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Molecular phylogenetic of Hipposiderids (Chiroptera: Hipposideridae) and Rhinolophids (Chiroptera: Rhinolophidae) in China based on mitochondrial cytochrome *b* sequences

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A b s t r a c t . Phylogenetic relations among five species of Hipposideridae and seven species of Rhinolophidae including one endemic species (*Rhinolophus rex*) were examined by partially sequencing of the mitochondrial cytochrome *b* gene (528 bp). Analyses of the cytochrome *b* sequences of Hipposideridae and Rhinolophidae suggest that each formed a monophyletic group. All phylogenetic analyses indicate that *Aselliscus* should remain as a genus within Hipposideridae, with the mean percentage sequence differences (16.43%) and transition: transversion ratios (2.032) between *Aselliscus* and *Hipposideros*. Within *Hipposideros*, *H. armiger* shows close affinity to *H. larvatus* although it possesses superficial similarity morphological characters to *H. pratti*. Genetic distance values suggest that *H. larvatus* and *H. armiger* diverged from each other approximately 1.7–4.3 million years ago, and *H. pratti* diverged from the *larvatus-armiger* clade approximately 2.1–5.2 million years ago.

Key words: China, cytochrome *b*, Hipposideridae, mitochondrial DNA, phylogeny, Rhinolophidae

Introduction

The family Hipposideridae, which includes 65 species, inhabits tropical and subtropical regions in Africa and Southern Asia, east to the Philippine Islands, the Solomon Islands, and Australia (C o r b e t & H i l l 1991, K o o p m a n 1994). Generally it has been accepted that the family originated in the Old World tropics, probably in Africa or Asia (K o o p m a n 1970). Recently, B o g d a n o w i c z & O w e n (1998) suggested that Hipposideridae, like their sister-family Rhinolophidae (B o g d a n o w i c z & O w e n 1992), most likely originated in Asia. Although Hipposideridae is a widely distributed family and has numerous species, the familial status has remained open to question. The relationships between the genus *Aselliscus* and other genera in Hipposideridae and Rhinolophidae have attracted the attention of some taxonomists, but it has remained controversial (B o g d a n o w i c z & O w e n 1998).

In China, bats of the family Hipposideridae primarily are distributed in the south (Z h a n g 1997) and include three genera and six species. These are four species of *Hipposideros*: (*H. armiger*, *H. bicolor*, *H. larvatus* and *H. pratti*), one *Aselliscus* (*A. stoliczkanus*) and one *Coelops* (*C. frithi*). The phylogenetic relationships among species of *Hipposideros* have been examined by morphological analysis by H i l l (1963). However, H i l l ' s (1963) classification was intuitive, and whether the species groups reflect the true phylogenetic history was not certain.

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Most previous systematic studies of extant hipposiderids have been based on craniodental and postcranial morphology as well as bacular morphology and morphometrics (Hill et al. 1986, Kitchener et al. 1992, Flannery & Colgan 1993, Zubaid & Davison 1987). Other taxonomic studies have used electrophoretic, karyological and immunomolecular data (Andō et al. 1980, Pierson 1986). However, the analysis of certain morphological characters can be difficult, since such features may not necessarily reflect the true phylogenetic relationships of the bat species examined (Barratt et al. 1995). Recently, Rajan & Marimuthu (2000) used RAPD-PCR method to examine the genetic variation within and between populations of *Hipposideros speoris*. Kingston et al. (2001) also determined the relationship between two cryptic species of *Hipposideros* (131KHZ *H. bicolor* and 142KHZ *H. bicolor*) which acoustically are divergent, using morphological data, echolocation calls, and partial cytochrome *b* sequence data.

With the advent of the polymerase chain reaction (PCR) and the discovery of universal PCR primers for mitochondrial DNA sequences (Kocher et al. 1989, Thomas et al. 1989), the analysis on mitochondrial DNA has been developed for evolutionary studies of many animal species including Chiroptera (Meyer et al. 1990, Mindell et al. 1991, Barratt et al. 1995). Animal mtDNA is maternally inherited (Avise 1986, Wilson et al. 1985) and generally it evolves more rapidly than nuclear DNA (Brown et al. 1982). Cytochrome *b* is the best-known mitochondrial gene with respect to its structure and function, its sequences are widely used in phylogenetics (Esposti et al. 1993). It is thought to be sufficiently variable for population analyses and conservative enough for phylogenetic analyses among distantly related organisms (Irwin et al. 1991, Meyer & Wilson 1990, Wright et al. 1999).

We determine partial nucleotide sequences of the cytochrome *b* gene for five species of Hipposideridae, and five species of Rhinolophidae, including *R. rex* which is endemic to China. The first 528 base pairs of cytochrome *b* gene were examined as they provide sufficient resolution for addressing phylogenetic relations at family level (Sudman et al. 1994). Our goals were to examine the relationship between Hipposideridae and Rhinolophidae, the phylogenetic relationships of the genus *Aselliscus* with *Hipposideros* and *Rhinolophus*, and the evolutionary relationships of the species of *Hipposideros* in China. The results of these analyses are compared to those previously reported for morphological and karyological data.

Materials and Methods

Specimens: Tissues were obtained from 10 voucher specimens deposited in Institute of Zoology at the Chinese Academy of Sciences, Beijing. All specimens were from China. Specimens investigated in the present study are listed in Table 1.

DNA extraction: Fresh muscle samples were collected from 10 species and stored at -20 °C or in 70% ethanol. Total DNA was extracted from small amounts of muscle by overnight incubation at 37 °C in 10mM Tris.HCl pH8.0/10mM EDTA/100mM NaCl/0.1% SDS/ 50mM dithiothreitol/0.5mg/ml proteinase K. The DNA was purified by extracting twice with phenol, once with phenol/chloroform/isoamylalcohol (25:24:1 vol/vol), and once with chloroform/ isoamylalcohol (24/1). The sample was then concentrated by 1/10 vol 3M NaAc, 3vol ethanol (Maniatis et al. 1982).

PCR amplification: Amplification reactions were performed in 50 μl volume with 1.5mM Mg^{2+}, 0.2 μM concentrations of each primer, each dNTP at 200 μM and 1.25 units of Taq polymerase. Thermal cycling profiles included an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation (94 °C, 1 min), primer annealing (56 °C,

Table 1. Specimens investigated for phylogenetic analyses.

Specimen designation	Specimen no.	Collection site	Longitude/Latitude
Hipposideros armiger	990801015	Xiniu cave of Zhenning, G	105°45' E, 26°04' N
H. bicolor	01090801	Guanyin cave of Danzhou, Ha	109°35' E, 19°30' N
H. larvatus	990807042	Jiangjia cave of Anlong, G	105°12' E, 25°18' N
H. pratti	990811052	Feilong Cave of Xingyi, G	104°53' E, 24°59' N
Aselliscus stoliczkanus	990807045	Jiangjia cave of Anlong, G	105°12' E, 25°18' N
Rhinolophus affinis	990722004	Heshang cave of Guiyang, G	106°46' E, 26°37' N
R. ferrumequinum	BJ995006010	Xipo Cave of Fangshan, B	115°45' E, 39°45' N
R. macrotis	HN99006	Batcave of Xixia, He	111°24' E, 33°18' N
R. pearsoni	990824076	Xianren Cave of Jiangkou, G	108°27' E, 27°40' N
R. rex	990726019	Xiniu cave of Zhenning, G	105°45' E, 26°04' N

Geographic abbreviations: G, Guizhou province; Ha, Hainan province; He, Henna province; B, Beijing; Tissue; ep, ethatol preserved

1 min), and polymerase extension (72 °C, 1 min). A final extension at 72 °C for 10 min reduced the number of partial sequences. PCR products were electrophoresised on 2% agarose gels and visualized with ethidium bromide stain. A specific region of cytochrome *b* gene and a portion of the adjacent $tRNA^{Glu}$ in the mtDNA genome were amplified. Primers for amplification and sequencing were L14724 and H15275 (S u d m a n et al. 1994). L and H refer to the 3' position of the primers in relation to human mitochondrial DNA light and heavy strand, respectively (A n d e r s o n et al. 1981).

Sequencing and sequence analysis: The purified double strand DNA was sequenced using the S a n g e r et al. (1977) method and an automated ABI DNA Sequencer (model 377). Sequence alignments were generated with the multiple alignment program CLUSTAL W (T h o m p s o n et al. 1994) and corrected by hand for alignment errors. Maximum parsimony and distance analyses were used to estimate phylogenetic relationships using four species of Pteropodidae as outgroups. All analyses were performed with the MEGA 1.02 computer program (K u m a r et al. 1994). For the parsimony analyses, we conducted heuristic searches with 15 random input orders and uniformative characters were excluded. All characters were equally weighted. Genetic distance values were calculated using the Kimura 2-parameter model of evolution (K i m u r a 1980). The neighbor-joining method (S a i t o u & N e i 1987) was used to construct two phylogenetic trees, one using the entire cytochrome *b* dataset, and the other included only the transversion differences. The stability of inferred topologies was assessed with bootstrap methods (F e l s e n s t e i n 1985). Four species of Pteropodidae (*Pteropus vampyrus*, *P. dasymallus, Rousettus amplexicaudatus* and *Eonycteris spelaea)* were used as outgroups to define the polarity of character-state change.

Results

For all taxa, 528 bp of the cytochrome *b* gene were examined. Sequences were deposited in GenBank under accession numbers (AF451332-AF451338, AF451340, AF460975, AF460976). Sequences of *R. monoceros* and *R. pumilus* were obtained from GenBank (accession numbers: AF406806, AB061526), as were four sequences for the four outgroup taxa (accession numbers: AB046323, AB046326, AB046329, NC_002612). The empirical base composition of the mitochondrial DNA sequenced was: A (27.3%), C (30.9%), G (16.1%), T (25.6%). Frequencies of A and T (52.9%) were slightly higher than those of C and G (47%), while C was present more than G. In addition, base compositions at the three

codon positions differed greatly: 1st positions had an unbiased composition of nucleotides, 2nd positions had a higher frequency of T (36.8%), whereas 3rd positions were depauperate in G (4.5%). The compositional bias were similar to that of some species of mammals (I r w i n et al. 1991). Of the 528 nucleotide positions examined, 198 positions (37.5%) were variable and 161 positions (30.5%) were phylogenetically informative.

Percentage sequence divergence and transition:transversion rates obtained from pairwise comparisons were shown in Table 2. All species possessed predominantly transitional changes at third position sites. The percent sequence divergence for pairwise comparisons within Hipposideridae ranged from 8.61% (*H. larvatus* versus *H. armiger*) to 17.23% (*H. larvatus* versus *A. stoliczkanus*), with a mean of 11.66% sequence divergence. Within Rhinolophidae, percent sequence divergence ranged from 3.12% to 13.32%, and the average percentage was 9.91%. Sequence divergence between *Aselliscus* and *Hipposideros* was from 15.54% to 17.23%, with a mean of 16.43%, whereas the average divergence value between *Aselliscus* and *Rhinolophus* was 16.55%. The average sequence divergence between *Hipposideros* and outgroup taxa was 20.27%. Similarly, the sequence divergence between *Rhinolophus* and outgroups was 21.27%.

The transition:transversion ratio generally decreases with increasing percent sequence divergence: the average transition:transversion ratio within *Hipposideros* was 6.254 and within *Rhinolophus*, 5.334. The mean ratio between *Aselliscus* and *Hipposideros* was 2.032, and was higher than that of *Aselliscus* and *Rhinolophus* (1.159).

All tree-building procedures resulted in the monophyletic clustering of both Hipposideridae and Rhinolophidae (Figs 1 and 2). The four species of Pteropodidae used as outgroup taxa grouped together at the base. Maximum-parsimony analysis, using equally weighted characters, resulted in three most-parsimonious trees of 541 steps in length, consistency index (CI) = 0.555, retention index (RI) = 0.563, rescaled consistency index (RCI) = 0.412 (Fig. 1). Parsimony analysis indicated that *H. bicolor* was the sister taxon to *A. stoliczkanus*. This clade was then grouped with the other species of *Hipposideros*. Within the clade of Rhinolophidae, *R. rex* and *R. macrotis* were firstly grouped with a clade consisting of *R. monoceros* and *R. pumilus*. Bootstrap supporting values (BS) were high for all nodes except those uniting *H. bicolor* and *A. stoliczkanus* (BS = 66%), *R. pearsoni* and *R. affinis* (BS = 66%).

Two neighbor-joining trees (entire dataset and transversions only) were generated using Kimura 2-parameter genetic distances. Both trees revealed that the four species of *Hipposideros* formed a clade, followed by the addition of *Aselliscus*. Members of the Rhinolophidae formed a 2nd clade. The only difference between the two analyses involved the placement of *R. ferrumequinum*, *R. pearsoni* and *R. affinis*. The neighbor-joining tree, derived from analysis of only transversions at three codon positions (Fig. 2), showed that *R. ferrumequinum* grouped with *R. affinis*, followed by the clades containing *rex-macrotis* and *monoceros-pumilus* respectively. *R. pearsoni* was placed at the base of the Rhinolophidae. However, in the neighbor-joining tree derived from analysis of all substitutions at three codon positions, species of Rhinolophidae were separated into two clades, one clade contained *R. ferrumequinum*, *R. pearsoni*, and *R. affinis*, the other clade contained the remaining four species. The topology of the parsimony and neighbor-joining trees differed in the placement of *A. stoliczkanus*, *H. bicolor*, and some species of Rhinolophidae.

Discussion

Data from the cytochrome *b* gene reveals that substitution rates of the three codon positions were in the order of 3rd>1st>2nd, which agree with other studies of this gene (K i m u r a

Table 2. Percent sequence divergence (below diagonal) and transition:transversion ratio (above diagonal) for all pairwise comparisons of cytochrome *b* nucleotide sequences (528 base pairs). Percent sequence divergence was calculated using Kimura 2-parameter model of evolution (K i m u r a 1980).

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *H. armiger*		2.875	13.000	6.286	1.586	1.200	1.438	1.394	1.485	1.290	1.176	1.250	0.667	1.116	1.043	0.981
2. *H. bicolor*	13.01		3.467	4.727	2.174	1.387	1.441	1.621	1.483	1.645	1.267	1.464	1.000	1.333	1.421	1.040
3. *H. larvatus*	8.61	14.23		7.167	2.077	1.056	1.515	1.176	1.412	1.406	1.257	1.121	0.830	1.167	1.133	0.887
4. *H. pratti*	10.59	13.35	10.15		2.292	1.132	1.297	1.500	1.441	1.441	1.371	1.455	0.939	1.075	1.122	0.796
5. *A. stoliczkanus*	15.92	15.54	17.23	17.02		1.316	1.171	1.250	0.868	1.500	1.143	0.696	1.106	0.980	0.865	1.059
6. *R .affinis*	16.32	15.65	15.59	17.27	19.03		6.714	5.125	5.875	7.833	6.857	5.857	0.611	0.936	0.917	0.962
7. *R. ferrumequinum*	16.61	17.83	17.85	18.29	19.24	11.29		4.000	4.636	3.538	4.400	4.000	0.696	1.106	0.980	0.865
8. *R. macrotis*	16.85	16.17	15.61	18.34	17.30	10.11	11.45		5.000	4.250	5.800	5.333	0.655	1.000	0.959	0.962
9. *R. monoceros*	17.60	15.19	17.58	17.83	14.85	11.50	13.10	7.25			5.100	6.600	0.887	1.217	1.149	0.959
10. *R. pearsoni*	14.92	17.63	16.37	17.83	20.86	11.07	12.35	13.32	12.89		5.000	4.091	0.855	1.318	1.149	0.679
11. *R. pumilus*	15.61	14.22	16.82	17.81	14.39	11.52	11.23	6.83	3.12	11.25		7.000	0.778	1.085	1.063	0.920
12. *R. rex*	15.15	14.48	14.66	17.35	15.84	9.90	10.31	3.72	7.70	11.68	6.41		0.611	0.936	0.917	0.962
13. *Eonycteris spelaea*	19.35	19.93	21.14	20.67	21.86	21.89	20.60	19.60	21.92	22.42	20.87	18.61		1.419	1.533	1.531
14. *Pteropus vampyrus*	19.72	19.79	19.74	17.74	21.70	20.97	21.74	20.94	22.57	22.61	21.48	19.67	15.89		5.000	1.429
15. *Pteropus dasymallus*	20.45	20.07	20.99	18.73	23.28	20.67	21.70	20.93	22.28	22.28	21.73	19.91	16.15	3.51		1.382
16. *Rousettus amplexicaudatus*	22.73	22.49	21.92	18.89	23.28	22.68	21.15	22.98	20.93	19.11	20.91	22.46	17.36	18.32	17.33	

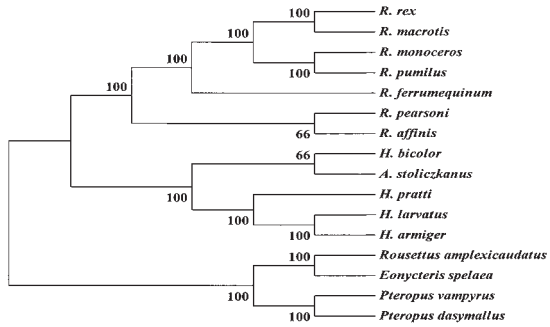

Fig. 1. Phylogenetic tree of 16 species of bat based on cytochrome *b* sequence (528 bp), using MEGA package. Strict consensus tree based on three most-parsimonious trees (541 steps) from a heuristic search, analysis of the entire cytochrome *b* dataset. All characters were equally weighted, consistency index (CI) = 0.555, retention index (RI) = 0.563, rescaled consistency index (RCI) = 0.412. Numbers above the branches denote the bootstrap values derived from 500 bootstrap iterations.

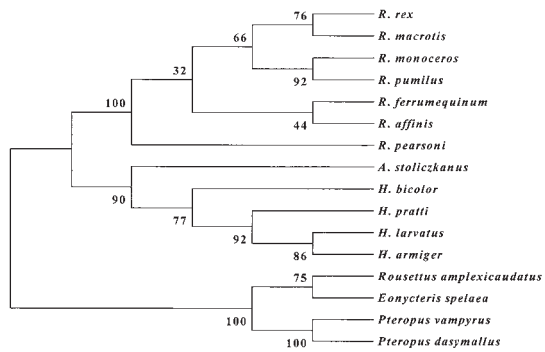

Fig. 2. Phylogenetic tree of 16 species of bat based on cytochrome *b* sequence (528 bp), using MEGA package. Neighbor-joining tree, generated using Kimura-2 parameter model of evolution: analysis of only transvertions at codon positions. Numbers above the branches denote the bootstrap values derived from 500 bootstrap iterations.

1983, Y a n g 1996). Most 3rd position and some 1st position substitutions were silent and should evolve faster because of relatively weak purifying selection, whereas 2nd position substitutions are nonsynonymous and should be under strong purifying selection (N e i 1987). Our data also indicate that amino acid sequences deduced from the nucleotide sequences imply that synonymous mutations were more numerous than non-synonymous ones, and a high transition bias was observed. These results agree with B r o w n et al. (1982) and B r o w n (1985) for the initial high transition bias of mtDNA, gradually decreases over time as transversions are accumulated.

Members of Rhinolophidae and Hipposideridae predominantly are distributed in Southeast Asia, which was alleged to be a center of the speciation of the two families (A n d ō et al. 1980). Hipposideridae is related closely to Rhinolophidae, but was first distinguished by M i l l e r (1907), based on its advanced specializations of the shoulder girdle and feet. In addition, the shape of the nose-leaf also is different, and the lower small premolar is lost in *Rhinolophus*. Some authors proposed that the ancestral lineage of *Hipposideros* was derived from a rhinolophid ancestor (A n d ō et al. 1980, S r e e p a d a et al. 1993). The progressive specialized morphological characters of *Hipposideros* which are not found in *Rhinolophus* may be a result of environmental effects such as clinal aridity and productivity stresses affecting the two taxa in different ways. However, the familial

status of these groups is debatable. Some researchers considered Rhinolophidae and Hipposideridae as two separate families (Corbet & Hill 1991, Lekagul & McNeely 1977), whereas others suggested that Hipposideridae is a subfamily of Rhinolophidae (Ellerman & Morrison-Scott 1966, Koopman 1984, 1993, 1994, Simmons 1998). The hypothesis that they represent different families is further supported by recent anatomical and immunological data: cranial, dental and external characters examined by Bogdanowicz & Owen (1998). Pierson (1986) found that although species of *Rhinolophus* and *Hipposideros* were morphologically similar, they were immunologically as distinct as were other taxa placed them in separate families.

Many studies propose that molecular sequence data holds potential for resolving the higher–level relationships of the bats (Hollar & Springer 1997, Sudman et al. 1994). Our arrangement provides convicing evidence to categorize Hipposideridae and Rhinolophidae as sister taxa (BS = 98–100%). This conclusion agreed with karyotypical and morphological analyses (Bogdanowicz & Owen 1998, Hand & Kirsch 1998). The molecular data indicated that Hipposideridae and Rhinolophidae formed two monophyletic groups which were closely related. Compared with the relative high transition: transversion ratio within Hipposideridae (4.565) and Rhinolophidae (5.334), the average transition: transversion ratio between the two taxa is only 1.846. The average sequence divergence between Hipposideridae and Rhinolophidae is 16.71%, which is greater than that within Rhinolophidae (9.91%) or Hipposideridae (11.66%).

Aselliscus contains only two species, and is distinguished from other genera in Hipposideridae by the uniquely shaped noseleaf. However, Pierson (1986) concluded that *Aselliscus* was aligned more closely with *Rhinolophus* than with *Hipposideros*. In this study, analyses of the molecular data suggest that *Aselliscus* is aligned with *Hipposideros*, but not with *Rhinolophus*. Strong bootstrap support was found for this arrangement. Neighbor-joining analyses of cytochrome *b* data indicate that *A. stoliczkanus* lies at the base of the *Hipposideros* lineage. Parsimony analysis also results in the inclusion of *A. stoliczkanus* within the *Hipposideros*. Meanwhile, this arrangement also is supported by the relatively low ratio of transitions to transversions (1.159 between *Aselliscus* and the species of *Rhinolophus* versus 2.032 between *Aselliscus* and the species of *Hipposideros*), the value between *Aselliscus* and *Hipposideros* (2.032) is consistent with genus level values in Molossidae (Sudman et al. 1994) and Vespertilionidae (Wang hui per com), whereas sequence divergence value between *Aselliscus* and *Hipposideros* is only a little lower than that between *Aselliscus* and *Rhinolophus*.

Hill (1963) grouped *Hipposideros* species into three primary divisions and recognized seven species groups within the genus *Hipposideros*. Most of the recent researchers (e.g., Jenkins & Hill 1981, Kock & Bhat 1994, Koopman 1994) either have accepted Hill's (1963) view or have made only minor changes to his classification. The four *Hipposideros* species examined herein were in the different species groups, *H. bicolor*, *H. armiger*, *H. larvatus* and *H. pratti* were arranged in *bicolor*, *armiger*, *speoris*, and *pratti* species groups, respectively. The last three groups were in the third division of the species of *Hipposideros* (Hill 1963). Our arrangement supports this classification. The basal placement of *H. bicolor* shows its distant relationship with other species of *Hipposideros* in our studies. The distance values between *H. bicolor* and other species of *Hipposideros* are greater than values within the three species. *H. armiger* has the same general size and appearance as *H. pratti*. However, the two species were not closely related based on morphological characters, such as shape of the skull, characterization of the noseleaves, and length of the tibia (Allen 1938). Our results agree with Allen's (1938) arrangement

that *H. larvatus* and *H. armiger* are closely related. The percent sequence divergence between *H. armiger* and *H. larvatus* is 8.61%, which is lower than those between *H. armiger* and other species of *Hipposideros* in China.

Vertebrate mitochondrial DNA has been estimated to accumulate mutations at a rate of 2–5%/million years (Lewis-Oritt et al. 2001, Arbogast & Slowinski 1998, Shields & Wilson 1987, Brown et al. 1979). Using this value as a rough estimate of the age at which lineages diverged, our molecular data imply that *Aselliscus* and *Hipposideros* in China diverged about 3.29–8.22 million years ago, *H. armiger* and *H. larvatus* diverged from each other approximately 1.72–4.31 million years ago, and *H. pratti* from the *larvatus-armiger* clade approximately 2.07–5.19 million years ago.

All phylogenetic trees produce the monophyletic clade of Rhinolophidae. The *macrotis-rex* clade is a sister group of the *monoceros-pumilus* clade. The grouping of *R. rex* and *R. macrotis* agrees with the classification of Hill (1972) that they are both recognized as the most primitive members of *philippinensis* group (Tate & ArchBold 1939, Bogdanowicz & Owen 1992). However, the arrangement of *R. ferrumequinum*, *R. pearsoni* and *R. affinis* differs greatly based on the method of analysis. The low bootstrap values suggest that the relationships of these species depicted here are not well supported.

This is the first report on the molecular phylogeny of bat species in China, inferred from the mtDNA sequences. There are six species of Hipposideridae in China, and unfortunately we have not obtained the sequence of *Coelops frithi*. We determine the molecular phylogenetic tree based on the cytochrome *b* sequences and analyzed the relationships of species within and between Hipposideridae and Rhinolophidae. However, the probability that the gene tree deduced from cytochrome *b* or any other single gene and species coincidence is greatest only when the time between lineage divergence is large and ancestral population size is small (Nei 1987). It is possible that the phylogenetic tree obtained from one gene does not accurately reflect evolutionary relationships among taxa. So it is necessary to use other mtDNA region and collect more species inside and outside China so that we can clarify more clearly the relationship between Hipposideridae and Rhinolophidae as well as the inter-genera relationships within Hipposideridae.

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