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Molecular Phylogenetics among Three Families of Bats (Chiroptera: Rhinolophidae, Hipposideridae, and Vespertilionidae) Based on Partial Sequences of the Mitochondrial 12S and 16S rRNA Genes

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Xiao-Ming Gu, Shu-Yan He, and Lei Ao (2008) Molecular phylogenetics among three families of bats (Chiroptera: Rhinolophidae, Hipposideridae, and Vespertilionidae) based on partial sequences of the mitochondrial 12S and 16S rRNA genes. *Zoological Studies* **47**(3): 368-378. Extensive morphologic and molecular analyses of the phylogenetics of bats have been carried out, but controversies still exist. In order to further deduce the phylogenetic relationships among the families Vespertilionidae, Hipposideridae, and Rhinolophidae of microbats (Chiroptera), partial mitochondrial 12S and 16S rRNA gene sequences (2400 bp) for 32 species of the 3 families were obtained, among which the sequences of 19 species were amplified in this study and the other 13 were retrieved from GenBank. Meanwhile, those of 3 species in the family Pteropodidae and 1 species in the family Molossidae were also obtained from GenBank. The phylogenetics of all 5 families were assessed using a maximum-parsimony (MP) analysis. Second, the intrafamily relationships of the Vespertilionidae as well as the intra- and interfamily relationships of the Rhinolophidae and Hipposideridae were addressed using Bayesian, minimum-evolution (ME), and Neighbor-joining (NJ) methods. The following results were clearly demonstrated. (1) The paraphyly of microbats was revealed. (2) The subfamily Miniopterinae could not be elevated to family status, and it was the 1st clade separated from the Vespertilionidae. (3) *Myotis* should be elevated to the subfamily Myotinae, which was sister to a clade containing the Kerivoulinae and Murininae. (4) In the genus *Myotis*, all assayed New World and Old World species were respectively placed together in clades, and the 3 subgenera were not closely phylogenetically related. (5) As for *Pipistrellus*-like bats, 3 genera (*Ia*, *Scotomanes*, and *Eptesicus*) were successively placed together in clades, and were sister to the clade containing *Pipistrellus*, indicating a higher probability that the genus *Ia* belongs to Eptesicini than to *Pipistrellus*. (6) The Rhinolophoidae and Hipposideridae should be treated as separate families. (7) In the genus *Hipposideros*, *Hipposideros armiger* was first placed together with *H. larvatus* in a clade, then with *H. pratti*, and *H. bicolor* was the 1st branch separated from other species of *Hipposideros*. (8) Finally, *R. ferrumequinum* and *Rhinolophus* sp. were the 1st branch separated from other Rhinolophids.
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Key words: Phylogeny, Bats, Mitochondrial rRNA.

The order Chiroptera contains about 1100 species, making up more than 20% of extant mammals (Simmons 2005). Among them, there are about 120 species in China (Wang et al. 2003). Phylogenetic relationships of bats have been extensively analyzed, but many controversies still exist. Traditionally, the microchiroptera was considered a monophyly with complex laryngeal echolocation systems (Simmons and Geisler 1998). Porter et al. (1996) and Hutcheon et al. (1998) proposed the paraphyly of micochiroptera, and the hypothesis was supported by Teeling et al. (2000 2002 2005), whose research indicated that the superfamily Rhinlolphoidea was sister to the

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megabats and who proposed 2 new suborders: the Yinpterochiroptera and Yangochiroptera. The former contains the megabats and the superfamily Rhinolophoidea of micorbats, and the latter contains all other microbats. To the present, many research results have supported Teeling's suggestion based on cytogenetic and molecular data (Springer et al. 2001, Eick et al. 2005, Ao et al. 2007). As for relationships between the Rhinolophidae and Hipposideridae, Koopman (1994) and Simmons and Geisler (1998) considered the Hipposideridae to be a subfamily of the Rhinolophidae, but some authors regard them as 2 parallel families (Bogdanowicz and Owen 1998, Wang et al. 2003). As to intrafamily relationships of the Rhinolophidae and Hipposideridae, most previous research was restricted to morphologic analyses, and molecular phylogenetic relationships for only a few species were defined by Sakai et al. (2003) and Wang et al. (2003), leaving many problems unresolved, especially the phylogenetic positions of some Rhinolophids endemic to China. In the family Vespertilionidae, the largest family in the order Chiroptera, some phylogenetic uncertainties still exist, such as the status of the subfamily Miniopterinae and the genus *Myotis*, and the phylogenetic positions of *Ia* and *Scotomanes* among *Pipistrellus*-like bats.

Guizhou Province is located in southwestern China, and about 40 bat species, all of which are microbats except for *Rousettus leschenaultia*, inhabit this province (Luo et al. 1993, Wang et al. 2003). We collected 19 species representing 3 families (Rhinolophidae, Hipposideridae, and Vespertilionidae) from Guizhou Province, and used partial sequences of mitochondrial 12S and 16S rDNA in order to investigate intra- and interfamily relationships of these 3 families.

MATERIALS AND METHODS

Taxonomic sampling

Nineteen species representing 3 families (Rhinolophidae, Hipposideridae, and Vespertilionidae) collected from natural populations in Guizhou Prov., China, are listed in table 1. Among them, *Myotis altarium*, *Rhinolophus rex*, *R. yunnanensis*, and *R. sinicus* are endemic to China (Wang and Xie 2005). Partial sequences of their mitochondrial 12S and 16S rRNA genes were amplified. The corresponding sequences of 13 species from the same 3 families were retrieved from GenBank along with those of 3 Pteropodidae and 1 Molossidae species. Sequences of 3 other species (Laurasiathera: Bovidae) from GenBank were selected as outgroups for constructing the maximum-parsimony (MP) tree.

Genetic analysis

Genomic DNA from muscle tissue samples were extracted with standard phenol methods (Longmire et al. 1997). A polymerase chain reaction (PCR) cocktail (with a 50 μl reaction volume) included 2 μl DNA extract, 1.5 μl of each primer, 4 μl dNTP (2.5 mM of each dNTP), 5 μl 10× PCR buffer, and 0.5 μl *Taq* polymerase (0.1 U/μl). Amplification included initial denaturation at 94°C for 5 min, followed by 37 cycles of 94°C for 40 s, 52°C and 54°C for 12S and 16S rRNA genes, respectively, for 2 min, and 72°C for 3 min; with a final extension at 72°C for 30 min. The PCRs were carried out with the primer pairs of 12S (12S-1: 5′-TTTCATCTTTTCCTTGCGGTAC-3′ and 12S-2: 5′-AAAGCAAARCACTGAAAATg-3′) and 16S (16-1: 5′-CYGGAAAGTGTGCTTGGA-3′ and 16-2: 5′-gCAATTACCGRRCTCTGCCA-3′) rDNA (Van Den Bussche and Hoofer 2000). The PCR products were purified and sequenced by Shanghai DNA Bio Technologies Co, Ltd (Shanghai, China). The fragments obtained were put into the NCBI database to search for sequences homologous. Since the 2 fragments of 16S rDNA did not overlap (the gaps were 200-300 bp), we separately aligned the 2 fragments of 16S rDNA and 12S rDNA by ClustalX, incorporating the default settings, and modified the alignments according to the secondary structures of 12S and 16S rDNA (Springer and Douzery 1996). Regions of ambiguous alignments were removed from the sequences.

Phylogenetic reconstruction

An MP analysis (using heuristic searches with 10 random input orders of taxa, TBR branch swapping, and equally weighted characters) for all 5 families was conducted with PAUP4.0 (Swofford 2003) using 3 species of Laurasiatherians (GenBank) as outgroups. Levels of repeatability of the branching patterns were assessed with 1000 bootstrap replicates. Bayesian, minimum-evolution (ME), and Neighbor-joining (NJ) analyses for the family Vespertilionidae were performed using Mrbayes 3.0 (Huelsenbeck and Ronquist

Table 1. Locality and GenBank accession numbers for the mitochondrial 12S and 16S rRNA genes of the specimens investigated here

	Locality	GenBank accession nos. number		Voucher number
		12S rDNA	16S rDNA	
Family Vespertilionidae				
Subfamily Vespertilioninae				
Ia io	Xingyi, Guizhou, China	DQ989600	DQ989618	GZNU200314
Ia io	Guiyang, Guizhou	DQ989603	DQ989616	GZNU200317
Scotomanes ornatus	GenBank	AY495537	AY495537	-
Eptesicus brasiliensis	GenBank	AY495464	AY495464	-
Pipistrellus javanicus	GenBank	AY495525	AY495525	-
Pipistrellus pipistrellus	GenBank	AF326105	AF326105	-
Pipistrellus abramus	Xiuwen, Guizhou	[a]	DQ989620	GZNU200323
Subfamily Myotinae				
Myotis petax	Anshun, Guizhou	DQ989592	DQ989617	GZNU200306
Myotis siligorensis	GenBank	AY495508	AY495508	-
Myotis altarium	Guiyang, Guizhou	DQ989602	DQ989621	GZNU200316
Myotis myotis	Guiyang, Guizhou	DQ9895601	DQ989610	GZNU200315
Myotis davidii	Chishui, Guizhou	DQ9895604	DQ989626[b]	GZNU200318
Myotis yumanensis	GenBank	AY495512	AY495512	-
Myotis velifer	GenBank	AY495509	AY495509	-
Subfamily Kerivoulinae				
Kerivoula pellucida	GenBank	AY495476	AY495476	-
Kerivoula hardwickii	GenBank	AF345928	AF345928	-
Subfamily Murininae				
Murina huttoni	GenBank	AY495492	AY495492	-
Subfamily Miniopterinae				
Miniopterus fuliginosus	Anshun, Guizhou	DQ9895605	DQ989622	GZNU200319
Miniopterus australis	GenBank	AY395864	AY395864	-
Family Molossidae				
Molossus molossus	GenBank	AY495455	AY495455	-
Family Rhinolophidae				
Rhinolophus ferrumequinum	Guiyang, Guizhou	DQ989594	DQ989619	GZNU200308
Rhinolophus sinicus	Anshun, Guizhou	DQ989599	DQ989614	GZNU200313
Rhinolophus affinis	Guiyang, Guizhou	DQ989596	DQ989612	GZNU200310
Rhinolophus rex	Anshun, Guizhou	DQ989598	DQ989615	GZNU200312
Rhinolophus monoceros	GenBank	AF406806	AF406806	-
R. yunnanensis	Anlong, Guizhou	DQ989587	[a]	GZNU200301
Rhinolophus sp.	Shuicheng, Guizhou	DQ989589	DQ989611	GZNU200303
Rhinolophus pusillus	Guiyang, Guizhou	[a]	DQ989613	GZNU200322
Family Hipposideridae				
Aselliscus stoliczkanus	Guiyang, Guizhou	DQ989595	DQ989625[b]	GZNU200309
Hipposideros pratti	Jinsha, Guizhou	DQ989588	DQ989623[b]	GZNU200302
Hipposideros larvatus	Anlong, Guiyang	DQ989590	DQ989609	GZNU200304
Hipposideros armiger	Guiyang, Guizhou	DQ989591	DQ989608	GZNU200305
Hipposideros galeritus	GenBank	HGU93054	AF203738	-
Hipposideros bicolor	Anlong, Guizhou	DQ989607	[a]	GZNU200321
Family Pteropodidae				
Rousettus aegyptiacus	GenBank	AB205183	AB205183	-
Pteropus scapulatus	GenBank	AF321050	AF321050	-
Pteropus dasymallus	GenBank	AB042770	AB042770	-
Family Bovidae				
Bos taurus	GenBank	AY676873	AY676873	-
Bos indicus	GenBank	AY126697	AY126697	-
Bos grunniens	GenBank	AY684273	AY684273	-

The classification system follows Simmons (2005). [a]Indicates that the amplified gene could not be sequenced. [b]Denotes that 16S rDNA was only sequenced using 1 primer. 16S rDNA genes were submitted to GenBank as “gapped sequences”.

2001) and PAUP4.0, respectively, with the GTR+G model of DNA evolution and 2 Rhinolophids as outgroups. The Bayesian analysis included 1×10^6 generations of Markov chain Monte Carlo (Altekar et al. 2004), and the burn-in value was set to 1100. The corresponding analyses for the Rhinolophidae and Hipposideridae were also conducted under the GTR+I+G DNA evolutionary model using 2 species of the subfamily Miniopterinae as outgroups.

We used parsimony K-H (Kishino and Hasegawa 1989) and Templeton tests (Templeton 1983), available in PAUP4.0, to test some alternative hypotheses, such as the monophyly of microbats.

RESULTS

Characteristics of the 12S and 16S rRNA gene datasets

Partial sequences of the mitochondrial 12S rRNA gene of 17 bat species (with the exception of *Rhinolophus pusillus* and *Pipistrellus abramus*) were sequenced and deposited in GenBank (see accession nos. in table 1). The sequence lengths were from 624 to 812 bp, and the overall nucleotide composition was biased toward a deficit of guanine residues (with a G+C content of 42.6%). Combining the corresponding sequences obtained from GenBank, 831 bp was retained after alignment, and taxa shorter than 831 bp were supplemented by adding Ns to them (Ruedi and Mayer 2001). Of the 831 bp, the constant and parsimoniously informative sites were 287 and 222 bp long, respectively, with a G+C content of 43.3%.

In addition to *H. bicolor* and *R. yunnanensis*, the 16S rRNA genes of 14 species were sequenced with both primers while *M. davidii*, *H. pratti*, and *A. stoliczkanusi* were sequenced with only a single primer. The 16S rRNA sequences were also deposited in GenBank as “gapped sequences”. The fragment sequenced with 1 primer was from 618 to 758 bp with a G+C content of 36.6%. After alignment, 828 bp was retained, shorter ones were supplemented with Ns, and constant and parsimoniously informative sites were 344 and 178 bp, respectively, with a G+C content of 43.3%. Similarly, the fragment sequenced with the other primer was from 664 to 853 bp with a G+C content of 43.0%. After alignment, 811 bp was retained, and shorter ones were also supplemented with Ns. Invariable and parsimoniously informative sites were 273 and 212 bp, respectively, with a G+C content of 35.9%.

The 12S and 16S rRNA genes are adjacent to each other, and they can be combined into a concatenated dataset. First, 2 fragments of the 16S rRNA gene (828 and 811 bp, respectively) were combined. Because of a lack of 1 fragment for *M. davidii*, *H. pratti*, and *A. stoliczkanusi*, we replaced these missing values by Ns. Second, the 12S rDNA (831 bp) and 16S rDNA (1639 bp) fragments were combined. Fragments of the 12S rRNA gene for *R. pusillus* and *P. abramus* as well as those of the 16S rRNA gene for *H. bicolor* and *R. yunnanensis* were also replaced by Ns.

Phylogenetic analyses

The 12S rDNA, 16S rDNA, and concatenated datasets of all 5 families were respectively analyzed

Table 2. Bootstrap support or posterior probability for the important clades of the different analyses

	MP (Unweighted)			Bayesian (GTR+G)	ME (ml) (GTR+G)	ME (lodget) (GTR+G)	NJ
Clade	12S rDNA	16S rDNA	Con	Con	Con	Con	Con
A	85	93	98	1.00	99	100	98
B	54	62	83	1.00	68	72	91
C	80	86	100	1.00	100	100	100
D	55	60	70	1.00	76	86	89
E	70	74	74	1.00	81	80	93
F	83	72	93	1.00	76	81	61
G	95	93	99	1.00	95	99	99
K	80	82	82	1.00	92	90	87

The letters represent clades described in figure 1. Con, concatenated dataset; MP, maximum parsimony; ME, minimum evolution: NJ, Neighbor-joining.

using the MP method. Generally, bootstrap values of separate datasets were lower than that of the concatenated one (Table 2), thus the phylogenetic relationships were analyzed mainly using the tree obtained from the concatenated data set (MP tree, Fig. 1). In the MP tree, the ingroups were divided into clades E and H with very high bootstrap support. Clade E was made up of the families Molossidae and Vespertilionidae, which belong to the suborder Yangochiroptera, while clade H consisted of the families Rhinolophidae, Hipposideridae, and Pteropodidae, which belong to the Yinpterochiroptera (Teeling et al. 2005), with each of the 5 families exhibiting monophyly. In the family Vespertilionidae, 2 species of the subfamily Miniopterinae were the 1st clade separated from other Vespertilionids, and the clade was not sister to *Molossus molossus*. Clade C indicated that the subfamily Vespertilioninae was paraphyletic relative to the genus *Myotis*, because *Myotis* was sister to a clade which contained the subfamilies Murininae and Kerivoulinae in clade B. *Pipistrellus*-like bats, including the genera of *Ia*, *Scotomanes*, *Eptesicus*, and *Pipistrellus*, were grouped into clade A, which was sister to clade B. In the genus *Myotis*, species from the New World (clade M, including *M. velifer* and *M. yumanensis*) and Old World (clade N including *M. daubentonii*, *M. siligorensis*, *M. altarium*, *M. myotis*, and *M. davidii*) formed respective monophyletic clades. In the suborder Yingochiroptera, clade G indicated that the Rhinolophidae and Hipposideridae were 2 parallel families. For the family Rhinolophidae, *R. ferrumequinum* and *Rhinolophus* sp. were placed together in a clade which formed the 1st branch separated from other Rhinolophids, followed by a branch containing *R. affinis*. In the 3rd branch, *R. sinicus* grouped with *R. rex*, as did *R. yunnanensis*, *R. monoceros*, and *R. pusillus*. As for the Hipposideridae, clade K clearly demonstrated that *A. stoliczkanus* belongs to the Hipposideridae not the Rhinolophidae. In the genus *Hipposideros*, *H. bicolor* and *H. galeritus* were the 1st 2 separate branches formed, suggesting they are distantly

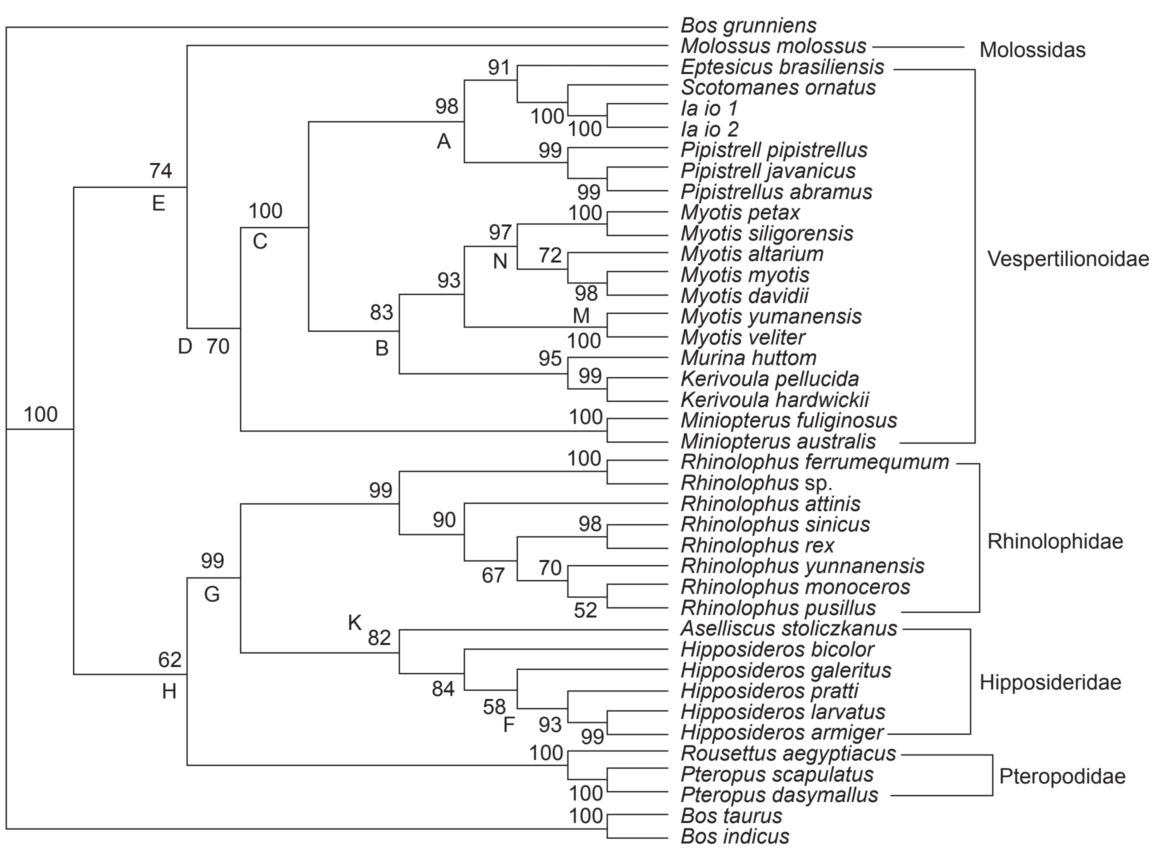

Fig. 1. Maximum-parsimony (MP) tree for 37 taxa of 5 families: the Rhinolophidae, Hipposideridae, Vespertilionidae, Molossidae, and Pteropodidae. The Pteropodidae belongs to the megachiroptera and the other 4 are in the microchiroptera. The MP tree was created using heuristic searches with 10 random input orders of taxa, TBR branch swapping, and equally weighted characters. The figure indicates the paraphyly of the microchiroptera and the interrelationships of the 5 families.

related to clade F consisting of *H. pratti*, *H. larvatus*, and *H. armiger*.

The Bayesian analysis showed that the family Vespertilionidae (Fig. 2) was almost identical to clade E in the MP tree (Fig. 1). Some important bootstrap values of the ME and NJ analyses are listed in table 2 (clades A-D), and these results further confirmed the results from the MP tree and Bayesian analyses.

For the Rhinolophidae and Hipposideridae, the results of the Bayesian analysis (Fig. 3) were congruent with that of clade H in the MP tree (Fig. 1). The bootstrap values of clades E-K for the ME and NJ analyses are also listed in table 2, and these results were identical to those of the MP and Bayesian analyses.

Test of some hypotheses

Using the concatenated dataset, K-H and Templeton tests were performed to check certain conclusions in the MP analysis (Table 3). These tests further verified the MP results, except that the precise phylogenetic position of the subfamily Miniopterinae was left unresolved.

DISCUSSION

The order Chiroptera has traditionally been divided into 2 monophyletic suborders: the megachiroptera and microchiroptera. All microbats have complex laryngeal echolocation systems, while megabats have enhanced visual

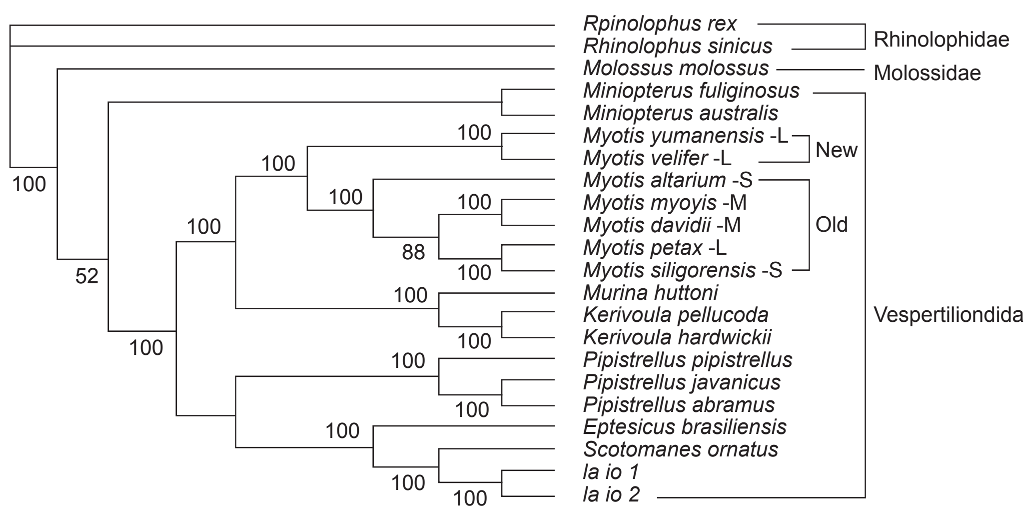

Fig. 2. Bayesian tree for the family Vespertilionidae, including 22 taxa. The tree was created using the GTR+G model of DNA evolution and 2 Rhinolophids were chosen as outgroups. The figure tries to explain the phylogenetic positions of the subfamily Miniopterinae and the genera *Myotis* and *Ia*.

Table 3. Test of alternative hypotheses compared to the most parsimonious trees (Templeton test) using concatenated datasets

	Kishino Hasegawa test (MP)			Templeton test (MP)	
Constraint	Extra steps	Standard deviation	*p* value	*n*	*p* value
a	9	10.05028	0.3706	101	0.4260
Microbats monophyletic	130	13.75625	< 0.0001*	193	< 0.0001*
c	77	11.60406	< 0.0001*	137	< 0.0001*
d	78	16.42090	< 0.0001*	233	< 0.0001*

a, ((*Miniopterus australis*, *Miniopterus fuliginosus*), *Molossus molossus*); c, (*Ia io* 1, *Ia io*, *Pipistrellus javanicus*, *Pipistrellus pipistrellus*, *Pipistrellus abramus*); d, represents *Aselliscus stoliczkanus* groups with the family Rhinolophidae instead of the Hipposideridae. * Indicates a significant difference at $p < 0.05$. MP, maximum parsimony.

abilities, and both were traditionally regarded as being monophyletic. Simmons and Geisler (1998) analyzed 180 morphological characteristics and 12 restriction sites of Chiroptera, and the results suggested the monophyly of microbats. However, recent comprehensive molecular phylogenetic analyses of the order indicated that the superfamily Rhinolophidae of microbats was phylogenetically more closely related to megabats than the rest of the microbats, suggesting the paraphyly of microbats (Teeling et al. 2000 2005, Springer et al. 2001, Eick et al. 2005). The concatenated MP tree (Fig. 1) in the present study obviously indicates that the Rhinolophidae and Hipposideridae are more closely phylogenetically related to the Pteropodidae which belongs to the megachiroptera than to other microbats, supporting the paraphyly of microbats, and the K-T and Templeton tests of the MP tree further confirmed Teeling's (2000 2005) conclusion ($p < 0.0001$) (Table 3).

The Vespertilionidae is the largest family in the order Chiroptera, and the tremendous amount of diversity in numbers and kinds within the family has hampered efforts to provide adequate assessments of long-standing genealogic hypotheses. With rapid developments of molecular phylogeny, phylogenetic positions of some taxa in this family, especially the subfamily Minopterinae, the genus *Myotis*, and *Pipistrellus*-like bats, have been vigorously debated.

Traditionally, the Vespertilionidae was divided into several subfamilies according to morphological characters, and the widely accepted ones are the Miniopterinae, Kerivoulinae, Murininae, and Vespertilioninae (Koopman 1994, Simmons and Geisler 1998). Mein and Tupinier (1977) challenged the traditional view and proposed that the subfamily Miniopterinae should be separated from the Vespertilionidae and elevated to family status based on the observation that the Miniopterinae, instead of the Vespertilionidae, possesses a supplementary vestigial tooth between the upper canine and 1st premolar. This proposition was supported by Hoofer et al. (2003) who analyzed 3 adjacent mitochondrial genes (12S rDNA, 16S rDNA, and $tDNA^{Val}$) of 17 genera and 110 species in the family Vespertilionidae, but was rejected by Volleth and Heller (1994) who described the banded karyotypes of 50 species representing 23 genera and all subfamilies of the Vespertilionidae, indicating that the subfamily Miniopterinae was the 1st branch separating from the other Vespertilionids and did not represent a separate family.

The concatenated MP (Fig. 1), the Bayesian tree for the Vespertilionidae (Fig. 2), and the ME and NJ analyses (clade D in table 2) support the view of Volleth and Heller (1994), that considered the Miniopterinae a subfamily of the Vespertilionidae. But the K-T and Templeton tests could not completely reject the alternative hypothesis ($p > 0.05$, Table 3, constraint a) of elevating the subfamily Minopterinae to the family Minopteridae. Furthermore, the cytogenetic analysis was also unable to verify the status of the subfamily Minopterinae (Ao et al. 2006). Thus, the phylogenetic position of the subfamily Miniopterinae remains uncertain.

With about 90 species spread all over the world, the genus *Myotis* represents one of the most diverse and successful radiations among mammals. Because the genus has a rather undifferentiated morphology, shares many plesiomorphic characters (Menu 1987), and has very conserved karyotypes, its precise position in the Vespertilionidae and the taxonomic subdivision

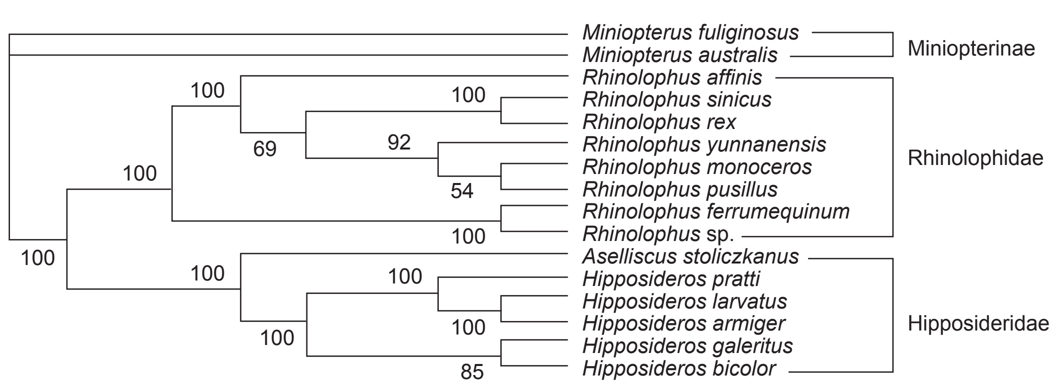

Fig. 3. Bayesian tree for the families Rhinolophidae and Hipposideridae with 14 taxa. The tree was created using the GTR+I+G model of DNA evolution, and 2 species in subfamily Miniopterinae were used as outgroups. The tree was constructed to elucidate the intra- and interfamily relationships of the 2 families.

of the genus are controversial. On the basis of a karyotype analysis, Volleth and Heller (1994) first promoted the genus *Myotis* to the subfamily Myotinae. The hypothesis was supported by a morphologic analysis by Simmons and Geisler (1998) and by a molecular phylogenetic analysis by Hoofer et al. (2003). According to the analyses of DNA sequences from the von Willebrand factor (vWF) gene and short interspersed elements (SINEs) of 38 species in the Vespertilionidae, Kawai et al. (2002) proposed a close association between *Myotis* and the Murininae, admitting to the subfamily status of *Myotis*. The concatenated MP and Bayesian trees in figures 1 and 2 and other related analyses (clade B in table 2) support *Myotis* being sister to the clade consisting of the Kerivoulinae and Murininae, which was identical to the proposition of Hoofer and Van Den Bussche (2003).

Findley (1972) used numerical taxonomy on cranial and external characters to classify most described species of *Myotis* and divided them into the 3 subgenera of *Selysius*, *Myotis*, and *Leuconoe*. However, Ruedi and Mayer (2001) reconstructed the phylogenetic history of 13 American, 11 Palaearctic, and 6 other species of *Myotis* using cytochrome *b* and NADH dehydrogenase subunit 1 genes, and the results clearly demonstrated that none of the 3 subgenera of Findley (1972) constituted monophyletic clades, but instead strongly supported 2 monophyletic clades: all New World species plus the Old World species *M. brandtii*, and the rest of the sampled Old World species. This conclusion was supported by Hoofer and Van Den Bussche (2003), and their results indicated that the genus *Myotis* should be grouped according to geography, supporting a primary divergence between New and Old World species, and deduced that morphologic and ecologic similarities defining each of the 3 subgenera represented convergent evolution. All the related analyses in the present study followed the suggestions of Ruedi and Mayer (2001) and Hoofer and Van Den Bussche (2003). As indicated in the Bayesian analysis of figure 2, none of 3 subgenera (*Selysius*, *Myotis*, and *Leuconoe*) constituted a monophyletic clade. In fact, species from the New (clade M in the MP tree) and Old (clade N in the MP tree) World comprised respective monophyletic clades.

To the present, the phylogenetic status of *Ia* and *Scotomanes* has been uncertain. Tate (1942) and Simpson (1945) regarded *Ia* as being closely related to *Pipistrellus* in morphology, but some authors recognized *Eptesicus* and *Ia* as being 2 closely related genera according to morphological and karyotypic studies (Topal 1970, Gu et al. 2003). In Simmon's (2005) system, *Ia* and *Scotomanes* were respectively placed into the tribes Vespertilionini and Nycticeiini. The concatenated MP tree (clade A in figure 1) and Bayesian tree for the Vespertilionidae (Fig. 2) in our study support *Ia* being more closely related to *Scotomanes* and *Eptesicus* than *Pipistrellus*, and the K-T and Templeton tests also excluded the possibility that *Ia* is closely related to *Pipistrellus*. However, without the Pipistrellini species, we cannot decide into which groups the genus *Ia* should be put, and the only conclusion that can be drawn is that the probability that the genus *Ia* belongs to the Eptesicini is higher than that it belong to *Pipistrellus*.

The Hipposideridae is closely related to the Rhinolophidae, but was first distinguished by Miller (1907) based on morphological characteristics. Some researchers regarded the Hipposideridae as a subfamily of the Rhinolophidae (Ellerman and Morrison-Scott 1966, Koopman 1994, Simmons and Geisler 1998, Teeling et al. 2002 2005), whereas others suggested that the Hipposideridae and Rhinolophidae were 2 separate families (Pierson 1986, Corbet and Hill 1991, Bogdanowicz et al. 1998, Volleth and Owen 2002, Simmons and Gesler 1998). Our results in the concatenated MP and Bayesian trees in figures 1 and 3 both support the Hipposideridae and Rhinolophidae being separate families, which is identical to the latest classification system of Simmons (2005).

The family Rhinolophidae is comprised of a single genus, *Rhinolophus*, containing 76 species (Simmons 2005). All earlier studies of the phylogenetic relationships of *Rhinolophus* were based on traditional taxonomic characters, primarily the shape of the noseleaf, the position of the 3rd upper premolar, and overall size (Qumsiyen 1988). To the present, a few studies of molecular phylogenetic relationships within the Rhinolophidae have been done (Sakai et al. 2003, Wang et al. 2003); therefore, the phylogenetics of the Rhinolophidae remain mostly uncertain, especially for species endemic to China.

Rhinolophus ferrumequinum, *R. affinis*, and *R. sinicus* belong to the *philippinensis* group; *R. pusillus* and *R. monoceros* are in the *lepkdus* group; whereas *R. rex* and *R. yunnanaensis* are in the *macrotis* group (Andersen 1905a, 1905b, 1918). The topologies of the Rhinolophidae in the concatenated MP and Bayesian trees in figures

1 and 3 were almost identical: *R. ferrumequinum* (2n = 58, Tate et al. 1943) and *Rhinolophus* sp. (2n = 62, unpublished data) were placed together in a clade, and *R. sinicus* (2n = 36, Gu et al. 2003) was grouped with *R. rex* (2n = 62, Gu et al. 2003), as were *R. yunnanensis* (2n = 44, Gu 2006), *R. monoceros* (2n = 62，Ando et al. 1980), and *R. pusillus* (2n = 62, Harada and Owen 1985). The present cladogram of the Rhinolophidae agrees with the suggestions of Bogdanowicz and Owen (1998) and Haiduk et al. (1981) that only a few groups identified by early morphologists were well defined, and non-differentially stained karyotypes were unreliable indicators of relationships. The results of phylogenetic relationships in the present study indicate that *R. ferrumequinum* and *Rhinolophus* sp. are distantly related to other assayed Rhinolophids, and that *R. sinicus* and *R. rex* are close to each other, as are *R. yunnanensis*, *R. monoceros*, and *R. pusillus*. However, more species and genes should be used to further confirm our results and elucidate the phylogenetics of the Rhinolophidae.

The family Hipposideridae is composed of 80 species in 9 genera, and *Hipposideros* is the largest genus in this family (Simmons 2005). In China, there are 3 genera (*Hipposideros*, *Aselliscus*, and *Coelops*) and 10 species in this family (Wang 2003). The concatenated MP and Bayesian trees in figures 1 and 3, and other related analyses (clade K in table 2 and constraint d in table 3) all supported *Aselliscus* undoubtedly belonging to the Hipposideridae. In the MP tree, *H. bicolor* and *H. galeritus* were the 1st and 2nd branches to separate from other *Hipposideros* species, and in figure 3, these 2 species were placed together in a clade which was sister to the clade consisting of the other *Hipposideros* species. To some extent, our results support Hill's (1963) proposition that the 2 species belong to the group *bicolor* and are distantly related to other *Hipposideros* species. The arrangement of *H. armiger*, *H. larvatus*, and *H. pratti* was identical to the classification of Wang et al. (2003).

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