* (Francis et al., 1999). However, molecular data place *H. ridleyi* and *H. rotalis* in different clades (this study; Guillen and Francis, 2006).

4.4.3. Diadema group

H. diadema is one of the larger species in the genus, is relatively common, and is distributed throughout Southeast Asia and into New Guinea, Australia and the Solomon Islands (Simmons, 2005). There have been several partial reviews of *H. diadema* and its allies based on morphology, which have designated no fewer than 14 subspecies and raised at least three additional subspecies to specific status (*Hipposideros demissus*, *H. inexpectatus*, and *Hipposideros inornatus*; Bonaccorso, 1998; Flannery, 1995a, 1995b; Kitchener et al., 1992; Laurie and Hill, 1954). Although, several of the subspecies that are currently recognized within *H. diadema* are of questionable status, there are island populations that have not yet been evaluated and may represent additional diversity (Simmons, 2005).

This study included *H. diadema* from Laos, peninsular Malaysia, Borneo and Sulawesi, and *H. pelingensis* from Kabaena Island in Southeast Sulawesi. *H. pelingensis*, a member of the *diadema* species group, was previously considered a subspecies of *H. dinops,* but raised to specific status based on geographic separation: *H. pelingensis* is endemic to Sulawesi while *H. dinops* occurs in the Solomon Islands (Flannery, 1995b; Simmons, 2005). The results of this study suggest that *H. diadema* is paraphyletic with respect to *H. pelingensis* (Fig. 4). Strikingly, there was a well-supported sister relationship between *H. pelingensis* and *H. diadema* from peninsular Malaysia, while the form currently referred to *H. diadema* from Sulawesi was more basal in the clade. Consistent with genetic data, *H. pelingensis* and *H. diadema* from peninsular Malaysia are similar in body size whereas *H. diadema* from Sulawesi is considerably smaller (T. Kingston, pers. obs.). However, *H. pelingensis* lacks the white spots on the dorsal pelage that typify *H. diadema* (T. Kingston, pers. obs.). While resolution of relationships in the *diadema* group awaits additional sampling on other Indonesian islands and inclusion of additional forms such as *H. dinops* and *H. inexpectatus*, the current data do not support species status for *H. pelingensis*. Likewise, the lack of a sister relationship between lineages from Sulawesi suggests a complex biogeographic history, in which *H. pelingensis* is a relatively recent arrival from the Sunda shelf, whereas the small-bodied *H. diadema* derives from an earlier colonization event.

4.4.4. Larvatus group

As with other hipposiderid species, there has been much confusion in the taxonomy of *H. larvatus* and its allies. The results of this and other studies suggest that there is more than one species within *H. larvatus* as it is currently defined. Kitchener and Maryanto (1993) reviewed the taxonomy of *H. larvatus* in the Greater and Lesser Sunda Islands and discriminated four distinct species using morphological characters: *H. larvatus*, *Hipposideros sumbae*, *H. madurae*, and *H. soren-*

Table 5
Percent uncorrected (below the diagonal) and corrected (above the diagonal) genetic distances for ND2 among *H. armiger* (*H.ar*), *H. diadema* (*H.di*),*H. pelingensis* (*H.pe*), *H. lylei* (*H.ly*), *H. scutinares* (*H.sc*), and *H. larvatus* (*H.la*). The labels for individuals include the capture site: Sulawesi (Sul), Sabah (Sabah), Sarawak (Sar), Central Kalimantan (C.Kal), Malaysia (Mal), and Laos (Lao).

	H.di Sul	H.ar Lao	H.ar Mal	H.di Sab	H.di Lao	H.di Mal	H.la1 Lao	H.la2 Lao	H.la3 Lao	H.la C.Kal	H.la Mal	H.ly Mal	H.pe Sul	H.sc Lao
H.di Sul	–	21.9	23.7	7.8	10.4	8.5	24.1	29.8	30.1	31.3	22.8	18.2	10.1	20.3
H.ar Lao	12.2	–	1.8	20.6	19.3	20.1	7.7	9.2	10.1	14.3	7.2	23.2	18.2	23.2
H.ar Mal	12.4	2.0	–	22.0	20.2	22.7	6.9	8.7	9.6	12.5	5.7	21.3	19.4	21.3
H.di Sab	6.7	11.2	11.4	–	6.2	2.9	23.9	28.2	27.8	31.2	22.6	13.2	3.8	15.6
H.di Lao	8.1	10.6	10.8	5.3	–	7.1	19.0	21.6	23.2	26.3	18.4	14.3	7.7	14.8
H.di Mal	7.3	11.0	11.6	2.9	5.9	–	26.3	30.7	24.5	27.7	22.1	14.4	2.7	16.8
*H.la*1 Lao	12.4	6.1	5.7	12.0	10.4	13.0	–	4.3	11.8	13.2	4.4	20.4	25.1	21.7
*H.la*2 Lao	14.1	7.1	6.9	13.4	11.4	14.3	3.9	–	11.0	14.5	5.4	23.1	28.0	23.2
*H.la*3 Lao	14.3	7.5	7.1	13.4	12.0	12.4	8.3	8.1	–	11.9	9.7	23.7	25.9	20.6
H.la E.Kal	14.9	9.6	8.8	14.3	13.0	13.4	9.0	9.8	8.6	–	12.0	25.2	27.8	22.3
H.la Mal	12.2	5.9	4.9	11.8	10.4	11.6	4.1	4.9	7.5	8.8	–	20.5	21.3	20.5
H.ly Mal	11.6	12.2	11.2	9.2	9.4	9.8	11.2	12.2	12.2	13.2	11.4	–	13.5	3.8
H.pe Sul	8.1	10.2	10.4	3.7	6.3	2.8	12.6	13.6	12.8	13.4	11.2	9.4	–	16.4
H.sc Lao	12.8	12.4	11.4	10.4	9.8	11.0	11.8	12.4	11.4	12.2	11.6	3.7	10.8	–

seni. These authors also suggested that the population in Thailand is specifically distinct, and proposed the name *Hipposideros grandis* Allen, 1936. More recently, a morphologically cryptic but acoustically and genetically divergent species within *H. larvatus* was described from northeastern India (*H. khasiana*: Thabah et al., 2006).

The current study included three ecomorphs from Laos (*H. larvatus*-1, -2, -3), which differ in size and echolocation call frequency (C.M. Francis, pers. obs.). Genetic divergence among *H. larvatus*-1, *H. larvatus*-2, and *H. larvatus* from peninsular Malaysia was relatively low, whereas the sister taxa *H. larvatus*-3 and *H. larvatus* from Borneo (E. Kalimantan) were divergent from the other *H. larvatus* lineages, and from each other (Table 5; Fig. 4). In all phylogenetic analyses, *H. larvatus* was paraphyletic with respect to *H. armiger* (Figs. 2–4). However, this relationship was poorly supported and could be resolved by recognition of two distinct species within *H. larvatus*: peninsular Malaysia *H. larvatus* plus Laos ecomorphs 1 and 2, and Borneo *H. larvatus* plus Laos ecomorph 3. Additional sampling of *H. larvatus* from Laos and elsewhere in mainland SE Asia (e.g. Thailand and Vietnam) will be essential to clarifying the status of this taxon.

5. Conclusions

The results of this study contribute to understanding the systematics and diversity of Old World leaf-nosed bats at four taxonomic levels. First, the relationship between *Rhinonicteris* and the remainder of Hipposideridae was ambiguous. This result contrasts with morphometric phylogenies, which consistently support monophyly of Hipposideridae. We suggest that the monophyly of Hipposideridae should be reevaluated with complete taxon sampling and additional molecular markers. Second, also in contrast to morphology-based phylogenies, monophyly of *Hipposideros* was well-supported. Third, with a few exceptions, the major *Hipposideros* clades recovered in our analysis were generally concordant with Hill's classification scheme of species groups (Hill, 1963), suggesting that divergence of the major hipposiderid lineages coincided with differentiation of basic cranial, skeletal and external attributes that are conserved among extant species. Fourth, widespread paraphyly and polyphyly among conspecific lineages indicates that current taxonomy fails to capture evolutionary diversity within *Hipposideros*, supporting the results of Francis et al. (2010). Available data for echolocation call frequencies implicate a close association between ecological and evolutionary diversification. For widespread species with multiple allopatric lineages (e.g. *H. galeritus*, *H. diadema*), it is likely that geographic isolation has played the major role in ecological differentiation. However, at least three species in the *bicolor* group (i.e. *H. bicolor*, *H. cineraceus*, *H. pomona*) contain co-distributed lineages. Pronounced differences in echolocation call frequency, together with subtle variation in facial morphology and body size, suggest that ecological selection has promoted diversification in these small-bodied, forest-adapted hipposiderids, possibly similar to the harmonic hopping in *Rhinolophus philippinensis* (Kingston and Rossiter, 2004). Anecdotal evidence supports a similar pattern of ecological diversification in *H. larvatus*. Finally, phenotypic similarities among non-sister lineages (e.g. *H. bicolor*-131 vs. -142, *H. bicolor* Philippines vs. other *H. bicolor*, *H. pomona*-1 vs. other *H. pomona*) suggest that morphological convergence is a common feature of hipposiderid evolution. While these subtle patterns of phenotypic divergence and convergence have undoubtedly contributed to taxonomic confusion, they support the notion that Southeast Asian hipposiderid bats represent an adaptive radiation that is no less spectacular for its cryptic nature.

Appendix A

Species names, locality, and GenBank accession numbers for samples included in the phylogenetic analysis. Voucher numbers are provided for individuals that were collected: FMNH (Field Museum, Chicago, USA), HNHM (Hungarian Natural History Museum, Budapest, Hungary), HZM (Harrison Zoological Museum, Kent, UK), MZB (Muzeum Zoologicum Bogoriense, Bogor, Indonesia), ROM (Royal Ontario Museum, Toronto, Canada), SEN (Senckenberg Museum, Frankfurt, Germany), and USNM (Smithsonian Institute, Washington, DC, USA). For individuals that were not collected, wing punches were used and a field identification number or wing band number (THK or MBCRU) is presented when available. All specimens and wing punches from

Species	Locality	Voucher/ Field #	GenBank ND2	RAG1
Rhinolophus stheno	Malaysia: Pahang State	THK 8800	JN714742	–
Rhinolophus creaghi	–	–	–	AF447511[a]
Aselliscus stoliczkanus	Laos: Khammouan	SEN 86209	JN714743	JN714690
Coelops frithii	Laos: Khammouan	SEN 85775	JN714744	JN714691
Coelops robinsoni	Malaysia: Pahang State	MBCRU 0051	JN714745	–
Rhinonicteris aurantia	Australia	–	AY504533[a]	–
H. armiger	Laos: Khammouan	SEN88031	JN714746	JN714692
H. armiger	Malaysia: Perlis State	THK 8839	JN714747	JN714693
H. ater	Borneo: Sabah	SEN 83700	JN714748	JN714694
H. ater	Borneo: E. Kalimantan	MZB 26345	JN714749	JN714695
H. ater	Philippines: Luzon I.	FMNH 166410	JN714750	JN714696
H. bicolor	Borneo: Sabah	SEN 83691	JN714754	JN714700
H. bicolor	Philippines: Luzon I.	FMNH 180193	JN714755	JN714701
H. bicolor-131	Malaysia: Pahang State	THK 41736	JN714751	JN714697
H. bicolor-142	Thailand: Krabi	–	JN714752	JN714698
H. bicolor-142	Malaysia: Perlis State	THK 40819	JN714753	JN714699
H. boeadii	SE Sulawesi: Kendari	MZB 28253	JN714799	–
H. cervinus	SE Sulawesi: Buton I.	20020437	JN714758	JN714703
H. cervinus	Borneo: Sabah	SEN 83683	JN714756	JN714702
H. cervinus	Borneo: C. Kalimantan-b	–	JN714757	–
H. cervinus	Malaysia: Pahang State	THK 40836	JN714759	JN714704
H. cineraceus	Borneo: C. Kalimantan-b	–	JN714762	JN714707
H. cineraceus	SE Sulawesi: Lintea I.	20040259	JN714765	JN714710
H. cineraceus-A	Malaysia: Perak State	–	JN714760	JN714705
H. cineraceus	Myanmar: Mon State	HZM 3.34873	JN714764	JN714709
H. cineraceus	Laos: Vientiane	ROM 118043	JN714763	JN714708
H. cineraceus-B	Malaysia: Pahang State	THK 41702	JN714761	JN714706
H. diadema	Laos: Champassak	ROM 118135	JN714767	JN714712
H. diadema	SE Sulawesi: Buton I.	20000458	AY504530[a]	–
H. diadema	Borneo: Sabah	SEN 83680	JN714766	JN714711
H. diadema	Malaysia: Pahang State	THK 3857	JN714768	JN714713
H. doriae	Borneo: C. Kalimantan-a	MS040527.1[b]	JN714769	JN714714
H. doriae	Malaysia: Pahang State	MBCRU 1459	JN714795	JN714739
H. dyacorum	Malaysia: Kelantan St.	THK 40460	JN714770	JN714715
H. fulvus	Sri Lanka: Wavulpane	HZM 3.28778	JN714771	JN714716
H. galeritus	Borneo: Sabah	SEN 83692	JN714772	JN714717
H. galeritus	Borneo: Sarawak	USNM 590268	JN714773	JN714718
H. galeritus	Borneo: E. Kalimantan	MZB 26766	JN714774	JN714719
H. galeritus	Malaysia: Pahang State	THK 40454	JN714776	JN714721
H. galeritus	Laos: Champassak	HNHM 2005.82.4	JN714775	JN714720
H. galeritus	SE Sulawesi: Buton I.	–	AY504532[a]	–
H. halophyllus	Malaysia: Perlis State	THK 22866	JN714777	JN714722
H. khaokhouayensis	Laos: Vientiane	ROM 116791	JN714778	JN714723
H. larvatus	Malaysia: Kelantan St.	THK 41321	JN714783	–
H. larvatus	Borneo: E. Kalimantan	–	JN714782	JN714727
H. larvatus-1	Laos: Champassak	ROM 118149	JN714779	JN714724
H. larvatus-2	Laos: Khammouan	SEN 94012	JN714780	JN714725
H. larvatus-3	Laos: Khammouan	ROM 106510	JN714781	JN714726
H. lylei	Malaysia: Perak State	–	JN714784	JN714728

Appendix A (*continued*)

Species	Locality	Voucher/ Field #	GenBank	
			ND2	RAG1
H. obscurus	Philippines: Luzon I.	FMNH 177464	JN714785	JN714729
H. pelingensis	SE Sulawesi: Kabaena I.	–	JN714786	JN714730
H. pomona-1	Laos: Champassak	ROM 118170	JN714788	JN714732
H. pomona-2	Laos: Khammouan	HNHM 2005.82.46	JN714789	JN714733
H. pomona	China: Yunnan Prov.	–	JN714787	JN714731
H. pomona	Malaysia: Perlis State	THK 41952	JN714791	JN714735
H. pomona[c]	Myanmar: Mon State	USNM 583861	JN714790	JN714734
H. ridleyi	Borneo: Sabah	SEN 83689	JN714792	JN714736
H. ridleyi	Malaysia: Pahang State	MBCRU A4549	JN714793	JN714737
H. rotalis	Laos: Khammouan	ROM 106530	JN714794	JN714738
H. scutinares	Laos: Khammouan	SEN 88023	JN714796	JN714740
H. species A	SE Sulawesi: Buton I.	20000035	JN714798	–
H. species B	Borneo: E. Kalimantan	MZB26346	JN714797	JN714741

[a] Sequences obtained from GenBank.
[b] Specimen located at the University of Palangkaraya, Central Kalimantan.
[c] Originally identified as *H. bicolor*.

peninsular Malaysia were collected by A. Zubaid, and the specimens and duplicate wing punches were deposited at UKM (Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia).

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.10.021.

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