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Phylogenetic relationships among megachiropteran species from the two major islands of the Philippines, deduced from DNA sequences of the cytochrome *b* gene

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Abstract: Complete cytochrome *b* gene sequences (1140 base pairs) in species of Megachiroptera were ascertained in order to deduce their phylogenetic relationships, using samples of *Cynopterus brachyotis*, *Eonycteris spelaea*, *Ptenochirus jagori*, *Pteropus vampyrus*, and *Rousettus amplexicaudatus* collected from the islands of Luzon and Mindanao in the Philippines. Genetic divergence between samples of *R. amplexicaudatus*, *E. spelaea*, and *C. brachyotis* was very small. On the other hand, a large genetic distance was detected between species of Megachiroptera. The phylogenetic tree using neighbor-joining, parsimony, and maximum-likelihood methods generated similar topologies, reflecting the evolutionary associations among megachiropteran species. We estimated that Megachiroptera separated from Microchiroptera 50.2 million years ago (MYA), and split further approximately 32.4 MYA, forming three lineages: *E. spelaea*, *R. amplexicaudatus*, and *P. vampyrus* and the *P. jagori* – *C. brachyotis* cluster. The third lineage, composed of *P. vampyrus* and the *P. jagori* – *C. brachyotis* cluster, branched out 31.9 MYA. We hypothesize that *R. amplexicaudatus* diverged from the three members of the subfamily Pteropodinae examined, and its phylogenetic relationship with *E. spelaea* remains unclear.

Résumé : Les séquences complètes du gène cytochrome *b* (1140 paires de base) chez des mégachiroptères ont été étudiées pour déterminer leurs liens de parenté phylogénétiques, au moyen d'échantillons de *Cynopterus brachyotis*, *Eonycteris spelaea*, *Ptenochirus jagori*, *Pteropus vampyrus* et *Rousettus amplexicaudatus* recueillis dans les îles de Luzon et de Mindanao, Philippines. La divergence entre les échantillons de *R. amplexicaudatus*, *E. spelaea* et *C. brachyotis* s'est avérée très étroite. Par ailleurs, le espèces de mégachyroptères apparaissent séparées par une grande distance génétique. Les méthodes de construction d'arbres phylogénétiques, celle du voisin le plus proche, la méthode basée sur le principe de parcimonie et celle de la plus grande vraisemblance ont généré des topologies semblables reflétant l'association évolutive entre les espèces de mégachiroptères. Nous avons estimé que la séparation entre les mégachiroptères et les microchiroptères a eu lieu il y a 50,2 millions d'années (MYA) et que les mégachiroptères se sont divisés de nouveau il y a environ 32,4 MYA pour former trois lignées : *E. spelaea*, *R. amplexicaudatus*, et le groupement *P. vampyrus* et *P. jagori* – *C. brachyotis*. La troisième lignée, formée du groupement *P. vampyrus* et *P. jagori* – *C. brachyotis* s'est individualisée il y a 31,9 MYA. Nous croyons que *R. amplexicaudatus* s'est éloigné des trois membres de la sous-famille des Pteropodinae examinés et sa relation phylogénétique avec *E. spelaea* reste mal comprise.

[Traduit par la Rédaction]

Introduction

The order Chiroptera is clustered into two suborders, Megachiroptera and Microchiroptera, commonly referred to as the megabats and microbats. Megabats are pollen, nectar, and (or) fruit feeders, lack an echolocation system except for the genus *Rousettus* (Hill and Smith 1984), and form a single family Pteropodidae with diverse numbers of genera and

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Table 1. Specimens of megachiropteran species (megabats) from the two major islands of the Philippines, Luzon and Mindanao, with their common names and collection sites.

	Common name	Collection site
Cynopterus brachyotis	Dog-faced fruit bat or short-nosed fruit bat	
Luzon		Mt. Banahao, Samil, Lucban, Quezon Province (1)
Mindanao		Tibungol, Panabo, Davao del Norte Province (6)
Eonycteris spelaea	Dawn bat	
Luzon		Mt. Banahao, Palola Manglit, Lucban, Quezon Province (2)
Mindanao, site 1		Tuburan, Mawab, Compostela Valley Province (4)
Mindanao, site 2		Poblacion, Carmen, Davao del Norte Province (5)
Ptenochirus jagori (Luzon)	Musky fruit bat	Mt. Banahao, Samil, Lucban, Quezon Province (1)
Pteropus vampyrus (Mindanao)	Large flying fox	Tagbayagan, Rosario, Agusan del Sur Province (3)
Rousettus amplexicaudatus	Rousette fruit bat	
Luzon		Mt. Banahao, Samil, Lucban, Quezon Province (1)
Mindanao, site 1		Liklik, Babak, Island Garden City of Samal (8)
Mindanao, site 2		Ma-a, Talomo District, Davao City (7)

Note: Numbers in parentheses are location numbers (see Fig. 1).

approximately 200 species (Koopman 1994), whereas microbats are insect feeders known for their complex laryngeal echolocation system (Altringham 1999). At the supra-generic level, Andersen (1912) categorized megabats into five sections (= tribes) based on morphology. These are rousettine, epomophorine, and cynopterine bats in the subfamily Pteropodinae and eonycterine and notopterine bats in the subfamily Macroglossinae. However, recent molecular evolutionary analyses, i.e., restriction fragment length polymorphism analysis of ribosomal DNA (Colgan and Flannery 1995), DNA hybridization (Kirsch et al. 1995), and mitochondrial DNA sequencing (Hollar and Springer 1997; Alvarez et al. 1999), have shown that this taxonomic classification has some discrepancies.

In the subfamily Pteropodinae, the taxa within three of the five sections proposed by Andersen (1912) have been the subject of controversy concerning their phylogenetic placement. One issue that remained unresolved is the obscure monophyletic relationships between the genera *Rousettus* and *Pteropus* within the rousettine section, leaving the genera *Cynopterus* and *Ptenochirus* to the cynopterine section, with the epomophorine section strictly an African assemblage (Bergmans 1997). Added to this uncertainty was the close similarity of the morphological features of *Rousettus* and *Eonycteris* and their identical chromosome number, $2n = 36$ (Rickart et al. 1989). Both genera are members of two distinct sections and subfamilies of megabats. However, in his taxonomic proposal concerning Recent Megachiroptera, Bergmans (1997) tentatively included *Eonycteris* in the taxonomic group containing *Rousettus*, but this seems to need more investigation.

To better understand the enigmatic phylogeny within this group of megabats, molecular information was employed to address inconsistencies. Thus, we present new molecular evidence derived from complete DNA sequences of the cytochrome *b* (cyt *b*) gene among species of Megachiroptera, and assess the monophyletic relationships of *Cynopterus*, *Ptenochirus*, *Pteropus*, and *Rousettus* at the suprageneric level. Furthermore, the relationship between *Rousettus* and *Pteropus* as members of the rousettine section (Andersen 1912) was ascertained, as well as the phylogenetic relationship of *Eonycteris spelaea* with the four species of Pteropodinae. The genetic distances between samples of three widespread Asian species collected from different sites on the two major islands in the Philippines were also determined.

Materials and methods

Species examined

The specimens of megachiropteran species used in this investigation were collected on the two major islands in the Philippines, Luzon and Mindanao. *Cynopterus brachyotis*, *Ptenochirus jagori* (Philippine endemic), *Pteropus vampyrus*, and *Rousettus amplexicaudatus* are representatives of the subfamily Pteropodinae. *Eonycteris spelaea*, in the subfamily Macroglossinae, was included in the analysis. Three of the five species, *C. brachyotis*, *E. spelaea*, and *R. amplexicaudatus*, are widely distributed in Asia and were collected from both islands. The geographic locations of the sampling sites for each species are presented in Table 1 and Fig. 1. Two species of the suborder Microchiroptera, *Chiroderma villosum* and *Uroderma bilobatum* (Baker et al. 1994), were used as the outgroup.

DNA extraction

Total cellular DNA was extracted from dried skin (about 1 cm^2) taken from stuffed museum (UPLBMNH) specimens of megabats or from specimens preserved in either ethanol (95%) or 10% neutralized formalin (Baker's calcium formalin solution). The specimens preserved in ethanol or neutralized formalin were air-dried and washed with 100 mM EDTA (pH 8.0) solution several times prior to extraction. The DNA was extracted by proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation following the standard procedure described by Sambrook et al. (1989).

Amplification and sequencing of the cyt *b* gene

The entire mitochondrial cyt *b* gene (1140 base pairs (bp)) was amplified via the polymerase chain reaction (PCR) prepared in 100 μL reaction using 250–500 ng total cellular DNA. The amount of reagent used in PCR amplification was in accordance with the manufacturer's instructions (TaKaRa *Taq*, Takara Biomedicals, Shiga, Japan). Double-stranded amplifications were performed using the following primers:

H15915R, 5′-GGAATTCATCTCTCCGGTTTACAAGAC-3′ (Irwin et al. 1991);

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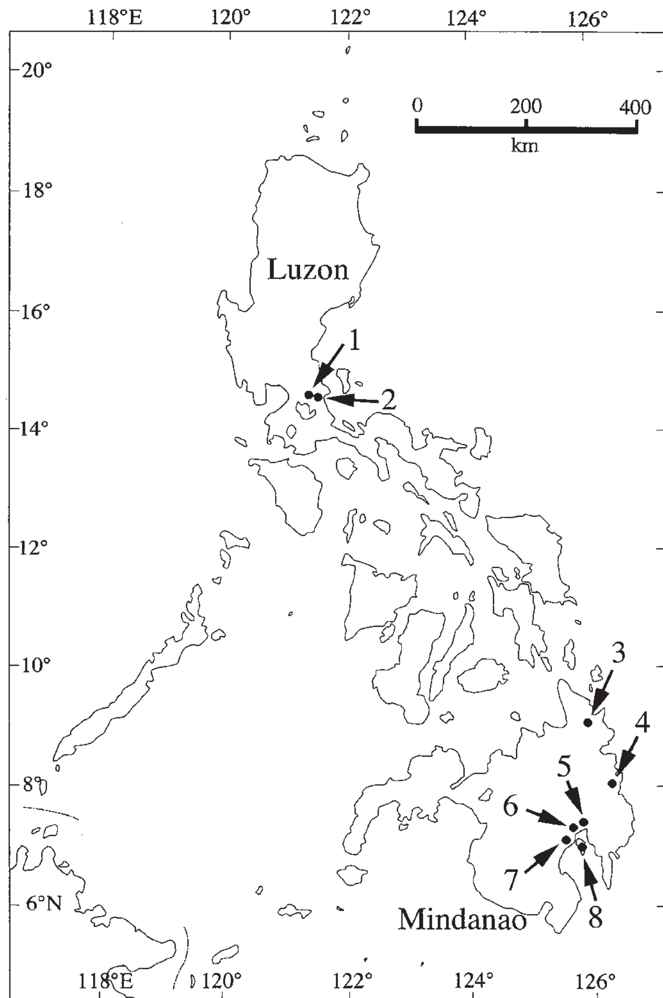
Fig. 1. Map of the Philippines showing the locations where the megachiropteran specimens were collected (see Table 1 for locality names and species).



L14724, 5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′ (Paabo 1989);

H15149, 5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′ (Kocher et al. 1989);

and the sequence corresponding to H15576 was that of M102, 5′-TAGGCGAATAGGAAATATCATTC-3′ (Chikuni et al. 1994).

Primer names indicate the DNA strand (H is heavy and L is light) and the position of the 3′ end of the oligonucleotide sequence corresponds to the human sequence (Anderson et al. 1981). PCR amplification was carried out using the Takara PCR Thermal Cycler (Takara Biomedicals). The following PCR conditions were used: denaturation at 94°C for 1 min, then for 40 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and final extension at 72°C for 5 min. A negative control was included in each amplification and all mixtures were covered with light white mineral oil (Sigma Chemical Company, St. Louis, Mo., U.S.A.). Two sets of individual fragments were amplified for the cyt *b* gene. The first amplification covered the whole gene (approximately 1200 bp) and the second covered the middle region of the gene (between 400–700 bp), using two primer sets of Kocher et al. (1989) and Chikuni et al. (1994). To check the band of interest, the PCR products were electrophoresed in 1.5% agarose (Seakem GTG, FMC Bioproducts, Rockland, Maine, U.S.A.), stained with ethidium bromide, and viewed via ultraviolet illumination.

The double-stranded PCR products were purified using a Microspin S400 HR Column (Amersham Pharmacia Biotech, Piscataway, N.J., U.S.A.). The sequencing reaction of the purified PCR product was carried out using the Dye Terminator Cycle DNA sequencing kit (PE Applied Biosystem, Foster City, Calif., U.S.A.) as explained in the manufacturer's manual, using the same primer set as used in the PCR amplification. Then the product was dissolved in 25 µL of Template Suppression Reagent (PE Applied Biosystem), denatured for 2 min at 95°C, and sequenced by using the automated sequencer Model 310 Genetic Analyzer (PE Applied Biosystem). All amplified PCR fragments were sequenced with both the light- and heavy-stranded primers.

DNA sequence alignment and saturation analysis

DNA sequencing of the entire region of the cyt *b* gene was done for alignment, connection, and homology comparison using the DNASIS software (Hitachi Software Engineering Co., Yokohama, Japan). To check the homology of each sequence, published sequences of mammals were used (Irwin et al. 1991; Wright et al. 1999; Nikaido et al. 2000).

We tested our cyt *b* sequence data for saturation at three different codon positions. Following the methods of Griffiths (1997), the total amount of sequence divergence (uncorrected) was plotted against the total number of substitutions in the first, second, and third codon positions, both in transition and in transversion. The data were considered saturated when there was an indication of plateauing or most of the ingroup exceeded the outgroup. Two microchiropteran species from the DNA data bank, *U. bilobatum* (L28943) and *C. villosum* (L28941) by Baker et al. (1994), were included as the outgroup in this analysis.

Estimate of divergence

In mammals, sequence differences based on transversion alone showed a linear relationship with time (Miyamoto and Boyle 1989; Irwin et al. 1991). Using a constant transversion rate of 0.2% per million years (Miyamoto and Boyle 1989), we calculated the divergence time between megabat species and the outgroup (*C. villosum* and *U. bilobatum*). The times of divergence are shown in the phylogenetic tree (Fig. 3).

Phylogenetic analysis

To infer relationships among taxa, two different approaches were applied in order to obtain the ideal topology, using three different methods of phylogenetic reconstruction. First, we generated a phylogenetic tree from the complete nucleotide sequences. Second, we carried out further phylogenetic analysis using the sequences from the first and second codon positions because the third codon position transition was saturated. In all these analyses we employed the neighbor-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981), and parsimony methods with 1000 bootstrap replications (Felsenstein 1985), which were all found in PHYLIP version 3.572c (Felsenstein 1993). The neighbor-joining tree was based on genetic distances calculated from the two-parameter model of Kimura (1980) using DNADIST software in PHYLIP. The parsimony tree was constructed after employing SEQBOOT (1000 replicates), then DNAPARS with 1000 multiple data sets and CONSENSE to generate a consensus tree by means of majority rule and strict consensus in the PHYLIP package. Support values for internal nodes in the maximum-likelihood tree were estimated in 100 datasets.

Results

Characteristics of cyt *b* DNA sequences

The entire cyt *b* gene of the megachiropteran species used in this investigation contained 1140 nucleotides. All sequences

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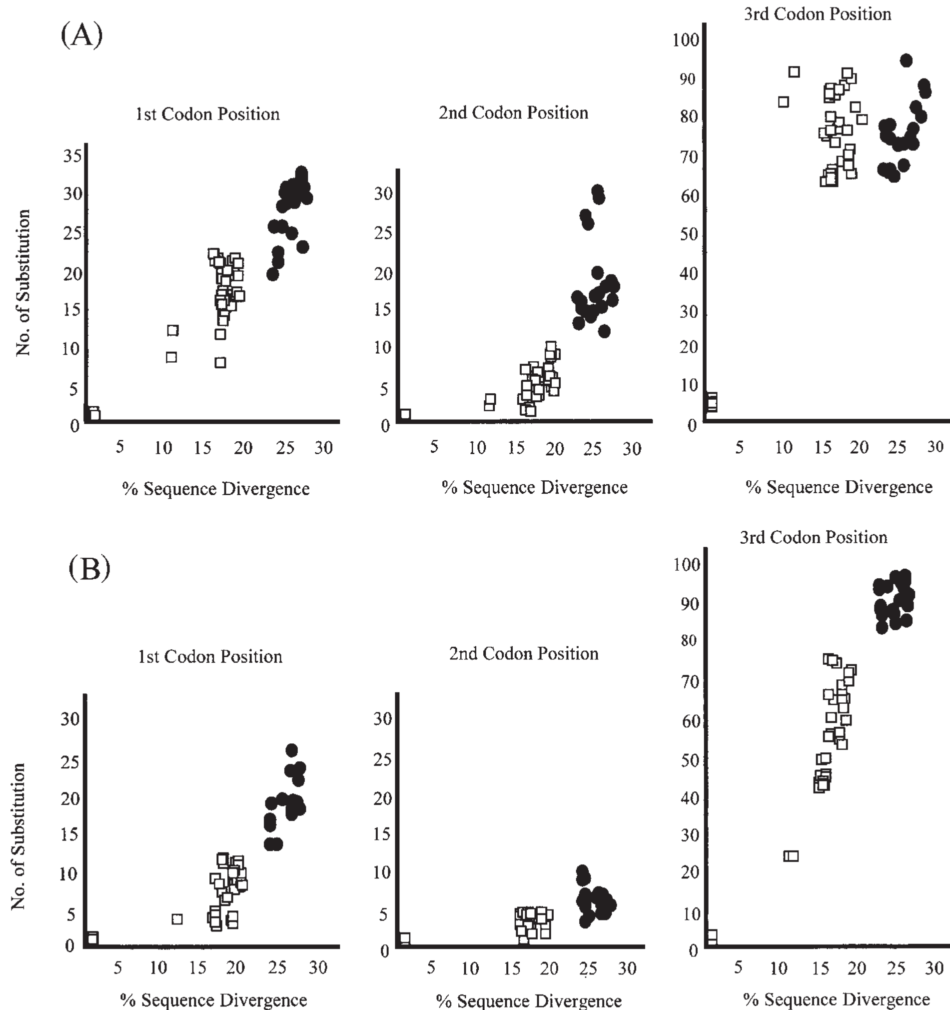
Fig. 2. Saturation-test graphs illustrating the nucleotide substitutions in the first, second, and third codon positions that occurred through transition (A) or transversion (B) in the complete DNA sequences of the cyt *b* gene of the bats. Each cluster of squares represents a comparison within megachiropteran species and the circles represent a comparison between megabats and the outgroup (microbats).



had an initial codon of ATG and ended up with codon AGA encoding 379 amino acids in length. The identical sites accounted for 812 bp (71.23%), and 328 positions (28.77%) were variable in the whole cyt *b* gene. Of these variable positions, 52 (15.85%) were found in the first codon position, 16 (4.88%) in the second, and 260 (79.27%) in the third. These sequences are available at DDBJ/EMBL/GenBank with accession numbers AB046320 to AB046329.

Saturation analysis

The results of the saturation analysis in the three different codon positions, both transition and transversion, are presented in Fig. 2. When the degree of saturation was assessed graphically, the transition at the first and second codon positions and transversion at all positions were not saturated. Only the third codon position had an indication of plateauing and was declared saturated.

Sequence comparison, genetic distance, and divergence time

The percentage of sequence divergence among five megachiropteran species in all pairwise comparisons revealed a wide range of nucleotide values. The substitution that occurred among the megabats for transition proved to be 56%, while it was 44% for transversion. There was high nucleotide substitution in the third codon position, but the substitution rate of amino acid replacement remained silent (i.e., unchanged). This study affirmed that 41 variable sites of the amino acid replacements had transpired: among others, these are lysine, alanine, valine, threonine, methionine, and isoleucine. The highest percentage of amino acid sequence divergence within the five species examined was found between *R. amplexicaudatus* and *P. vampyrus* (7.79–8.38%). The species having the least divergence fell between *C. brachyotis* and *P. jagori* (1.06–1.33%). *Rousettus amplexicaudatus* had a lower percentage of divergence from *E. spelaea* (4.07–5.12%) than the other two species that were classified within the subfamily Pteropodinae, *P. jagori* (6.34–6.92%) and *C. brachyotis* (5.76–6.63%).

To determine genetic divergence among taxa, the two-parameter model (Kimura 1980) for detecting genetic distance was used in the analysis (Table 2). A very short genetic distance (see Table 2) was found among the samples of *E. spelaea*, *R. amplexicaudatus*, and *C. brachyotis* collected

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from different sites on the islands of Luzon and Mindanao. Of the four members of the subfamily Pteropodinae, *R. amplexicaudatus* had least nucleotide sequence divergence from *E. spelaea* (16.26–17.47%). On the other hand, *E. spelaea* had fairly similar genetic distances from *P. vampyrus* and *R. amplexicaudatus*. Within the species of Pteropodinae, *C. brachyotis* and *P. jagori* reflected closer genetic affinity.

We estimated that about 50.2 million years ago (MYA) the megabats diverged from the outgroup (*C. villosum* and *U. bilobatum*). Thus, radiation within megabat species happened 32.4 MYA, while *Pteropus* diverged from *Cynopterus*–*Ptenochirus* about 31.9 MYA. Subsequently, *Cynopterus* and *Ptenochirus* separated 12.3 MYA. The time of divergence of *Rousettus* or *Eonycteris* from the group of megabats, or within species of *Cynopterus*, *Eonycteris*, and *Rousettus*, was not clear.

Phylogenetic relationships

We performed three methods of phylogenetic analysis (neighbor-joining, parsimony, and maximum likelihood) for the cyt *b* sequences of megabats (see Fig. 3) using the two microchiropteran species *C. villosum* and *U. bilobatum* (Baker et al. 1994) as the outgroup. The topology generated from the neighbor-joining and parsimony methods with bootstrap analysis (1000 replications) had comparable features in terms of branching and clustering of taxa. The difference was the positioning of *E. spelaea* in the maximum-likelihood tree compared with the neighbor-joining and parsimony trees. However, when we used bootstrap analysis (1000 replicates) to test the robustness of the internal nodes, we found a low support value (<50%) at the node shared by *Eonycteris* and *Rousettus* in the maximum-likelihood tree. Once this node with a low bootstrap value was collapsed, the branching pattern in the topology became similar to the phylogeny generated by the other two methods of phylogenetic reconstruction.

Since our saturation analysis at the third codon position transition showed saturation, we explored phylogenetic analysis further by utilizing the sequences of the first and second codon positions. The topology produced from the analyses of the methods of neighbor joining, parsimony with 1000 bootstrap iterations, and maximum likelihood (with 100 replications) was a single kind of phylogenetic tree that was identical with the topology produced using the other approach. A very slight difference in their bootstrap values at each node joining the taxa was noted (see Fig. 3).

In the phylogenetic tree, three lineages emerged from the node as megabats diverged from the outgroup (microbats). Two of these lineages are *E. spelaea* and *R. amplexicaudatus*, and the third with high bootstrap values (see Fig. 3) branches into two: *P. vampyrus* and the *P. jagori* – *C. brachyotis* cluster. The phylogenetic relationship of *P. vampyrus* as sister taxon of *P. jagori* and *C. brachyotis* is well supported in all the phylogenetic analyses, and likewise the relationship between *P. jagori* and *C. brachyotis*. Our overall phylogenetic analysis had shown that *R. amplexicadatus* did not cluster with any of the megabat species classified within the subfamily Pteropodinae. The species collected from different sites on the two major islands in the Philippines showed very little genetic divergence, with 100% bootstrap values on the node adjoining them.

Table 2. Percentage matrix of genetic distances (below the diagonal), using the two-parameter model of Kimura (1980); values above the diagonal correspond to the total substitutions of transversion in the complete sequences of the cyt *b* gene (1140 bp) among the megachiropteran species and the outgroup.

	1	2	3	4	5	6	7	8	9	10	11	12
1. *Chiroderma villosum*[a]		39	102	113	111	114	119	113	119	106	110	109
2. *Uroderma bilobatum*[a]	14.00		119	120	118	116	122	121	126	103	114	114
3. *Cynopterus brachyotis* (Luzon)	24.21	26.49		3	85	84	85	26	73	80	78	82
4. *Cynopterus brachyotis* (Mindanao)	24.33	26.12	1.24		88	88	89	30	80	81	71	84
5. *Eonycterus spelaea* (Luzon)	24.57	27.14	17.72	18.27		4	6	86	68	52	51	54
6. *Eonycterus spelaea* (Mindanao, site 1)	24.14	26.72	17.38	17.72	1.24		0	84	64	50	50	55
7. *Eonycterus spelaea* (Mindanao, site 2)	24.40	26.99	17.62	17.95	1.42	0.18		84	64	51	51	56
8. *Ptenochirus jagori* (Luzon)	26.86	27.54	12.55	12.05	19.35	19.46	19.70		69	70	70	75
9. *Pteropus vampyrus* (Mindanao)	24.94	27.98	16.92	16.81	16.69	16.36	16.59	17.29		68	66	66
10. *Rousettus amplexicaudatus* (Luzon)	24.47	27.69	19.24	19.01	16.49	17.01	17.24	19.92	18.71		2	3
11. *Rousettus amplexicaudatus* (Mindanao, site 1)	24.07	27.27	18.76	18.54	16.26	16.56	16.78	19.45	18.49	0.35		3
12. *Rousettus amplexicaudatus* (Mindanao, site 2)	24.33	27.16	18.89	18.66	16.94	17.24	17.47	19.57	18.59	0.71	0.71	

[a]Microbats in the outgroup (Baker et al. 1994).

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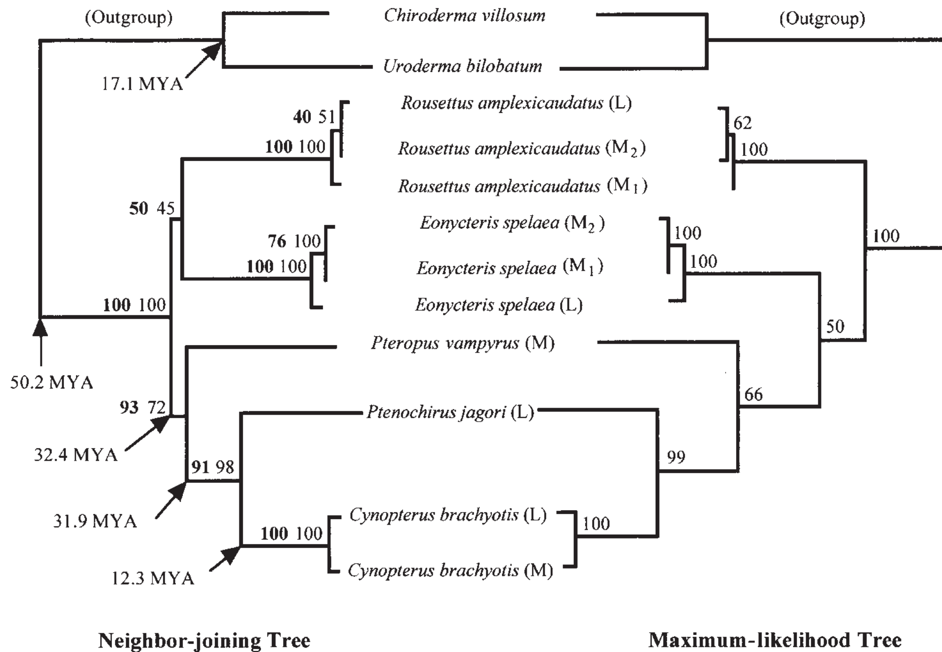
Fig. 3. Neighbor-joining and maximum-likelihood trees depicting the phylogenetic relationships of megachiropteran species deduced from complete DNA sequences of the cyt *b* (1140 bp) gene. The number(s) above each node in the topology indicate(s) the bootstrap probability (%) based on 1000 replications for the neighbor-joining tree ascertained by using the parsimony tree and on 100 replications for maximum-likelihood tree determined by the majority rule and strict consensus tree program. In the neighbor-joining tree, boldface numbers represent the bootstrap value when the first and second codon positions are used and lightface numbers represent the value when all the three codon positions are used. L, Luzon: M, Mindanao; M_1, Mindanao, site 1; M_2, Mindanao, site 2.



Discussion

Analysis of the complete cyt *b* gene sequences among the megachiropteran species from the two major islands in the Philippines provided evidence of their phylogenetic relationships. The positioning of some species in the phylogenetic tree that were grouped according to their suprageneric (rousettine, cynopterine, and eonycterine) and subfamilial (Pteropodinae and Macroglossinae) categories by Andersen (1912) was clarified. The use of two separate approaches, namely the complete nucleotide sequence data and the sequence data from the first and second codon positions, for deducing the phylogenetic relationships employing the neighbor-joining, parsimony, and maximum-likelihood methods with bootstrap analysis helped us to clarify the relationships. In the end, an overall phylogenetic analysis (Fig. 3) was arrived at, reflecting the evolutionary associations among the megabat species investigated.

In the phylogenetic tree (Fig. 3), the divergence between the group of megabats and the outgroup (microbats) in our calculation was 50.2 MYA. This is very close to the earliest fossil record of bats back the Early Eocene period (about 50–55 MYA), which shows substantial diversity (Simmons and Geisler 1998). As Nikaido et al. (2000) concluded, using analysis of complete sequences of the mitochondrial genome, the Jamaican fruit bat, *Artibeus jamaicensis* (a microbat) split from the Ryukyu flying fox, *Pteropus dasymallus* (a megabat), 57.7 ± 3.9 MYA.

Among the megabat species reported in this paper, divergence occurred 32.4 MYA. The phylogenetic relationships of *Eonycteris* with some species in the subfamily Pteropodinae (*Rousettus*, *Cynopterus*, *Pteropus*, and *Ptenochirus*) revealed them to have separated during this period; likewise, *R. amplexicaudatus* diverged from other members of the subfamily Pteropodinae (*Cynopterus*, *Pteropus*, and *Ptenochirus*). Subsequently, in a very short period of evolutionary time (approximately 0.5 million years after the megabats diversified), *Pteropus* split from *Cynopterus* and *Ptenochirus*. A trichotomy is reflected in the phylogenetic tree once the node with low bootstrap support (<50%) clustering *R. amplexicaudatus* and *E. spelaea* is collapsed. The large genetic distance of *Eonycteris* or *Rousettus* from *Cynopterus*, *Pteropus*, or *Ptenochirus* supports this finding. It further supports the idea of early diversification (Hollar and Springer 1997) and early radiation (Kirsch et al. 1995) of megabats.

At the suprageneric level, all methods of phylogenetic reconstruction disclosed that *Pteropus* formed separately from *Rousettus* and was excluded from the rousettine section. However, *Pteropus* was clearly reflected as a sister taxon of *Cynopterus* and *Ptenochirus*. The subfamily Pteropodinae became diphyletic when *Rousettus* was included in the *Cynopterus, Ptenochirus*, and *Pteropus* group. The relationship between *Cynopterus* and *Ptenochirus* as members of the cynopterine section (Andersen 1912) was sustained. The high bootstrap value found at the node adjoining *Pteropus* and the *Cynopterus–Ptenochirus* cluster and at the node shared by *Cynopterus* and *Ptenochirus*, as shown in Fig. 3, was confirmed by this molecular evidence. Moreover, the relationship between *Rousettus* and *Eonycteris* was not clearly defined in all our analyses. The radiation time of these two species remains uncertain. Furthermore, the species collected from different sites on the islands of Luzon and Mindanao in the Philippines had a very small genetic distances within them, and the divergence between samples was unclear in our analysis.

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