Molecular Phylogenetics and Evolution 53 (2009) 772–783

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

The phylogenetic relationships of cynopterine fruit bats (Chiroptera: Pteropodidae: Cynopterinae)

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ARTICLE INFO

Article history:
Received 10 February 2009
Revised 25 July 2009
Accepted 29 July 2009
Available online 4 August 2009

Keywords:
Pteropodidae
Cynopterinae
Molecular phylogeny
Biogeography
Southeast Asia
Megaerops

ABSTRACT

The subfamily Cynopterinae comprises ca. 24 species of pteropodid bats (Family Pteropodidae) distributed exclusively in South and Southeast Asia. Although some studies have supported monophyly of the subfamily, molecular analyses have produced contradictory results and there has been little agreement on relationships of cynopterines to other megabat groups. However, no previous studies have included a complete sampling of cynopterine genera. Here we describe a phylogenetic analysis of Cynopterinae based on more than 6000 bp from six different genes sampled in representatives of all 14 recognized genera. Our results support the monophyly of Cynopterinae but refute a close relationship of cynopterines with Nyctimeninae. Within Cynopterinae, our analyses consistently recovered two monophyletic clades, which we recommend be recognized formally as tribes: Cynopterini and Balionycterini. Biogeographic analyses indicate a Sundaland origin of the Cynopterinae and divergence date estimates suggest different timing of diversification of the two major cynopterine clades.

1. Introduction

Bats in the family Pteropodidae, the Old World Fruit Bats or megabats, comprise 186 currently recognized species in at least 42 genera distributed in the Paleotropical realm from West Africa to Polynesian Islands in the Western Pacific Ocean (Simmons, 2005). Pteropodidae includes the largest (>1 kg) as well some of the smallest bats in the world (10+ g). Pteropodids eat plant products, chiefly fruits, nectar, or pollen, depending on the group, and also flowers parts and leaf buds as dietary supplements (Kunz and Pierson, 1991). The subfamily Cynopterinae, a clade of short-faced fruit bats, includes approximately 24 species (but probably more) classified in 14 genera (Simmons, 2005). Species are distributed from the Indian subcontinent, into Southeast Asia, and nearby Islands, including the Philippines and the Moluccas (Corbet and Hill, 1992; Simmons, 2005). However, cynopterine bats are not found in Papua New Guinea nor Australia. While many species have a widespread distribution on the continent and across islands of the Sunda Shelf, some are endemic to a particular island or archipelago. These latter are particularly threatened by habitat destruction (Mickleburgh et al., 1992).

Cynopterines have been recognized as a distinct group since Andersen's (1912) revision of the family Pteropodidae. Andersen (1912) included 11 genera in his "*Cynopterus* section," which he divided into two subsections: Nyctimenina for *Nyctimene* and *Myonycteris*, and Cynopterina for the remaining genera. Subsequent studies raised questions about the affinities of *Nyctimene* and *Myonycteris* (see below), and several new cynopterine genera were described in the years following the publication of Andersen's (1912) monograph. In the most recent comprehensive classification of pteropodids, Bergmans (1997) recognized Cynopterinae as one of six subfamilies within Pteropodidae, and placed *Nyctimene* and *Myonycteris* in different subfamilies (Nyctimeninae and Epomophorinae, respectively). As defined by Bergmans (1997), Cynopterinae includes 14 genera: *Aethalops*, *Alionycteris*, *Balionycteris*, *Chironax*, *Cynopterus*, *Dyacopterus*, *Haplonycteris*, *Latidens*, *Megaerops*, *Otopteropus*, *Penthetor*, *Ptenochirus*, *Sphaerias*, and *Thoopterus.*

The absence of a close relationship between the African genus *Myonycteris* and cynopterines (i.e., the genera listed above) has received ample support from phylogenetic analyses of both molecular and morphological data (Juste et al., 1999; Jones et al., 2002; Giannini and Simmons, 2003, 2005). However, the nature of the relationship between cynopterines and *Nyctimene* remains a significant systematic problem. A monophyletic clade including cynopterines sensu Bergmans (1997) plus *Nyctimene* has been recovered in most analyses based on morphological characters

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doi:10.1016/j.ympev.2009.07.035

(Springer et al., 1995; Giannini and Simmons, 2005). However, an earlier study based on female reproductive tract characters found that *Nyctimene* did not cluster with cynopterines (Hood, 1989). The distinction between *Nyctimene* and other cynopterines had been noticed previously by Miller (1907), who proposed the monotypic subfamily Nyctimeninae for this enigmatic genus. Nyctimeninae was recognized as a distinct subfamily by Simpson (1945) and Corbet and Hill (1992), but other authors preferred to separate it at the tribal or subtribal level (e.g., Koopman, 1994; McKenna and Bell, 1997).

Contrary to the morphological evidence, reciprocal monophyly and a non-sister relationship of Nyctimeninae and Cynopterinae have been recovered in most molecular phylogenetic analyses of megabats. These analyses were based on a number of different molecular markers and techniques including restriction site variation, DNA–DNA hybridization, and nucleotide sequences (Colgan and Flannery, 1995; Kirsch et al., 1995; Hollar and Springer, 1997; Romagnoli and Springer, 2000). Most of these studies, however, included only two cynopterine genera, *Cynopterus* and *Thoopterus*.

Other molecular analyses including more cynopterine genera have reached different conclusions. Juste et al. (1999) used DNA sequences of the genes cytochrome *b* and 16S to study megabat phylogeny. They included four cynopterine genera in their analyses and found Cynopterinae to be polyphyletic in a combined analysis of the two genes. *Aethalops* and *Balionycteris* grouped with a clade formed by *Nyctimene* and *Macroglossus*, and *Cynopterus* and *Megaerops* formed a separate clade that clustered with other megabat genera (Juste et al., 1999). Colgan and da Costa (2002) found contrasting results between tree reconstruction algorithms in combined analyses of 12S and *c-mos* sequences. They included four genera, *Cynopterus*, *Chironax*, *Thoopterus*, and *Aethalops*, with the latter three clustering together in a monophyletic clade in all analyses. However, some analyses did not support the grouping of *Cynopterus* with the remainder of cynopterines.

In the most inclusive analysis of molecular data for pteropodids, Giannini and Simmons (2003) assembled all the available sequences in public databases to produce a dataset consisting of data from five loci (four mitochondrial and one nuclear gene) variously representing 43 species including exemplars from seven cynopterine genera. Analyses of this combined dataset recovered two most-parsimonious trees. Cynopterinae appeared as a monophyletic clade but it was not sister to *Nyctimene,* but rather to a clade consisting of *Macroglossus* + *Syconycteris* (Macroglossinae). Two main clades were observed within Cynopterinae, one consisting of *Cynopterus*, *Megaerops*, and *Ptenochirus*, and a second clade comprising *Thoopterus*, *Chironax*, *Aethalops*, and *Balionycteris*.

A subsequent reanalysis of these data with a few more sequences added (including additional data for *Chironax*) using different substitution to gap costs resulted in two different arrangements for cynopterine taxa: one tree in which Cynopterinae sensu Bergmans (1997) was monophyletic with *Macroglossus* + *Syconycteris* as its sister clade, and another in which the latter lineage nested within Cynopterinae (Giannini and Simmons, 2005). Inclusion of 236 non-molecular characters (mostly morphological) yielded slightly different but better supported trees, all of which recovered Cynopterinae as monophyletic (Giannini and Simmons, 2005; Fig. 1). Regardless of the gap-change costs employed, Nyctimeninae and Cynopterinae appeared as reciprocally monophyletic lineages, with neither closely related to Macroglossinae nor to each other (Giannini and Simmons, 2005). Two well-resolved clades, identical to those recovered in analyses of molecular data alone, were recovered within Cynopterinae (Fig. 1).

Although past studies have been successful in identifying which pteropodid genera do not belong to Cynopterinae, and in providing a preliminary framework for understanding relationships among a portion of the included genera, no phylogenetic analyses to date have included representative of all cynopterine genera. Here we present the first molecular study focused on the Cynopterinae subfamily. We obtained sequences of three nuclear and three mitochondrial genes for numerous specimens; for the species from which we could not obtain tissue/DNA samples, we gathered sequences from GenBank. In this way, we constructed a data set in which all cynopterine genera are represented by at least one species and two gene loci. Using several approaches to analyze the data, our goal was to investigate relationships within Cynopterinae. Based on the phylogeny obtained, we analyze biogeographic scenarios and the tempo of evolution in the group.

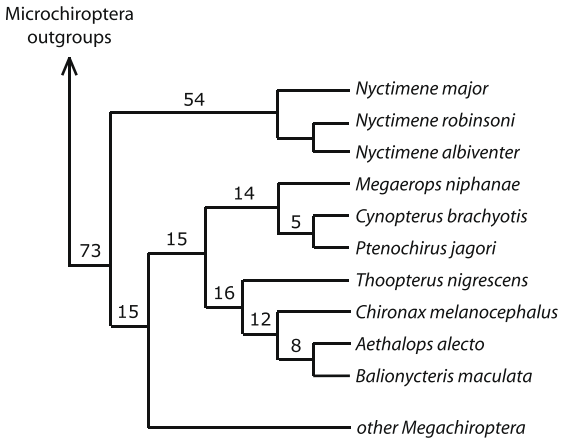

Fig. 1. Relationships of cynopterines obtained by Giannini and Simmons (2005) based on direct optimization of 5 genes and 236 non-molecular characters using an equal substitution to gap cost ratio.

2. Material and methods

2.1. Sampling

To study relationships among the cynopterine fruit bats, we obtained samples from a total of 16 species representing 13 currently recognized genera (Appendix A). Tissue samples were donated by several institutions and individuals (Appendix A). Among cynopterines, the only genus for which we could not obtain tissue samples was *Thoopterus*. For 10 of the cynopterine species sampled, two individuals were sequenced in an effort to reduce misidentification and contamination problems. We additionally obtained from GenBank partial sequences for three other cynopterine species (*Megaerops niphanae*, *Cynopterus horsfieldi*, and *Ptenochirus minor*) to increase our taxonomic sample.

Sequences from 10 non-cynopterine pteropodid species, representing nine genera, were also included in our data set. Two species of *Nyctimene* were included to help test previous suggestions of a relationship between this genus and the cynopterine genera, and to test the monophyly of the latter group. In the set of non-cynopterines, we included one member from each of the subfamilies and tribes recognized by Bergmans (1997), with the exception of the tribes Plerotini and Scotonycterini from the Epomophorinae, a subfamily represented by other members. Sequences for the non-cynopterine species were partly obtained from GenBank, partly obtained previously by ourselves (Giannini et al., 2006, 2008), and partly generated specifically for this study. As outgroups, we used sequences from GenBank for *Rhinopoma hardwickei* and *Hipposideros commersoni*.

2.2. Sequences

Six genes were sequenced for this study, including both nuclear and mitochondrial loci. The three nuclear loci included the exon 28 of the von Willebrand Factor gene (*vWF*, 1231 bp), partial Recombination Activating Gene 1 (*RAG1*, 1084 bp), and partial Recombination Activating Gene 2 (*RAG2*, 760 bp). The three mitochondrial sequences included the complete cytochrome *b* gene (*Cytb*, 1140 bp), partial rRNA 12S (1069 bp), and partial rRNA 16S (1330 bp). The combined sequence set encompassed a total of 6614 bp and was obtained for all the Cynopterinae species for which DNA samples were available. The only exception was the rare Indian endemic *Latidens salimalii*, for which only the *Cytb* and 12S sequences were obtained due to the degraded nature of the DNA sample available.

Total DNA was obtained from preserved tissue samples with the DNeasy tissue kit (Qiagen). PCR amplification was carried out using previously published primers (*RAG1* and *RAG2*: Teeling et al., 2000; *vWF*: Porter et al., 1996; *Cytb*: Bastian et al., 2002; 12S: Springer et al., 1995; 16S: Springer et al., 1995 and Romagnoli and Springer, 2000). To obtain both forward and reverse sequences for each gene region, internal primers were used for sequencing in addition to the PCR primers (for internal primers for *vWF* and *RAG1* see Giannini et al., in press). New primers were designed for the 12S gene: 12i (5′-GGATTAGATACCCCACTATGC) and 12j (5′-AAGCTCTCTATTC TTAATTTACTTC). All sequences were obtained with an automated ABI 3730XL sequencer. Sequence editing and prealignment were done with the Sequencher 4.2 software (Gene Codes). GenBank accession numbers and voucher information for taxa included in this study are provided in Appendix A.

2.3. Analyses

Prealignments of the three nuclear genes and *Cytb* sequences did not show any indels and alignment was done manually. The 12S and 16S fragments contained several indels and were aligned with the program MAFFT with gap opening penalty of 1.53 and gap extension penalty of 0.123 (default values). Saturation plots were obtained for transitions and transversions for each gene using the distance selected with Modeltest 3.7 (Posada and Crandall, 1998) based on the Akaike Information Criterion. When the selected model contained six or more parameters, we used the GTR + Γ + I distance. To test for a possible conflict between mitochondrial and nuclear data we used the partition homogeneity test (Farris et al., 1994). The test was implemented in PAUP* 4.10b (Swofford, 2002) using 500 searches with random stepwise addition and 30 replicates per search.

Phylogenetic inferences were done using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). These analyses were done for the complete combined dataset and for nuclear genes only. Reduced taxon datasets were analyzed with only mitochondrial genes to determine relationships within specific cynopterine clades and the placement of taxa for which only mtDNA sequences were available.

The MP searches were done in PAUP* 4.10b (Swofford, 2002) with 2000 random sequence additions followed by tree bisection reconnection branch swapping (TBR). Clade support was assessed using Bremer decay values (Bremer, 1994) and bootstrap. ML analyses were performed using the GTR + Γ model with the program RAXML (Stamatakis, 2006). Parameters were estimated from the data using a rapid bootstrap procedure with 150 replicates. ML search was done using a starting tree obtained by maximum parsimony followed by 10 rounds of slow likelihood optimization. Statistical support was obtained with 1000 bootstrap replicates. Bayesian search was done using MrBayes 3.1.2 using the GTR + Γ model (Huelsenbeck and Ronquist, 2001). Two replications were used with four chains and three million generations. Trees were sampled once every 1000 generation and the first 600 trees were discarded.

2.4. Biogeography

The cynopterines are endemic to the Indomalayan Region, which extends East from the Indus River Basin to the Lydekker's line—the biogeographic boundary drawn between the Moluccas and New Guinea which roughly represents the eastern distributional limit of Asian taxa (Corbet and Hill, 1992). We based our biogeographic analyses of cynopterines on the geographic distributions described primarily in Corbet and Hill (1992) and Simmons (2005), with some recent updates. Two analyses were carried out to elucidate cynopterine biogeographic history: a Weighted Ancestral Area Analysis (WAAA; Hausdorf, 1998) and a partial Fitch optimization as implemented in the program TNT (Goloboff et al., 2003, 2008).

In the WAAA, we used a very simple subdivision of the areas occupied by cynopterine species, including four main regions: India (including Nepal, Bhutan, Bangladesh and Northern Myanmar), Sunda Shelf (including Indochina, Sumatra, Java and Borneo), the Philippines, and Sulawesi plus the Moluccas. In this analysis, each area receives a score, which is obtained by the ratio of gains (when assuming that the area is derived) over losses (when assuming that the area is ancestral) of clades as suggested by the tree topology (Hausdorf, 1998). Gains and losses are weighted according to the number of nodes between the "gained" or the "lost" clades and the root of the tree. The closer to the root, the higher is a gain or a loss weighted. In this way, higher scores are associated with a higher likelihood that the group originated in that particular area.

For the Fitch optimization, we made a detailed partition of the Indomalayan Region based on the natural subdivisions used by Corbet and Hill (1992). Corbet and Hill (1992) divided the Indomalayan Region into six subregions, many of which were characteristically composed of divisions. These are listed in Table 1. Most but not all the Divisions are meaningful in the context of a biogeographic analysis of cynopterine bats. Our analysis included those biogeographic units in which our cynopterine terminals were reported to occur, and consisted of mapping a single multistate, unordered character on the ingroup tree topology obtained in the combined ML analysis. Using the downpass-only option for mapping (the TNT command *map:*), transformations based on states assigned to the internal nodes can be interpreted in terms of reconstructed dispersal–vicariance events (e.g., Ronquist, 1997).

2.5. Divergence times

Divergence times were estimated using a Bayesian framework and the MULTIDIVTIME package (Thorne and Kishino, 2002). We followed the methods of Rutschmann (2004) and first used *baseml* (PAML; Yang, 1997, 2007) to obtain ML parameters for each nuclear gene based on its individual tree and the $F_{84} + \Gamma$ model. Then we used *estbranches* (from the MULTIDIVTIME package) to calculate the branch lengths on the combined ML tree (nuclear genes only) and a variance–covariance matrix of the parameters. Posterior distributions of substitution rates and divergence times were approximated using a Markov Chain Monte Carlo procedure as implemented by *multidivtime*. We chose to use only nuclear genes because sequences were more conserved and likely less homoplastic, but yet presented enough variation to show resolution in the phylogenetic analyses. Therefore, the taxa for which nuclear data were not available were not included in these analyses.

Table 1
Biogeographic division of the Indomalayan Region used in the dispersal–vicariance analysis of the cynopterine evolutionary history, based in Corbet and Hill (1992). Areas not occupied by cynopterine species (indicated with asterisks) were excluded from the analysis.

Subregion	Division	Code
Indian		A
	Indus[a]	A1
	Peninsular Indian	A2
	Sri Lanka	A3
	Maldive and Laccadive Islands[a]	A4
Himalayan		B
Indochinese		C
	Indochinese	C1
	Southern Chinese[b]	C2
	Central Chinese[a]	C3
	Taiwan[a]	C4
	Ryukyu Islands[a]	C5
	Andaman and Nicobar Islands[c]	C6
Sundaic		D
	Malayan	D1
	Sumatran	D2
	Mentawai[d]	D3
	Javan	D4
	Bornean	D5
	Palawan	D6
Philippine		E
Wallacean		F
	Sulawesi	F1
	Lesser Sunda	F2
	Moluccan	F3

[a] Excluded from the analysis due to the lack of cynopterine records.
[b] Subsumed under the Indochinese Division.
[c] Subsumed under the Sumatran Division.
[d] Subsumed under the Sumatran Division.

Ideally, at least one internal calibration point should be used besides the basal ones to improve accuracy of divergence date estimates. However, given that there is no reliable divergence date based on geological events or fossil data for Pteropodidae (megabats), we had to rely solely on calibration points in the microchiropteran outgroups. We used two calibration points that have been previously used for molecular dating of bats (Teeling et al., 2003, 2005). One is the split of hipposiderids and rhinolophids for which the oldest fossils date is in the Middle Eocene. Therefore, we constrained the node of *Hipposideros commersoni* and *Rhinolophus creaghi* to a minimum of 37 million years ago (Mya). The second point is the split between *Megaderma lyra* and *Rhinopoma hardwickei*, based on megadermatid fossils from the late Eocene. This node was constrained to a minimum age of 34 Mya. Both nodes were constrained to a maximum age of 55 Mya. These intervals are very conservative, since 55 Mya is in the upper confidence interval (CI) for the split between the two clades constrained here (Teeling et al., 2005). In this analysis, *Artibeus jamaicensis* was included to root the tree.

We also performed a second analysis constraining, additionally, the node of the crown pteropodids to a minimum of 20 Mya and a maximum of 29 Mya. This node has been consistently dated with different molecular markers to 24/25 Mya (20–29 95% CI; Kirsch et al., 1995; Teeling et al., 2003, 2005). The Teeling et al. (2005) estimate was obtained using a large sampling of Chiroptera, including representatives of all extant families, and six calibration points based on fossils. Nevertheless, pteropodids were represented by only four species. Although it is important to stress that this constraint is based on an estimate, we wanted to test the effect of constraining the pteropodid node based on the divergence time estimates previously generated using a large sampling of bats. The primary goal of both of our analyses was to compare divergence time estimates within the major ingroup clades.

3. Results

3.1. Sequence characteristics

Sequence statistics for each gene are shown in Table 2. Mitochondrial genes were more variable and had more parsimony-informative sites than nuclear genes, although the latter also showed a substantial amount of informative variation. Most of the genes did not show saturation within pteropodids (Fig. 2). The exception was *Cytb*, which suggests that among its large number of parsimonious informative sites many are probably homoplastic. The plot for *Cytb* showed substitution saturation even within Cynopterinae, affecting both transitions and transversions, but especially the former. The partition homogeneity test showed no significant conflict between nuclear and mitochondrial genes ($p = 0.442$).

3.2. General phylogenetic relationships of cynopterines

The results of the MP analyses using the combined data set are shown in Fig. 3. This tree is the strict consensus of two most-parsimonious trees with 7604 steps (CI = 0.419, RI = 0.561). Cynopterine megabats form a monophyletic clade with high bootstrap and Bremer support. This clade is not sister to the clade formed by the two species of *Nyctimene*. Similar results were obtained in the ML analyses (Fig. 3). The BI tree topology (not shown) was essentially the same as the ML tree topology and therefore we will not discuss these results further. Despite general agreement between different analyses, some differences were observed between the MP and the ML trees. Most of these differences, however, are in

Table 2
Sequence statistics.

Locus	RAG1	RAG2	VWF	Cytb	12S	16S	Combined
Base pairs	1084	760	1231	1140	1069	1332	6617
Invariable	850	578	836	616	652	824	4373
Pars. info.	134	112	226	458	324	393	1641
ML model	GTR + I + G	TrN + I + G	TIM + I + G	TVM + I + G	GTR + I + G	GTR + I + G	GTR + I + G
A	0.28	0.30	0.21	0.38	0.4	0.39	0.31
C	0.23	0.21	0.3	0.38	0.22	0.22	0.25
G	0.26	0.21	0.31	0.06	0.16	0.17	0.21
T	0.23	0.27	0.18	0.18	0.22	0.22	0.22
p. inv.	0.57	0.44	0.39	0.44	0.45	0.43	0.46
α	0.78	0.91	0.74	0.38	0.55	0.47	0.46
# trees MP	245	2277	6	10	2	33	2
Length MP	427	288	740	2533	1583	2105	7604
CI MP	0.642	0.684	0.616	0.338	0.399	0.386	0.419
RC MP	0.484	0.518	0.435	0.167	0.219	0.211	0.235

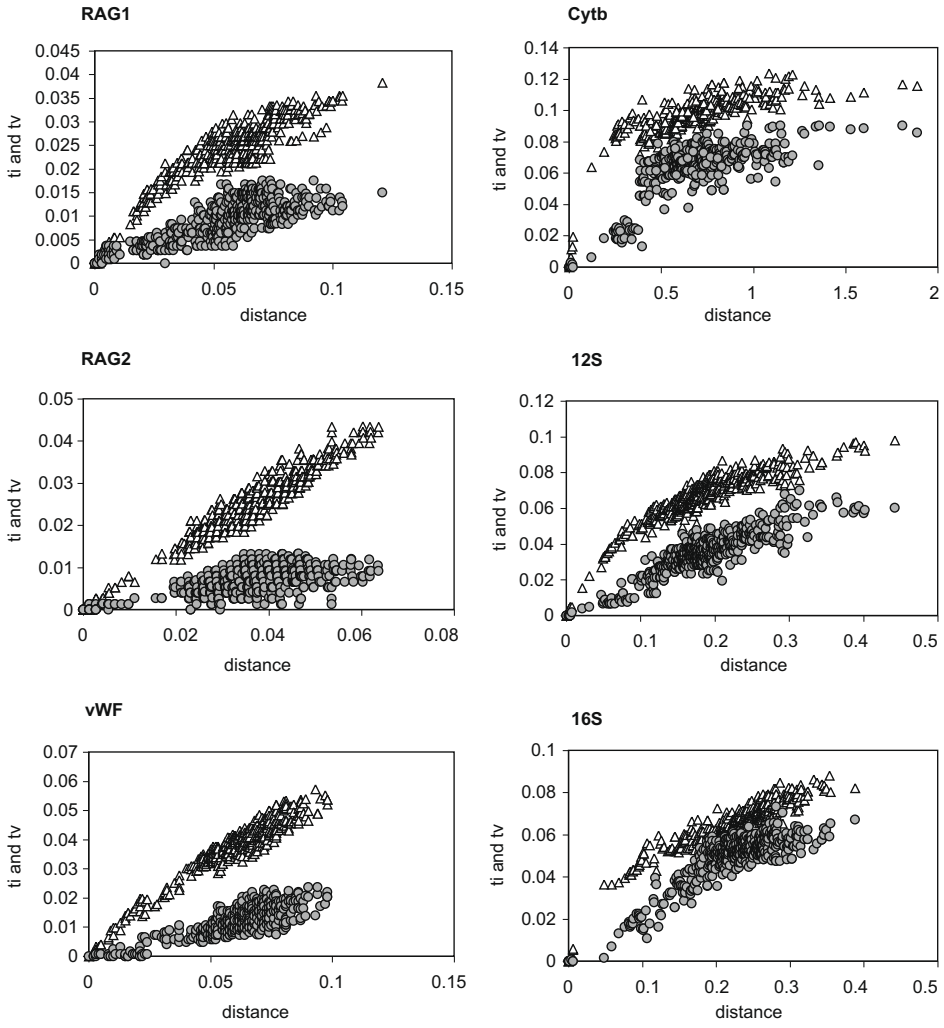

Fig. 2. Nucleotide substitution saturation plots.

the relationships among megabats other than cynopterines, many of which were poorly supported in both analyses. Since the sampling scheme used here was not designed to address relationships among Pteropodidae subfamilies, these differences are probably due to sampling bias.

Results of all analyses indicate a basal split of Cynopterinae into two main clades. These clades generally correspond to cynopterine clades previously recovered by Giannini and Simmons (2005) with a different set of genes and a smaller taxon sample. One of the clades, which we will call the "*Cynopterus* clade" for convenience, has very high support values and includes species of the genera *Megaerops*, *Ptenochirus*, and *Cynopterus*. All the remaining cynopterine genera are included in the second clade, which was not as highly supported in the MP analyses but had 99% bootstrap in the ML tree. This second clade will be referred to as the *Balionycteris* clade. Relationships within these clades will be described separately as follows.

3.3. Phylogenetic relationships within the Cynopterus clade

The *Cynopterus* clade includes the most speciose genera among the cynopterines. The genus *Megaerops*, represented by three species in the combined dataset, was not recovered as monophyletic. Instead, the species *M. wetmorei* was found to be more closely related to *Ptenochirus jagori* than to the other species of *Megaerops*. This result was obtained in both MP and ML analyses and in the analyses using only nuclear genes (Figs. 3–5). To further address this issue, we did a separate analysis of the *Cynopterus* clade including additional sequences available for species not included in our larger sample. This analysis was restricted to the *Cytb* gene for which sequences were available for all species of *Megaerops* and *Ptenochirus* as well as three out of seven *Cynopterus* species. The resulting tree confirms the findings based on the combined and nuclear datasets in recovering *Cynopterus* and *Ptenochirus* as monophyletic, and excluding *wetmorei* from *Megaerops* (Fig. 6). All the different analyses and dataset recovered the genus *Megaerops* (to the exclusion of *wetmorei*) as the most basal member of the *Cynopterus* clade.

3.4. Phylogenetic relationships within the Balionycteris clade

The second of the two internal clades of Cynopterinae, which we call the *Balionycteris* clade, was also recovered in both the combined and the nuclear genes only analyses (Figs. 3–5). Statistical support for this clade was not as high as for the *Cynopterus* clade, specially in the MP analyses. Within the *Balionycteris* clade, most intergeneric relationships were congruent across the different analyses, although ML searches showed generally higher support values for these clades than the MP analyses. Among the highly congruent relationships recovered was the position of *Dyacopterus* as the most basal genus, followed by *Sphaerias*, which were successive sisters to all remaining genera. Other consistently recovered

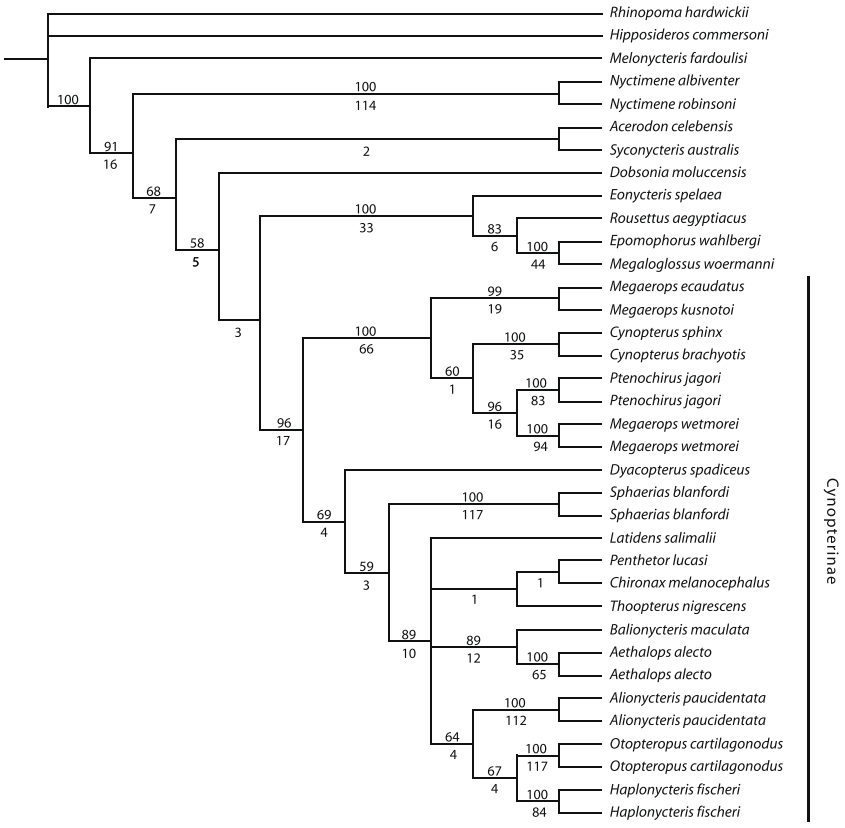

Fig. 3. Maximum parsimony tree based on the combined analysis of six genes (*vWF*, *RAG1*, *RAG2*, *12S*, *16S*, and *Cytb*). Numbers above branches represent bootstrap values and numbers below branches represent Bremer decay values.

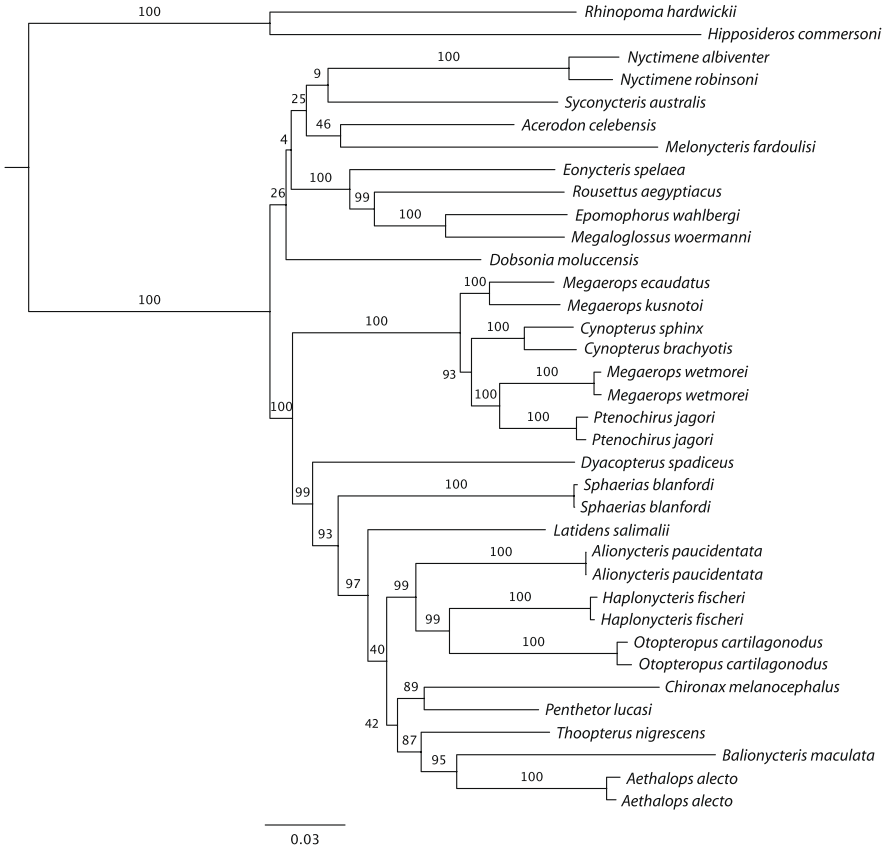

Fig. 4. Maximum likelihood tree based on the combined analysis of six genes (*vWF*, *RAG1*, *RAG2*, *12S*, *16S*, and *Cytb*). Numbers above branches represent bootstrap values.

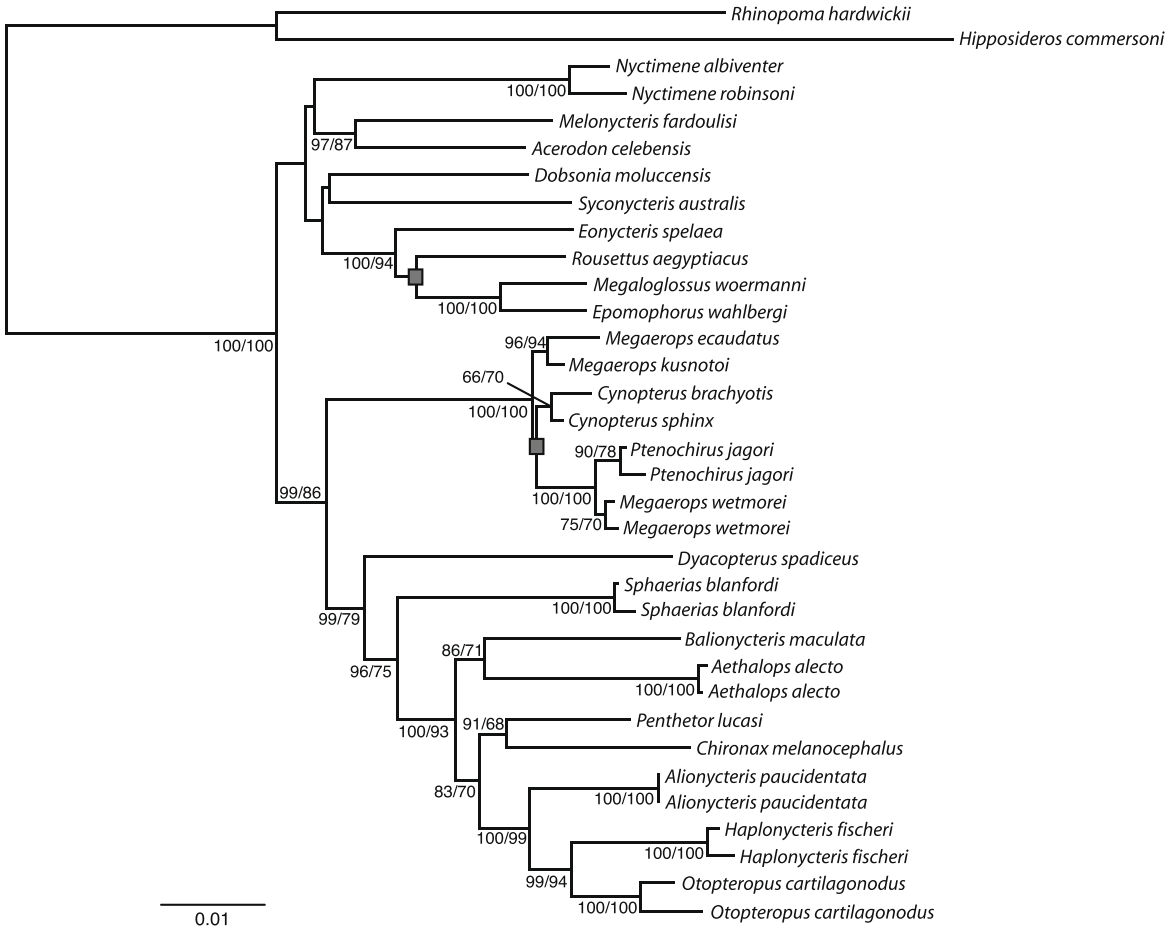

Fig. 5. Maximum likelihood tree based on the combined analysis of three nuclear genes (*vWF*, *RAG1*, and *RAG2*) with both ML and MP bootstrap values (ML/MP). Squares mark clades that were not recovered in the MP tree.

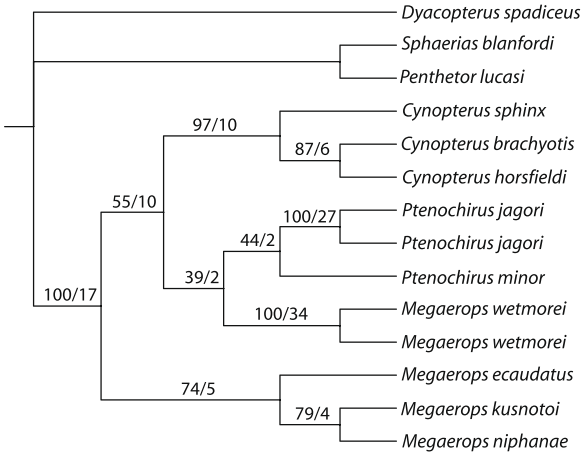

Fig. 6. Cytochrome *b* MP tree for the *Cynopterus* clade, with bootstrap values and Bremer decay indexes.

clades include *Penthetor* + *Chironax*, *Balionycteris* + *Aethalops*, and *Alionycteris* + [*Otopteropus* + *Haplonycteris*]. Relationships among these clades were not resolved in the MP combined analysis and were resolved but with no statistical support in the ML analysis.

The placements of *Thoopterus* and *Latidens* are controversial. *Latidens* did not show a close relationship with any other group within the *Balionycteris* clade although it clearly belongs within this clade. *Thoopterus* was associated with either *Penthetor* + *Chironax*, in the MP tree, or with *Balionycteris* + *Aethalops*, in the ML tree. While the former arrangement had no statistical support, the latter received relatively high ML bootstrap support (87%). Both *Thoopterus* and *Latidens* were represented by only two mitochondrial genes in our dataset and the large amount of missing data could be the cause for the uncertainty about their placements.

The analyses using nuclear genes only (Fig. 4) resulted in better resolved trees and higher support values for the clades within the *Balionycteris* clade when compared to the combined analyses. Also, MP and ML trees had completely congruent topologies in this more restricted dataset (Fig. 4). The nuclear only dataset excluded not only the more homoplastic mitochondrial genes, but also the taxa with missing data (*Thoopterus* and *Latidens*). Both factors could have affected the results of the all genes combined analyses.

3.5. Biogeography

The WAAA suggested that the cynopterines originated in the Sunda Shelf, the continental platform of SE Asia (score 1.30, against: India, 0.733; Philippines, 0.631; Sulawesi, 0.320). Fig. 7 shows the results of Fitch optimization based on divisions of the Indomalayan Region sensu Corbet and Hill (1992). With the caveat that a few of extant species remain to be included in a phylogenetic analysis, we interpret the biogeographic patterns of the cynopterine clade as follows. Three biogeographic states are assigned to the root of the cynopterine tree, the Malayan, Sumatran and Bornean Divisions of the Sundaic Subregion (Fig. 7). That is, the ancestral cynopterine bat is inferred to be an inhabitant of either one of

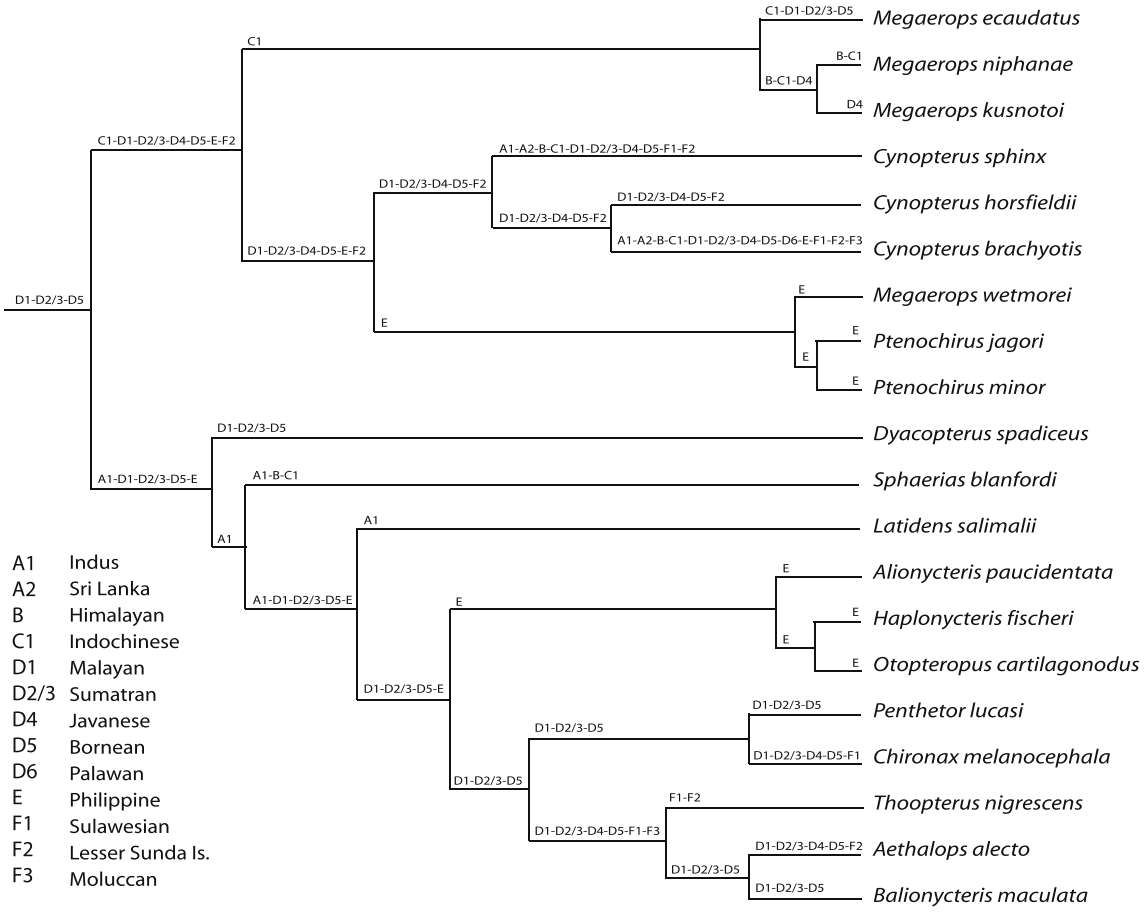

Fig. 7. Results of biogeographic analysis using Fitch partial optimization of areas of the Indomalayan Region (see text and Table 1).

three major regions of the Sunda Shelf, called hereafter the *core* Sunda Shelf (to the exclusion of Java). This conclusion is in accordance to the results of the WAAA. Next, there is the major split of the cynopterines into the *Cynopterus* and *Balionycteris* clades, which we will examine separately.

The *Cynopterus* clade is inferred to have experienced a Northern expansion beyond the Isthmus of Krau into Asian mainland as far as the Indochinese Division, a Southern expansion colonizing Java and the Lesser Sunda Is. beyond the Wallace Line, and a Eastern expansion reaching the Philippines. Next, a major vicariance event is reconstructed, with the ancestral typical *Megaerops* clade in the Indochinese Division. Members of this clade later on recolonized the core Sunda Shelf (*M. ecaudatus*) reaching Java (*M. kusnotoi*) or invaded the Himalayan Subregion. The sister clade experienced another major vicariance event with the ancestor of "*Megaerops*" *wetmorei* and *Ptenochirus* reaching in the Philippines and evolving there in isolation. In turn, members of *Cynopterus* either remained within the Sunda Shelf and Lesser Sunda Islands (*C. horsfieldii*) or spectacularly expanded their distribution to cover the entire Indomalayan Region (*C. brachyotis*) or nearly so (*C. sphinx*).

The reconstructed distribution of the ancestor of the *Balionycteris* clade included the Indus Subregion and three areas of the core Sunda Shelf. The first vicariance event was between the Indus region and the core Sunda Shelf and occurred in the next clade up the tree. Within the *Balionycteris* clade, the first radiation within the core Sunda Shelf was represented by *Dyacopterus*. Its sister clade is reconstructed as an Indian group. This clade gave rise to *Sphaerias*, a taxon that, from the Indus, expanded its distribution to the North reaching the Indochinese Subregion and the Himalayas, and its sister clade, which expanded South recolonizing the core Sunda Shelf and moving Eastward into the Philippines. Next, a vicariance event originated *Latidens*, which remained in the Indus, and a group that evolved in the core cynopterine areas of the Sunda Shelf and Philippines throughout most of its history, expanding East but never recolonizing the Asian continent. This group produced another endemic Philippine clade, inclusive of *Alionycteris*, *Haplonycteris* and *Otopteropus*. No exchange of taxa between these areas is inferred after this split, so clades essentially evolved in isolation on each side of the water barrier isolating the Philippines. Its sister group remained in the core Sunda Shelf. Finally, the evolution of the largely Sundaic clade inclusive of *Chironax*, *Balionycteris*, *Penthetor*, *Thoopterus*, and *Aethalops* included one major dispersal–vicariance event, the dispersal across the Wallace line and posterior split of the ancestor of *Thoopterus* versus *Balionycteris + Aethalops*, and a few dispersal events that represented expansions of distributional areas in established lineages (*Aethalops* expanding into Java and the Lesser Sunda Islands; *Chironax* invading Java and Sulawesi).

We performed an additional analysis at the genus level. The results (not shown) are remarkably similar with the important exception that the reconstructed ancestral areas of cynopterines included the divisions of the core Sunda Shelf plus the Philippines. The addition of the Philippines to the set of putative ancestral areas may represent an artifact of merging the distribution of individual species per genera. We favor the interpretation from the species-level analysis (Fig. 7), which can be tested against results including all cynopterine species individually in a future analysis.

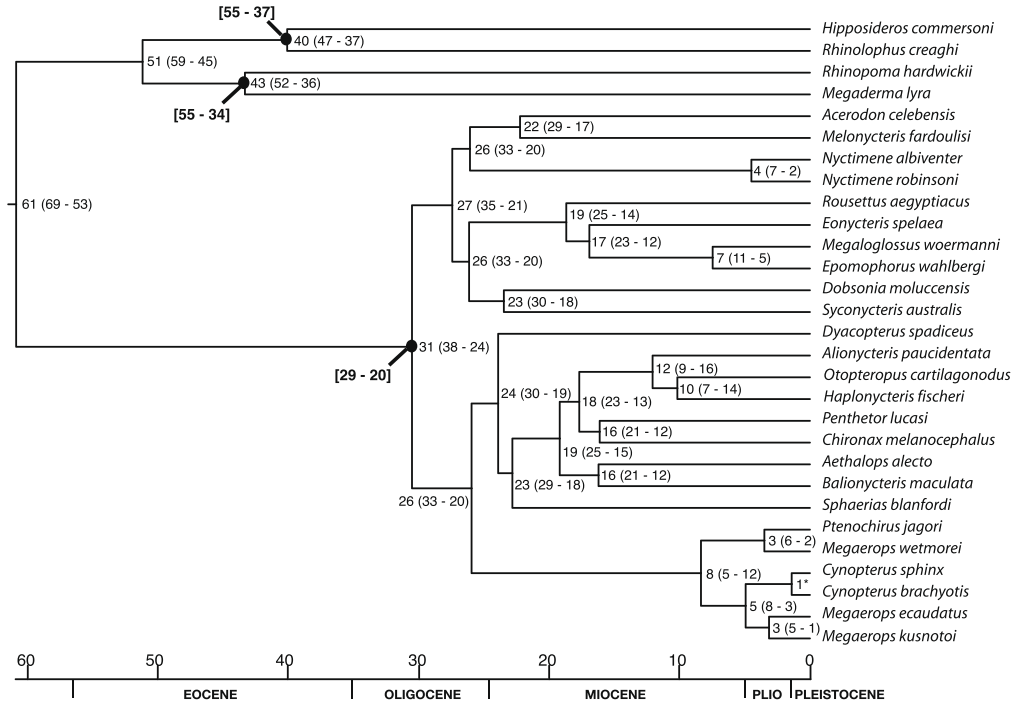

Fig. 8. Divergence date estimates. Marked nodes were used as calibration points and the numbers between brackets indicate upper and lower bounds for their age. Numbers on remaining nodes are the estimated average age with 95% confidence interval in parentheses. These estimates were obtained when using only two calibration points: the *Hipposideros–Rhinolophus* and the *Rhinopoma–Megaderma* splits (see text for details).

3.6. Divergence time estimates

The analyses using two or three (including a constraint for megabats) calibration points gave very similar results (Fig. 8). The posterior estimates obtained for the clades present in this analysis and the results obtained by Teeling et al. (2005) are roughly similar with overlapping confidence intervals. Not surprisingly, our analyses, which used a smaller number of calibration points, resulted in slightly larger confidence intervals. For instance, the divergence date for the *Hipposideros* and *Rhinolophus* clade was estimated by Teeling et al. (2005) at 39 Mya with 43–37 Mya 95% confidence interval. We obtained a divergence date of 40 Mya with a 47–37 Mya 95% confidence interval.

The inclusion of a large sample of pteropodids in our study gave an older estimate for the divergence of the crown clade Pteropodidae. Our estimates are 7 million years (31 Mya) older than the previous estimates (24 Mya; Teeling et al., 2005), which were based on a much smaller taxonomic sample. The inclusion of a constraint for this node (between 20 and 29 Mya) did not change the results. Another interesting result of our analyses was the difference in the diversification timing of the two main clades of Cynopterinae. While in the *Balionycteris* clade all the genera seem to have been originated more than 10 Mya, in the *Cynopterus* clade the extant diversity had its origins more recently (in the last 8.5 million years).

4. Discussion

4.1. Phylogenetic results

Our multigene analyses including representatives of all genera assigned to Cynopterinae *sensu* Bergmans (1997) confirmed the monophyly of the subfamily. There was a clear exclusion of *Nyctimene* from the group. Although there was little resolution of the relationships of this subfamily and other pteropodids, there was no indication that cynopterines are sister to nyctimenines or close allies. The low resolution in subfamily relationships was not surprising since the taxon sampling in the present study was not designed for that purpose. More inclusive studies will be necessary to determine higher-level relationships within Pteropodidae (see Giannini and Simmons, 2005).

Within Cynopterinae, intrageneric relationships were mostly well-resolved with statistical support, showing a clear basal split of the subfamily into two main groups, the *Cynopterus* and the *Balionycteris* clades. Although the placement of *Thoopterus* and *Latidens* in the *Balionycteris* clade seems unambiguous, their close evolutionary relationships are still not clear. Since mitochondrial genes provided less resolution likely due to a large number of homoplastic sites (as suggested by the consistency indexes), obtaining nuclear gene sequences for these two genera will be fundamental to determining their closest relatives.

Considerable work remains to be done at the species level within Cynopterinae. Recent intraspecific studies based on genetic markers have shown that several species in different genera are probably species complexes with clear genetic differentiation despite high morphological similarity (Helgen et al., 2005, 2007; Heaney et al., 2005; Roberts, 2006; Campbell et al., 2004). This is specially true for the genus *Cynopterus* (Campbell et al., 2004, 2006). It is likely that phylogeographic analysis of other genera will find similar results, particularly for those with multi-island distributions. The study of these intrageneric relationships will be important for further understanding the biogeography and the origins of cynopterine diversity.

4.2. Taxonomic implications

Our results strongly support the division of Cynopterinae into two main groups, the *Cynopterus* clade and the *Balionycteris* clade. We defer proposing new names (or adopting available names) for these groups until an adequate morphological diagnosis of each can be produced. Our results also strongly suggest that *Megaerops* is not monophyletic. Both nuclear and mitochondrial loci place

wetmorei as sister to *Ptenochirus* rather than in a clade with other *Megaerops* species. A number of morphological characters, most prominently the presence of a tail in *wetmorei*, and its absence thereof in all other species of *Megaerops* (see Corbet and Hill, 1992), separates these taxa. However, an external tail is plesiomorphic in Pteropodidae (Giannini and Simmons, 2005). This also shows the need for a thorough morphological review that is beyond the scope of this paper. Regardless, taxonomic changes are clearly warranted by our findings. The type species of *Megaerops* is *M. ecaudatus*. As a consequence, there are two options for solving the taxonomic problem of *M. wetmorei*. Either *M. wetmorei* should be transferred to *Ptenochirus*, or a new genus needs to be erected for *wetmorei*. We conclude that the latter option would be preferable in view of the clear morphological distinction between *M. wetmorei* and the two species of *Ptenochirus*, and the close morphological similarity between *P. jagori* and *P. minor*. The problem, however, is further complicated by the discovery of *M. wetmorei albicollis* (Francis, 1989) from Sundaic localities (Borneo, Peninsular Malaysia, and Sumatra; Simmons, 2005), a form that differs in a number of details from nominate *M. w. wetmorei* from Mindanao, Philippines (Corbet and Hill, 1992; Francis, 1989). This clearly requires further study and will be treated elsewhere.

4.3. Biogeography

The cradle of cynopterine bats was the Sunda Shelf and many evolutionary events continued to occur within the Sundaic Subregion throughout the history of the subfamily. Major islands of the Sunda Shelf (Sumatra, Java, Borneo), as well as most of the surrounding smaller islands, were connected at times with Peninsular Malaysia as recently as the late Pleistocene (Corbet and Hill, 1992; Bird et al., 2005). The overall biogeographic pattern of cynopterines seems to have been the origination of clades via vicariance followed by successive waves of expansion of successful descendant taxa over most of the Indomalayan Region in various directions, predominantly North and East. The speciation events were probably linked to isolation generated by fluctuating sea levels, perhaps more remarkably so in the two clades of Philippine endemics.

Besides changes in sea level leading to cycles of island connection and isolation, habitat fragmentation may have also played a role. The role of ecology in determining dispersal and gene flow on one hand and population genetic structure on the other has already been demonstrated in fruit bats (Heaney et al., 2005; Heaney, 2007). Many cynopterine species are restricted to close canopy forest or to certain altitudes (e.g., *Sphaerias*, *Chironax, Haplonycteris*). Inability to utilize open or lowland habitats may greatly decrease the potential for dispersal in species restricted to close canopy forests or montane habitats. There is evidence suggesting that climatic oscillations could have promoted isolation and fragmentation of forested areas in the Sundaland, perhaps contributing to vicariant events leading to the diversification of cynopterines (Heaney, 1991; Bird et al., 2005)

Range expansions following major vicariance splits apparently occurred in parallel and several times in the two major cynopterine clades. Major geographic accidents or events, chiefly the opening or flooding of the Isthmus of Krau, the Makassar strait, the straits in the Sulu and Celebes Sea that isolate the Philippines, the Strait of Malacca, and the Java Sea, can be clearly linked to specific cladogenetic events inferred in the cynopterine history, some of which involved necessary dispersals (e.g., across Wallace's Line and to the Philippines) and vicariance associated to fluctuating sea level (e.g., among islands within the Sunda Shelf or the Philippines). According to the phylogenetic trees obtained here, over-water dispersal to the Philippines occurred three times and to Sulawesi and the Moluccas at least many times, both involving dispersal–vicariance events and mere dispersals of individual taxa. It is interesting to notice that no "backward" flow is inferred (although we cannot totally rule out this possibility) from the Philippines, Sulawesi, or the Moluccas, to the Sundaic Region. Once a lineage reached those regions, speciation occurred locally among islands of archipelagos, particularly in the case of taxa restricted to the Philippines (see also Roberts, 2006).

4.4. Divergence times

Due to the dearth of pteropodid fossils and consequent lack of calibration points within the family, divergence date estimates may be biased and conclusions based on these estimates should be taken with care. Nevertheless, some interesting results deserve interpretation. According to our analyses, the diversification of Cynopterinae coincided with a time of warm temperatures that began at the end of the Oligocene and continued through the mid-Miocene, peaking between 15 and 17 Mya (Zachos et al., 2001). This warm phase in the Miocene was scattered with several brief glaciation events (Zachos et al., 2001) that could have lead to sea level fluctuations and vegetation shifts. This period coincides with our estimates for the diversification of much of the *Balionycteris* clade. After this period (at ~10 Mya), for several million years there was a trend toward cooler temperatures until a subtle warming in the early Pliocene (6 Mya) that preceded the glaciations of late Pliocene (3.2 Mya) and Pleistocene (Zachos et al., 2001).

Regardless of the accuracy of age estimates, it is evident that the colonization of islands by various cynopterine lineages happened several times independently and in different time periods. It is also quite clear that Pleistocene climatic variations accompanied by sea level changes cannot explain the generic diversification of cynopterine bats, since most of it seems to have taken place before this Era. Many recent studies of birds and mammals show that diversification in several taxa predate the Pleistocene (Klicka and Zink, 1997; Steppan et al., 2003; Lovette, 2004; Yoder and Yang, 2004; Steele et al., 2005), thus showing that Pleistocene glaciations and accompanying sea level shifts were not the only or main mechanism that produced extant diversity in the region. Island isolation dynamics as consequence of Pleistocene climate changes seems more likely to have affected differentiation at lower taxonomic levels (e.g., within genera rather than between them), such as the evolution of multiples species of *Cynopterus* and distinct island populations of *Haplonycteris* in the Philippines (Heaney et al., 2005; Roberts, 2006). Future work with more finely calibrated timescales and phylogenies of taxa and populations will be necessary to fully understand the effects of climate and ecological change on pteropodid bats.

Acknowledgments

We thank Lawrence Heaney (Field Museum of Natural History, Chicago), Jim Patton and Carla Cicero (Museum of Vertebrate Zoology, Berkeley), John Wible and Suzanne McLaren (Carnegie Museum, Pittsburgh), Burton Lim and Judith Eger (Royal Ontario Museum, Toronto), Denis O'Meally (Australian Museum, Sydney), Linda Gordon, Jeremy Jacobs, Louise Emmons, and James Mead (National Museum of Natural History, Smithsonian Institution, Washington, DC), and Paul Bates and Malcolm Pearch (The Harrison Institute, Sevenoaks) for access to tissue samples that made possible this contribution. Funding for this study was provided by the National Science Foundation (research awards DEB-9873663 and EF-0629811 to N.B.S.), a Vernay postdoctoral fellowship at the AMNH to F.C.A.

Appendix A

Species	Voucher ID	RAG1	RAG2	vWF	cytb	12S	16S
Acerodon celebensis	AMNH272877	EU617946	EU617896	EU617928	**GQ410231**	—	—
Acerodon celebensis	GenBank	—	—	—	—	U93071	AF293641
Aethalops alecto	ROM102164	**GQ410263**	**GQ410240**	**GQ410286**	**GQ410218**	**GQ410312**	**GQ410335**
Aethalops alecto	ROM102176	**GQ410264**	**GQ410241**	**GQ410287**	**GQ410219**	**GQ410313**	**GQ410336**
Alionycteris paucidentata	FMNH148095	**GQ410266**	**GQ410243**	**GQ410289**	**GQ410221**	**GQ410315**	**GQ410338**
Alionycteris paucidentata	FMNH148099	**GQ410267**	**GQ410244**	**GQ410290**	**GQ410222**	**GQ410316**	**GQ410339**
Balionycteris maculata	ROM102014	**GQ410272**	**GQ410249**	**GQ410295**	**GQ410227**	**GQ410321**	**GQ410337**
Chironax melanocephalus	ROM101945	**GQ410265**	**GQ410242**	**GQ410288**	**GQ410220**	**GQ410314**	**GQ410335**
Cynopterus brachyotis	ROM102015	**GQ410256**	**GQ410233**	**GQ410279**	**GQ410210**	**GQ410303**	**GQ410327**
Cynopterus horsfieldi	GenBank	—	—	—	EF201643	EF139873	—
Cynopterus sphinx	AMNH274354	EU617947	EU617897	DQ445697	DQ445703	**GQ410302**	**GQ410336**
Dobsonia moluccensis	AM M20735	EU617949	EU617899	EU617930	—	FJ18484	—
Dobsonia moluccensis	GenBank	—	—	—	AF144064*	—	AF179290*
Dyacopterus spadiceus	ROM102017	**GQ410275**	**GQ410252**	**GQ410298**	**GQ410230**	**GQ410324**	**GQ410347***
Eonycteris spelaea	GenBank	—	—	—	AB046322	U93059	AF044610
Eonycteris spelaea	MVZ176487	EU617951	EU617901	DQ445684	—	—	—
Epomophorus wahlbergi	FMNH177209	UE617953	EU617903	DQ445691	DQ445706	—	—
Epomophorus wahlbergi	GenBank	—	—	—	—	U93064	AF203744
Haplonycteris fischeri	FMNH146627	**GQ410270**	**GQ410247**	**GQ410293**	**GQ410225**	**GQ410319**	**GQ410342**
Haplonycteris fischeri	FMNH146632	**GQ410271**	**GQ410248**	**GQ410294**	**GQ410226**	**GQ410320**	**GQ410343**
Latidens salimalii	126433	—	—	—	**GQ410217**	**GQ410311**	—
Megaerops ecaudatus	ROM113028	**GQ410260**	**GQ410237**	**GQ410283**	**GQ410214**	**GQ410308**	**GQ410332**
Megaerops kusnotoi	ROM101944	**GQ410261**	**GQ410238**	**GQ410284**	**GQ410215**	**GQ410309**	**GQ410333**
Megaerops niphanae	GenBank	—	—	—	AF044647	—	AF044616
Megaerops wetmorei	FMNH146667	**GQ410258**	**GQ410235**	**GQ410281**	**GQ410212**	**GQ410306**	**GQ410330**
Megaerops wetmorei	FMNH146669	**GQ410259**	**GQ410236**	**GQ410282**	**GQ410213**	**GQ410307**	**GQ410331**
Megaloglossus woermanni	AMNH268358	EU617956	EU617906	DQ445702	DQ445710	—	—
Megaloglossus woermanni	GenBank	—	—	—	—	U93055	AF044620
Melonycteris fardoulisi	AMNH275744	EU617957	EU617907	DQ445699	—	—	—
Melonycteris fardoulisi	GenBank	—	—	—	AY847236*	U93056	AF293644
Nyctimene albiventer	GenBank	AF447514	AF447549	AF447531	DQ314264*	U61077	AF293640
Nyctimene robinsoni	AM M22990	**GQ410276**	**GQ410253**	**GQ410299**	AF144066*	**GQ410325**	**GQ410348**
Otopteropus cartilagonodus	FMNH175388	**GQ410268**	**GQ410245**	**GQ410291**	**GQ410223**	**GQ410317**	**GQ410340**
Otopteropus cartilagonodus	FMNH175391	**GQ410269**	**GQ410246**	**GQ410292**	**GQ410224**	**GQ410318**	**GQ410341**
Penthetor lucasi	ROM102183	**GQ410262**	**GQ410239**	**GQ410285**	**GQ410216**	**GQ410310**	**GQ410334**
Ptenochirus jagori	FMNH175395	EU617960	EU617910	DQ445696	FJ218480	**GQ410304**	**GQ410328**
Ptenochirus jagori	FMNH175398	**GQ410257**	**GQ410234**	**GQ410280**	**GQ410211**	**GQ410305**	**GQ410329**
Ptenochirus minor	GenBank	—	—	—	AY974702	—	—
Rousettus aegyptiacus	Uncataloged	EU617979	EU617927	DQ445688	DQ445713	—	—
Rousettus aegyptiacus	GenBank	—	—	—	—	AB205183	AB205183
Sphaerias blanfordi	AMNH274187	**GQ410274**	**GQ410251**	**GQ410297**	**GQ410229**	**GQ410323**	**GQ410346**
Sphaerias blanfordi	AMNH274189	**GQ410273**	**GQ410250**	**GQ410296**	**GQ410228**	**GQ410322**	**GQ410345**
Syconycteris australis	MVZ 140249	**GQ410278**	**GQ410255**	**GQ410301**	**GQ410232**	—	—
Syconycteris australis	GenBank	—	—	—	—	U93060	AF293650
Thoopterus nigrescens	GenBank	—	—	—	—	U93067	AF293646
Artibeus jamaicensis	GenBank	AY834655*	AY834663	AY834737*	DQ869515	—	—
Hipposideros commersoni	GenBank	AF203760*	AF203770	AF203778*	—	AY395856	AY395856
Rhinopoma hardwickii	GenBank	AF447518*	AY141026	AF447551*	AY056462*	AF263231	AF263231

In bold are sequences generated for this study. Sequences marked with an asterisk are partial, shorter than the fragment sequenced and analyzed for most sequences of the same gene.

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