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

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Abstract. Phylogenetic relations among five species of Hipposideridae and seven species of Rhinolophidae including one endemic species (Rhinolophus rex) were examined by partially sequencing 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 (Corbet & Hill 1991, Koopman 1994). Generally it has been accepted that the family originated in the Old World tropics, probably in Africa or Asia (Koopman 1970). Recently, Bogdanowicz & Owen (1998) suggested that Hipposideridae, like their sister-family Rhinolophidae (Bogdanowicz & Owen 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 (Bogdanowicz & Owen 1998).

In China, bats of the family Hipposideridae primarily are distributed in the south (Zhang 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 Hill (1963). However, Hill’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 craniodontal and postcranial morphology as well as bacalar morphology and morphometrics (Hill et al. 1986, Kitchen et al. 1992, Flannery & Colgan 1993, Zubaid & Davis 1987). Other taxonomic studies have used electrophoretic, karyological and immunomolecular data (Andry 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 (Avice 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 Mg²⁺, 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,
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 electrophoresed 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 (Sudman 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 (Anderson et al. 1981).

Sequencing and sequence analysis: The purified double strand DNA was sequenced using the Sanger et al. (1977) method and an automated ABI DNA Sequencer (model 377). Sequence alignments were generated with the multiple alignment program CLUSTAL W (Thompson 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 (Kumar et al. 1994). For the parsimony analyses, we conducted heuristic searches with 15 random input orders and uninformative characters were excluded. All characters were equally weighted. Genetic distance values were calculated using the Kimura 2-parameter model of evolution (Kimura 1980). The neighbor-joining method (Saitou & Nei 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 (Felsenstein 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

<table>
<thead>
<tr>
<th>Specimen designation</th>
<th>Specimen no.</th>
<th>Collection site</th>
<th>Longitude/Latitude</th>
</tr>
</thead>
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<tr>
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<td>01090801</td>
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<td>109°35' E, 19°30' N</td>
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<td>105°12' E, 25°18' N</td>
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<td>H. pratti</td>
<td>990811052</td>
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<td>104°53' E, 24°59' N</td>
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<td>Jiangjia cave of Anlong, G</td>
<td>105°12' E, 25°18' N</td>
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<tr>
<td>Rhinolophus affinis</td>
<td>990722004</td>
<td>Heshang cave of Guiyang, G</td>
<td>106°46' E, 26°37' N</td>
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<td>R. ferrumequinum</td>
<td>BJJ95006010</td>
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<td>Batcave of Xixia, He</td>
<td>111°24' E, 33°18' N</td>
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<tr>
<td>R. pearsoni</td>
<td>990824076</td>
<td>Xianren Cave of Jiangkou, G</td>
<td>108°27' E, 27°40' N</td>
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<td>R. rex</td>
<td>990726019</td>
<td>Xiniu cave of Zhenning, G</td>
<td>105°45' E, 26°04' N</td>
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</tbody>
</table>

Geographic abbreviations: G, Guizhou province; Ha, Hainan province; He, Henna province; B, Beijing; Tissue; ep, ethatol preserved
The compositional bias were similar to that of some species of mammals (Irwin 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. macrois 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-macrois 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 (Kimura 262
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 (Kimura 1980).

<table>
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<tr>
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<th>1</th>
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<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
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<td>17.83</td>
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<td>11.23</td>
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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 transversions at codon positions. Numbers above the branches denote the bootstrap values derived from 500 bootstrap iterations.

1983, Yang 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 (Nei 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 Brown et al. (1982) and Brown (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 (Andô et al. 1980). Hipposideridae is related closely to Rhinolophidae, but was first distinguished by Miller (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 (Andô et al. 1980, Sreepada 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 convincing 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 (Wanghui 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

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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-Oritte 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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**Literature**


