Genetic analysis on three South Indian sympatric hipposiderid bats (Chiroptera, Hipposideridae)

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Abstract

Genetic analysis on three South Indian sympatric hipposiderid bats (Chiroptera, Hipposideridae).— In mitochondrial DNA, variations in the sequence of 16S rRNA region were analyzed to infer the genetic relationship and population history of three sympatric hipposiderid bats, *Hipposideros speoris*, *H. fulvus* and *H. ater*. Based on the DNA sequence data, we observed relatively lower haplotype and higher nucleotide diversity in *H. speoris* than in the other two species. The pairwise comparisons of the genetic divergence inferred a genetic relationship between the three hipposiderid bats. We used haplotype sequences to construct a phylogenetic tree. Maximum parsimony and Bayesian inference analysis generated a tree with similar topology. *H. fulvus* and *H. ater* formed one cluster and *H. speoris* formed another cluster. Analysis of the demographic history of populations using Jajima's *D* test revealed past changes in populations. Comparison of the observed distribution of pairwise differences in the nucleotides with expected sudden expansion model accepts for *H. fulvus* and *H. ater* but not for *H. speoris* populations.

Key words: Chiroptera, *Hipposideros*, mtDNA, 16S rRNA, Phylogeny.

Resumen

Análisis genético de tres murciélagos hiposidéridos (Chiroptera, Hipposideridae) simpátricos del sur de la India.— Se analizaron las variaciones en las secuencias de la región del ARNr 16S del ADN mitocondrial, con el fin de deducir la relación genética y la historia de la población de tres murciélagos hiposidéridos simpátricos: *Hipposideros speoris*, *H. fulvus* e *H. ater*. Basándonos en los datos de las secuencias del ADN, observamos una diversidad de nucleótidos mayor y una diversidad haplotípica relativamente menor en *H. speoris* que en las otras dos especies. Las comparaciones por pares de la divergencia genética dio como resultado una relación genética entre los tres murciélagos hiposidéridos. Utilizamos las secuencias haplotípicas para construir un árbol filogenético. Los análisis de inferencia bayesiana y de máxima parsimonia dieron lugar a un árbol con una topología similar. *H. fulvus* e *H. ater* formaban un conglomerado, y *H. speoris* formaba otro conglomerado. El análisis de la historia demográfica de las poblaciones, utilizando el test *D* de Jajima, puso de manifiesto cambios de población sucedidos en el pasado. La comparación de la distribución observada de las diferencias de nucleótidos por pares con el modelo previsto de expansión súbita se acepta para las poblaciones de *H. fulvus* e *H. ater*, pero no así para las de *H. speoris*.

Palabras clave: Chiroptera, *Hipposideros*, ADNm, ARNr 16S, Filogenia.

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Introduction

Recent advances in molecular methods have added new insights into studies related to organismic evolution and have revealed unexpected levels of diversity in many vertebrate groups (Meyer et al., 1990; Roca et al., 2001). In many such studies, current patterns of genetic variation are used to infer historical events such as population expansions, past population selection from refugia and evolutionary relationship (Miller & Waits, 2003; Johnson & Dunn, 2006; Hoffmann et al., 2008). Comparing genetic structure among co–distributed species may provide significant insight to the extent to which extrinsic and intrinsic factors interact to influence the scale of population differentiation or speciation event (Arbogast & Kenagy, 2001). It is complicated to determine the exact identity of species that occupy a similar ecological niche and have very close morphological characters. Traditional morphological groupings have recently been questioned based on molecular data (Mayer & Von Helversen, 2001). Similarly, within well–studied genera, morphologically cryptic and genetically divergent species have been identified (Von Helversen et al., 2001; Thabah et al., 2006). In many regions, bats rank among the most rare and least known mammals due to very low local abundance of several species and their way of life (Hulva et al., 2004). Recent studies on molecular systematics of Chiroptera have unfolded the level of diversity in many species (Teeling et al., 2000; Campbell et al., 2004). However, many interspecific phylogenetic relationships remain poorly characterized, particularly in tropical regions where the diversity of bats is relatively high (Jones et al., 2002). Hence, it suggests that diversity within the order Chiroptera may be underestimated by the current taxonomy. Based on craniodental, postcranial morphology and other morphometric characters, the family Hipposideridae is systematically classified with 65 species, which mainly inhabit tropical and subtropical regions (Hill et al., 1986; Flannery & Colgan, 1993; Wang et al., 2003). Twelve of these species are found in India (Bates & Harrison, 1997). Species boundaries within Hipposideridae have been revised several times based on the cranial, morphological and acoustic characteristics, mtDNA and nuclear DNA data (Kingston et al., 2001; Thabah et al., 2006).

The hipposiderid bats *Hipposideros speoris*, *H. fulvus* and *H. ater* are non–migratory and they have a low natal dispersal system. *H. speoris* is the largest of the three [forearm length (FAL) 50–54 mm, body weight (BW) 8.5–12.0 g, constant frequency (CF) ca. 135 kHz] and it shares its day roost with *H. fulvus* (FAL 38–44 mm, BW 7–9 g, CF = ca. 157 kHz). The two species also share common foraging grounds, such as very close to the canopy, around bushes and trees, and very close to obstacles, but their foraging strategies are different (Habersetzer et al., 1984; Neuweiler et al., 1984). *H ater* (FAL 35–38 mm, BW 5–7 g, CF ca. 166 kHz) is smaller but superficially similar to *H. fulvus*. However, it does not share day roost with the other two species (Jones et al., 1994). Studies on the genetic variation and the genetic relationship of closely related species provide insights into their evolutionary history (Avise, 2004) as well as important information on biodiversity (Hedrick, 2004).

We surveyed *H. speoris* and *H. fulvus* populations in southern India and found these two species lived together in the same habitat. For example, they occupied a cave close to the Madurai Kamaraj University campus. However, there was a clear spatial partition in their roosting area (G. Marimuthu, personal observations), *H. ater* usually occupied a separate roost. The present study aimed to address the phylogenetic relationship and genetic diversity of these three sympatric species.

Materials and methods

Samples

The study was carried out between June 2006 and February 2007. Using a nylon mosquito net we captured *H. speoris* and *H. fulvus* on their predawn return flight into a cave in the Pannian hills (10º 50' N, 78º 43' E), about 10 km northwest of the Madurai Kamaraj University campus. *H. ater* was captured using the same method in an unused building in as the village of Puliyankudi (9º 18' N, 77º 40' E), about 80 km south of Madurai city. *Rhinolophus beddomei* was also captured using a similar method in a cave in the High Wavy mountains (9.49º 52' N, 77.22º 58' E), about 60 km west of Madurai city. All four species were identified based on their morphological characters (Bates & Harrison, 1997). Thirty individuals representing each species of hipposiderids and one sample from the out group taxa (*R. beddomei*) were used in the present study. Tissue biopsies (3 mm diameter) were obtained from the wing membranes of bats and preserved in extraction buffer (100 mM Tris–Cl; 10 mM EDTA; 1.5 M NaCl; 1.0 % CTAB; 0.2% mercaptoethanol). The bats were released at their roosting sites soon after completion of the sample collection. All the procedures adopted in the study were approved by Bharathidasan University Wild Animals Ethical Committee (BUWAEC), Bharathidasan University, Tiruchirappalli, India.

Amplification of mtDNA 16S rRNA

Total genomic DNA was extracted from the wing membrane samples following standard procedure (Sambrook et al., 1989). Approximately 600 bp of the 16S rRNA region from mtDNA was amplified with specific primer–light chain (L): TTACCAAAAACATCACCTCTAGC; heavy chain (H): CGGTCTGAACTCAGATCACGTA (Lin et al., 2002). A portion of the 16S rRNA region was amplified in 50 µL reaction mixture that contained 1 unit of *Pfu* DNA polymerase (Invitrogen Inc, USA), 1.5 mM Mg^{2+}, 0.2 µM of primers, 200 µM of each dNTP, and 20 ng template DNA. Amplifications were performed in a MJ–mini thermal cycler (Bio–Rad, CA, USA) by employing an initial denaturation (95ºC, 1 min), followed by 25 cycles of denaturation (94ºC, 1 min), annealing (56ºC, 1.5 min), and extension

(72ºC, 2 min) and a final extension at 72ºC for 5 min. Amplicons were excised from agarose gel, purified with spin column (RBC gel elution kit, Taiwan), and sequenced with L primer (MWG, India).

Phylogenetic analysis

All the obtained sequences were initially aligned with Clustal X software (Thompson et al., 1997) prior to further analysis. Genetic distances were calculated according to Kimura 2–parameter method in MEGA 4.0 (Tamura et al., 2007). Mitochondrial 16S rRNA haplotype sequence data set was used for the Maximum parsimony (MP) and Bayesian analyses. Based on the MP, we constructed phylogenetic relationships by using the programs MEGA 4.0 (Tamura et al., 2007), and Bayesian inferences implemented in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The MP branch support was assessed by bootstrap analysis (1,000 BS replicates). In order to find the best–fitting substation model for each data set, Model Test (3.06) was used to test the likelihood ratio (Posada & Crandall, 1998). Bayesian analysis was performed under the selected General Time–Reversible (GTR + I + G) model (Rodriguez et al., 1990) of nucleotide substitution parameters, which were estimated during the course of the run at the rate matrix: A–C = 2.99, A–G = 4.32, A–T = 1.94, C–G = 3.08, C–T = 8.57 and G–T = 1.0; nucleotide base frequencies A = 0.311, C = 0.235, G = 0.208 and T = 0.244; transition/transversion ratio = 3.128. We ran six Markov Chain Monte Carlo (MCMC) chains for 200,000 generations with trees sampled for every 100 generations. The first 30% of the sampled trees were discarded and Bayesian posterior probabilities (BPP) were estimated from the 50% majority–rule consensus tree of the retained trees.

Genetic diversity and population structure

Mitochondrial haplotype diversity (*h*) and nucleotide diversity (π) were used to calculate the levels of mtDNA diversity by using DNASP 4.5.0 (Rozas et al., 2003). First, the neutrality test (Tajima *D*) was carried out for the three species to test the population expansion hypothesis (Tajima, 1989). Afterwards, we used various methods, each with a particular sensitivity to one demographic scenario. Fu's *Fs* is sensitive to excess of recent mutations, which is a pattern typical for both demographic expansion and a selective sweep (Fu, 1997). In contrast, Fu and Li's *D** are designed to detect an excess of old mutations, a characteristic of population that has experienced a historical reduction in effective population size (Fu & Li, 1993). In addition, we tested the goodness–of–fit of distributions under a model of population expansion by calculating the sum of squared deviations (SSD), which was used in other non–migratory bats (Chen et al., 2006) with ARLEQUIN 3.11 (Excoffier et al., 2005). Past events of population such as demographic expansion and contractions were also tested, using the evolution model of departure from neutral (Ramos–Onsins & Rozas, 2002), which is implemented in DNASP 4.5.0 (Rozas et al., 2003).

Results

Phylogenetic relationships

The partial sequences of 16S rRNA gene of *H. speoris, H. fulvus* and *H. ater* were aligned, and 484 bp data set was considered for phylogenetic analysis. Parsimony analysis showed that 411 of 484 siteswere constant, 46 were variable and parsimony informative, and 27 (5.6%) were variable but parsimony uninformative. We found 24 distinct haplotypes and their sequences, deposited in GenBank, are available with the following accession number; *H. speoris* (FJ747652–656, FJ825630–631), *H. fulvus* (FJ 747658–666), *H. ater* (FJ747667–674) and *R. beddomei* (FJ009217). We obtained the six most parsimonious trees with 123 steps, consistency index (CI = 0.84); retention index (RI = 0.96) and composite index (CI = 0.88). Bayesian inference analysis generated a tree with 1 n *L* = –1375.97 (relative rate parameters estimated for this model was proportion of invariant site, I = 0.025; gamma shape, α = 1.271). The Maximum parsimony and Bayesian analysis generated tree showed a similar pattern (fig. 1A, 1B). Phylogenetic trees rooted with *R. beddomei* showed basal position of *H. speoris* with respect to *H. fulvus* and *H. ater.* Tree topology was not sensitive to transition to down–weighting or differential treatment of gap characters. The genetic relationship between *H. fulvus* and *H. ater* was supported by both models.

Population genetic structure

Analysis revealed similar haplotype and high nucleotide diversity with 21 polymorphic sites in *H. speoris* populations than *H. fulvus* and *H. ater* populations (table 1). The neutrality test (Tajima *D* test) showed negative value for *H. speoris, H. fulvus* and *H. ater* populations, which suggests possible occurrence of changes in population (*e.g.* expanding or linkage to a locus under directional selections). Fu's *Fs* test detected significant departure from the neutral/equilibrium expectation, whereas Fu and Li's *D** demonstrate nonsignificant, implicating demographic expansion in *H. speoris* population (table 1). While the mismatch distribution (fig. 2A) was not unimodal, the accumulations of low–frequency mutations were the characteristics of non–equilibrium population dynamics. Comparison of the observed distribution of pairwise differences with that expected under a population expansion hypothesis did not accept the sudden expansion model for *H. speoris*. The observed mismatch distribution of *H. fulvus* supports the long–term demographic equilibrium (fig. 2B). However, a significant value for Fu's *Fs*, but not for Fu and Li's *D** suggests an expansion in *H. fulvus.* We found strong support for demographic expansion in *H. ater* also. The smooth unimodal shape of mismatch distribution provided a good visual fit with expectation of the sudden expansion model for *H. ater* (fig. 2C). Likewise, a highly significant value of Fu's *Fs* and a nonsignificant value of Fu and Li's *D** supported demographic expansion (table 1). The obtained non–significant pairwise difference value from

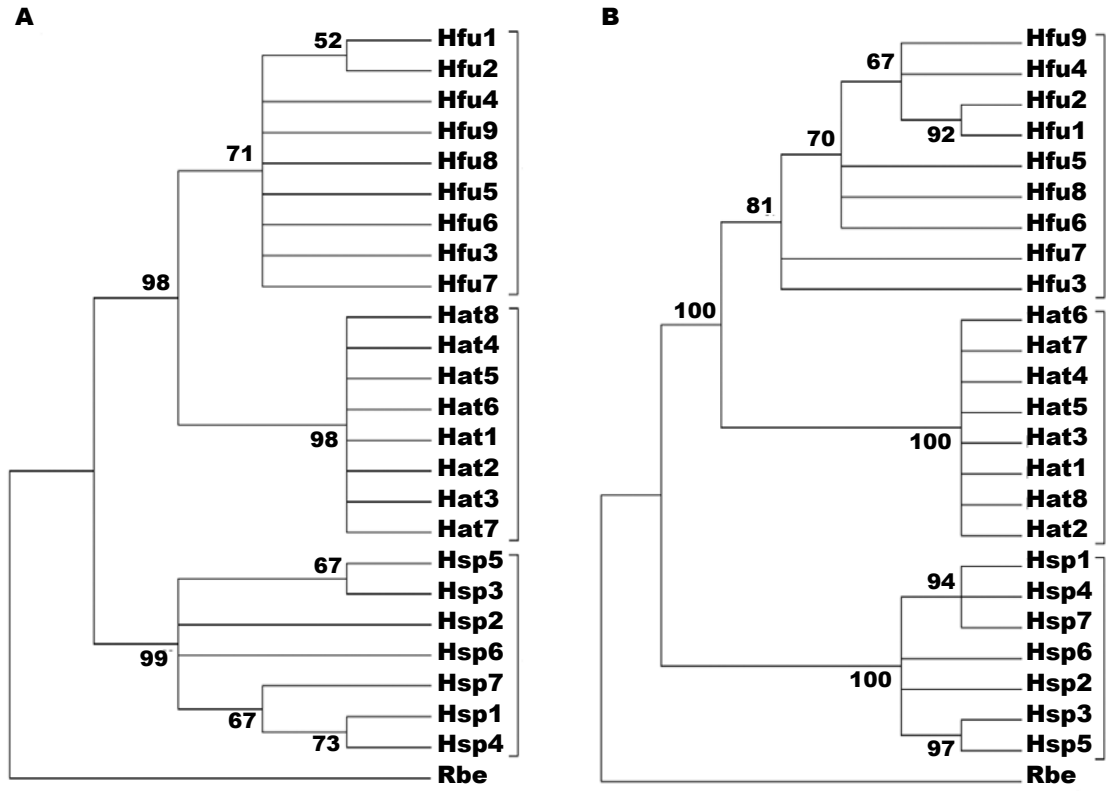

Fig. 1. Phylogenetic relationship between the sympatric hipposiderid bats *H. speoris* (Hsp), *H. fulvus* (Hfu), *H. ater* (Hat) and an out group, *R. beddomei (*Rbe*),* based on the haplotype sequences of 16S rRNA: A. Maximum parsimony tree, numbers at the nodes indicate bootstrap support as percent; B. Bayesian likelihood tree for the sympatric hipposiderid bats, the tree was created using the GTR+I+G model of DNA evolution.

Fig. 1. *Relación filogenética entre los murciélagos hiposidéridos simpátricos* H. speoris *(Hsp),* H. fulvus *(Hfu),* H. ater *(Hat) y un fuera del grupo,* R. beddomei *(Rbe), basada en las secuencias haplotípicas del ARNr 16S: A. Árbol de máxima parsimonia, las cifras de los nodos indican el apoyo "*bootstrap*" en porcentajes; B. Árbol de verosimilitud bayesiano para los murciélagos hiposidéridos simpátricos, este árbol se creó utilizando el modelo GTR+I+G de la evolución del ADN.*

Table 1. Mitochondrial DNAs 16S rRNA sequence divergence parameters for three sympatric hipposiderid bats.

Tabla 1. *Parámetros de divergencia de las secuencias de ARNr 16S de los ADNs mitocondriales de tres especies simpátricas de murciélagos hiposidéridos.*

	H. speoris	*H. fulvus*	*H. ater*
Number of variable site	21	16	6
Haplotype diversity (h)	0.911 (SD = 0.077)	0.978 (SD = 0.054)	0.956 (SD = 0.0059)
Nucleotide diversity (π)	0.01 (SD = 0.006)	0.008 (SD = 0.002)	0.004 (SD = 0.0049)
Tajma *D* test	–1.44030	–1.24610	–1.05965
Fu & Li's *D**	–1.65755 (*P* = 0.08)	–1.39100 (*P* = 0.10)	–0.8344 (*P* = 0.5)
Fu's *F*s	–0.664 (*P* = 0.002)	–4.258 (*P* < 0.001)	–4.819 (*P* < 0.001)
P_{SSD}	0.016	0.540	0.51

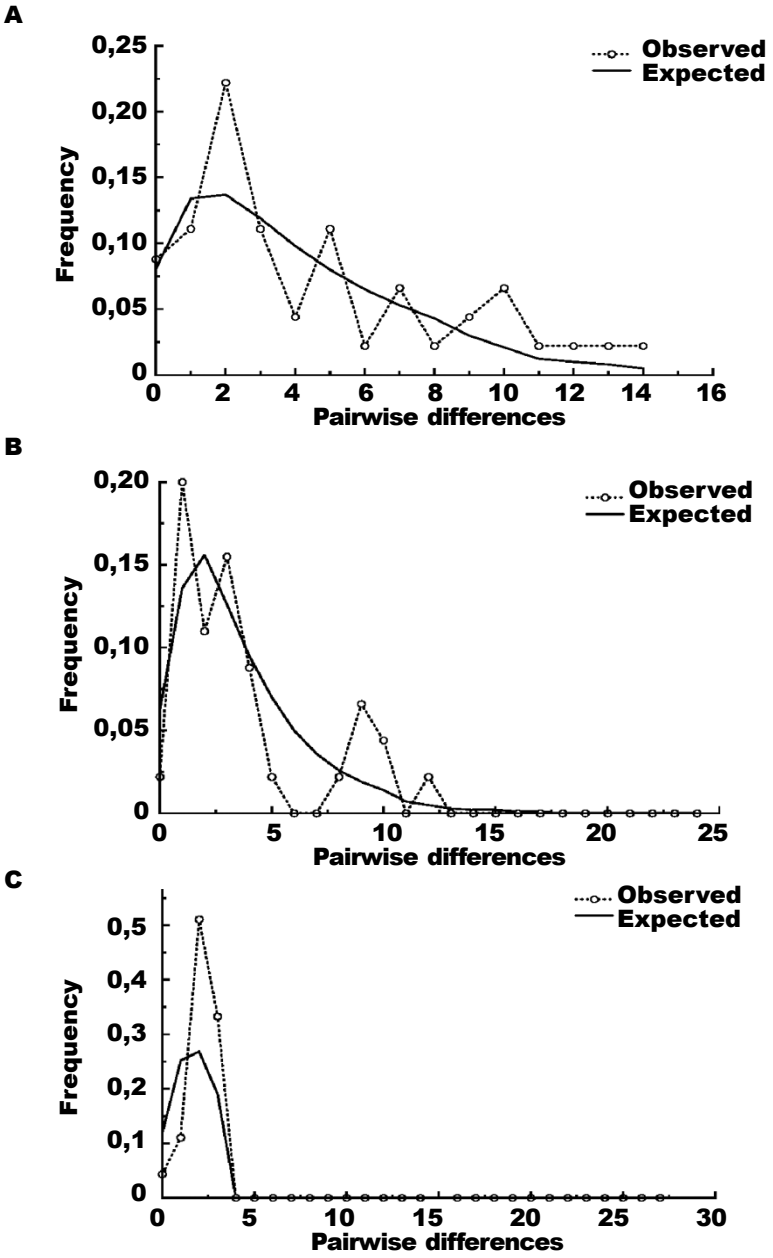

Fig. 2. Distribution of the number of pairwise nucleotide differences between *H. speoris (A)*, *H. fulvus* (B) and *H. ater* (C), based on 484 bp of 16S rRNA sequences. Solid lines represent the observed data, dashed lines represent the line fitted to the data under the expectations of the sudden expansion model, based on 1,000 simulated samples.

Fig. 2. *Distribución del número de diferencias de nucleótidos por pares entre* H. speoris *(A),* H. fulvus *(B) y* H. ater *(C), basada en 484 pares de bases de secuencias del ARNr 16S. Las líneas continuas representan los datos observados, y las líneas de puntos representan las líneas que concuerdan con los datos de las expectativas de un modelo de expansión súbita, basado en mil muestras simuladas.*

H. ater population provides additional support to the expansion hypothesis. Over all, the observed mismatch distribution for *H. speoris* and *H. fulvus* populations was multimodal, and did not confirm the expectations of historically stable populations. The estimated genetic distances within *H. speoris* and *H. ater* were 1.5% and 0.5%, respectively. The comparison between *H. speoris* and *H. ater* was 10.1% and between *H. ater*

Table 2. Measures of within (on diagonal bold) and between groups (below diagonal) genetic distances based on 484 bp of partial 16S rRNA sequences. The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distance.

Tabla 2. Medidas dentro de los grupos (en diagonal, en negrita) y entre grupos (por debajo de la diagonal) de las distancias genéticas, en base a secuencias parciales de ARNr 16S de 484 pares de bases. La presencia de n/c en los resultados se debe a la existencia de un caso en el que no fue posible estimar la distancia evolutiva.

	H. speoris	*H. fulvus*	*H. ater*	*R. beddomei*
H. speoris	**0.015**			
H. fulvus	0.092	**0.010**		
H. ater	0.101	0.036	**0.005**	
R. beddomei	0.149	0.128	0.146	**n/c**

and *H. fulvus* it was 3.6%, which is lower than other comparisons in the present analysis (table 2).

Discussion

The mechanism for diversification in bats remains unclear. Reports from both population genetic analysis (Burland & Wilmer, 2001) and studies combining molecular and phenotypic data (Barratt et al., 1997; Kingston et al., 2001) suggest that ecological changes may promote intraspecific divergence. The genus *Hipposideros* is primarily classified into three groups, with seven species in each group (Hill, 1962). Based on previous studies (Giannini & Simmons, 2003; Pestano et al., 2003), we used the 16S rRNA sequence data set to investigate the phylogenetic relationship and genetic diversity of three sympatric hipposiderid bats.

Maximum parsimony and Bayesian inference analysis produce phylogenetic trees with similar topology. The relationship between *H. fulvus* and *H. ater* was strongly supported by each tree. These two species are more closely related to each other than to *H. speoris.* Both maximum parsimony and Bayesian analysis with high boot strap and statistical analysis support an early divergence between *H. speoris* and the other two species. Based on kimura 2–parameter distance model in–group variation for 16S rRNA ranges from 0–7.9%. The observed variations for the three species fall in the same range. A study on the foraging behaviour of *H. speoris* and *H. fulvus* showed that they preferred to forage in a similar area with fine structural difference (Neuweiler et al., 1984). The distribution of species specific call frequencies with marginal overlap between the three hipposiderids is attributed to interspecific resource partitioning (Jones et al., 1994) with preference to different sized prey.

Changes in sizes of populations leave particular foot prints that can eventually be detected in their DNA sequences (Tajima, 1989; Slatkin & Hudson, 1991; Rogers & Harpending, 1992). Haplotype diversity within a species leads to the stochastic process of lineage extinction with a long demographic history (Campbell et al., 2006). The observed changes in *H. speoris* population based on the Tajima's neutrality test (*D*) may possibly be due to accumulation of low frequency mutations (Hall, 2004). Alternatively, the generated negative Tajima's *D* value in the neutrality test explains the possibility of population expansion by its recent growth (Campbell et al., 2006). Non–equilibrium and unstable demographic status of *H. speoris* population may possibly be due to its co–distribution and habitat association with *H. fulvus*. Similar demographic expansion was observed in the co–distributed megachiropteran species of *Cynopterus* (Campbell et al., 2006).

Our observations of non–significant value in goodness–of–fit distribution for *H. fulvus* and *H. ater* suggest that population expansion occurred recently (Rogers, 1995). Frequency distribution of pairwise difference shows a smooth or bell–shaped model that is due to the descent of alleles from one or few ancestral types (Rogers & Harpending, 1992). However, the negative and significant Fu's *Fs* statistical value observed in *H. fulvus* and *H. ater* populations provide strong evidence for past population expansion, and rule out the possibility of genetic hitching and background selection, and evolutionary force that produce a pattern similar to population expansion (Fu & Li, 1993; Fu, 1997; Okello et al., 2005). Beneficial genetic variation will generally be accumulated and maintained in a rapidly growing population (Su et al., 2001).

Our study appears to be the first report on the phylogenetic relationships and genetic diversity of the three sympatric hipposiderid bats. It is well known that *H. speoris* and *H. fulvus* share their day roost as well as foraging area (Neuweiler, 1990). Although, *H. ater* lives in the same area, it does not share day roost with them (G. Marimuthu and K. Emmanuvel Rajan, personal observations). As the details of the foraging strategy of

H. ater are still unknown, a study on this aspect would possibly provide valuable information to further update the relationships between the three hipposiderids.

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