Genetic divergence and echolocation call frequency in cryptic species of *Hipposideros larvatus s.l.* (Chiroptera: Hipposideridae) from the Indo-Malayan region

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The intermediate leaf-nosed bat (*Hipposideros larvatus*) is a medium-sized bat distributed throughout the Indo-Malay region. In north-east India, bats identified as *H. larvatus* captured at a single cave emitted echolocation calls with a bimodal distribution of peak frequencies, around either 85 kHz or 98 kHz. Individuals echolocating at 85 kHz had larger ears and longer forearms than those echolocating at 98 kHz, although no differences were detected in either wing morphology or diet, suggesting limited resource partitioning. A comparison of mitochondrial control region haplotypes of the two phonic types with individuals sampled from across the Indo-Malay range supports the hypothesis that, in India, two cryptic species are present. The Indian 98-kHz phonic bats formed a monophyletic clade with bats from all other regional populations sampled, to the exclusion of the Indian 85-kHz bats. In India, the two forms showed 12–13% sequence divergence and we propose that the name *Hipposideros khasiana* for bats of the 85-kHz phonic type. Bats of the 98-kHz phonic type formed a monophyletic group with bats from Myanmar, and corresponded to *Hipposideros grandis*, which is suggested to be a species distinct from *Hipposideros larvatus*. Differences in echolocation call frequency among populations did not reflect phylogenetic relationships, indicating that call frequency is a poor indicator of evolutionary history. Instead, divergence in call frequency probably occurs in allopatry, possibly augmented by character displacement on secondary contact to facilitate intraspecific communication. © 2006 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2006, **88**, 119–130.

ADDITIONAL KEYWORDS: echolocation – ecomorphology – resource partitioning.

INTRODUCTION

In recent years, the identification of increasing numbers of cryptic species of bats has led to the assertion that the number of bat species currently described might be a substantial underestimate of bat diversity (Jones, 1997; Jones & Barlow, 2004). The identification of cryptic species of bats has been driven largely by advances in the study of echolocation (Jones & van

Parijs, 1993) and by genetic sequencing studies (Mayer & von Helversen, 2001). Some cryptic species of echolocating bats differ substantially in their echolocation calls, whereas others do not (Jones & Barlow, 2004).

Cryptic species have been identified in several bat families; however, the Old World family Hipposideridae appears to exhibit particularly high levels of cryptic diversity (Pye, 1972; Jones *et al.*, 1993; Francis, Kock & Habersetzer, 1999; Kingston *et al.*, 2001). Jones & Barlow (2004) argued that acoustic diver-

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gence in cryptic species of echolocating bat was more likely to occur in species using narrowband echolocation calls, such as in the Hipposideridae, where acoustic signatures were reliable badges of species identity. Communication among bats of the same species would then be facilitated through each species echolocating within its own bandwidth of frequencies.

The intermediate leaf-nosed bat (*Hipposideros larvatus*) is a widespread bat species in Asia with a geographical distribution that includes Bangladesh, China, Indonesia, Malaysia, Myanmar, and north-east India (Bates & Harrison, 1997). Kitchener & Maryanto (1993) suggested that larger bats from Vietnam and Thailand had sufficiently distinctive penis and skull morphology to be classified as a separate species, *Hipposideros grandis*. This suggestion has not been widely cited, with most identification guides still treating *H. larvatus* as a single taxon over mainland south-east Asia (Bates & Harrison, 1997). Our initial hypothesis was that *H. larvatus* is represented by a single taxon in our study area.

In the present study, for the first time, we describe two cryptic forms of *H. larvatus*, sampled from a single cave system in north-east India. We use a combination of morphological, dietary and acoustic analyses to examine differences between the forms, and assess whether these provide evidence for resource partitioning. To resolve the taxonomic status of bats described as *H. larvatus* in southern Asia, we compare the echolocation call frequencies and phylogenetic relationships of both forms from India with *H. larvatus* populations sampled from southern China, Malaysia, and Myanmar. Finally, we investigate the extent to which call frequency differences among populations reflect true phylogenetic signals, and consider how conflicts between these patterns can help us to understand the possible causes of cryptic diversity in hipposiderid bats.

METHODS

STUDY AREAS

Hipposideros larvatus was sampled at 11 sites across the Indo-Malay region (Fig. 1). All individuals were identified following standard keys (Bates & Harrison, 1997). Our main study site was Tem-dibai cave at Sohbar, East Khasi Hills District in Meghalaya, northeast India (25°11′N, 91°37′E) where bats were sampled over three periods (March 2000, November 2001 to June 2002, and March to May 2003). This cave is located in a betel nut (*Areca catechu*) grove and serves as a temporary roost prior to the onset of the monsoon. In China, bats were studied in Guangdong, Guangxi, and Yunnan provinces at the following sites: Longmen, Guangdong, September 2002 (24°02′N, 112°42′E, *n* = 12); Guilin, Guangxi, September 2002 (25°28′N,
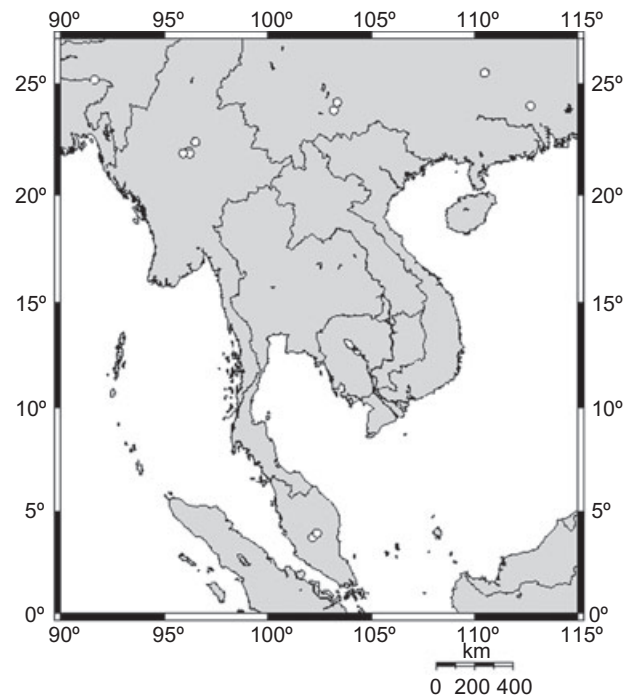
Figure 1. Indo-Malay region showing sites where bats were studied. Site details are given in the Methods section. The map was created using Online Map Creation (see http://www.aquarius.geomar.de/omc/omc_intro.html).

110°28′E, *n* = 2); Mile, Yunnan, September 2003 (24°11N, 103°21′E, *n* = 23); and Xiao Long Xi, Yunnan, September 2003 (23°50N, 103°11′E, *n* = 5). In Myanmar, fieldwork was conducted in March 2003 in Divisions of Mandalay and Sagaing, and in Shan State. Bats were captured at the following locations: Wangaber (Lahing Gu) caves, Yankin Hill, Mandalay and Inwa (Ava), Mandalay Division (21°59′N, 96°10′E, *n* = 8); Payataung (Pagoda Hill) cave, Patheingyi Township, Htonebo, Mandalay Division (21°53′N, 96°13′E, *n* = 10); Nan Dan Ya Monastery cave, Sagaing, Sagaing Division (21°54′N, 95°54′E, *n* = 32); and Saya San cave, Hokho village, Naunghkio (Nawngcho), North-west Shan State (22°24′N, 96°30′E, *n* = 1). In Malaysia, work was conducted in Pahang State: Kota Gelanggi, Tongkat Cave, Jerantut (3°56′N, 102°22′-E, *n* = 14) in January 2003 and Kuala Lompat Field Station, Krau Wildlife Reserve, Pahang (3°43′N, 102°10′E, *n* = 5) from September 1996 to January 1997.

MORPHOLOGICAL MEASUREMENTS, ECHOLOCATION CALL ANALYSIS, AND DIETARY ANALYSIS

All bats were captured after evening emergence using either harp-traps or mist-nets. Individuals were weighed using a Pesola scale (± 0.1 g) and forearm and

ear lengths were measured to the nearest 0.1 mm. Age (adult or juvenile) was established on the basis of epiphysial fusion of the finger joints, and reproductive status was determined. Wings were drawn by placing the ventral side of the bat on a plain sheet of paper, extending one wing, the uropatagium and half the body, and tracing around it (Kingston *et al.*, 2000). Wing area was measured using a Summagraphics SummaSketch III digitizing tablet and wing parameters were calculated in accordance with Norberg & Rayner (1987). Skull measurements were made under a binocular microscope fitted with an eyepiece graticule to the nearest 0.1 mm.

For all individuals, echolocation calls were recorded in the hand, held approximately 30 cm from the microphone. In India, recordings were made using an Ultra Sound Device S-25 bat detector (frequency response 57 ± 3 dB ref. 1 V/μ bar from 20–120 kHz) attached to an Ultra Sound Advice Portable Ultrasound Processor (PUSP, sampling rate 448 kHz) and a Sony Professional Walkman (WM-D6C). Calls from bats in Malaysia, Myanmar, and China were recorded with a Pettersson D-980 bat detector (Pettersson Elektronik) with time expansion at ×10, attached to either a Sony Professional Walkman as described above, or a Sony TCD-D8 DAT recorder. Because analyses were restricted to determining frequencies of most energy in echolocation calls (the constant frequency portion), all recording systems gave similar results. We used the software BatSound version 3 (Pettersson Elektronik) to determine the frequency of maximum energy (kHz) from power spectra (512 point FFT, Hanning Window), taken for one randomly selected call from each individual.

Droppings were collected from bats at Tem-dibai cave after bats were processed in March and April 2003. Faecal samples were dried in the sun and stored in airtight containers. An intact pellet was selected at random from each individual bat and softened by soaking in water. Pellets were teased apart with tweezers and identifiable insect fragments with distinct features were prepared as slides. Insect fragments were identified by observation under a low power binocular microscope (×10 magnification) and identified to order using keys available in the literature (McAney *et al.*, 1991) and by comparison with reference material collected in the field. Prey composition was estimated according to percentage volume.

TISSUE COLLECTION, DNA ISOLATION, AND SEQUENCING

Wing membrane samples were collected using 3-mm biopsy punches (Stiefel Laboratories), fixed in 90% ethanol and stored at −20 °C. Genomic DNA was extracted using Qiagen DNeasy Kits. A 516-base pair portion of mitochondrial control region was amplified using the primers ThrL16272 (Stanley *et al.*, 1996) and DLH 16750 (Wilkinson & Chapman, 1991). Polymerase chain reactions (PCRs) were undertaken in 15 μL of total reaction volumes, containing final primer concentrations of 0.667 μM. Reaction mixtures contained 5 μL of DNA extract, 10 mM dNTPs, 0.5–1 U *Taq* polymerase (Bioline), 10 × Taq buffer (Bioline), and 1.5 mM of MgCl$_2$. PCRs were performed on a DNA Engine Tetrad thermal cycler (MJ Research) with the following profile: 94 °C for 2 min; 34 cycles of 94 °C for 30 s; 55 °C (annealing temperature) for 30 s; 72 °C for 30 s; and 72 °C for 3 min. PCR products were cleaned with ExoSAP-IT (Amersham Pharmacia Biotech) following the manufacturer's instructions. Sequencing reactions contained 4 μL of template, 3 μL of Better-Buffer (Microzone Ltd), 1 μL BigDye Terminator Reaction Kit (Applied Biosystems), 1 μL water, and 0.1 μM primer. Sequences were run on an ABI 3700 automated sequencer.

PHYLOGENETIC RECONSTRUCTION

Sequences were aligned in BIOEDIT (Hall, 1999) and phylogenetic reconstruction was undertaken in PAUP (Swofford, 1991). A maximum likelihood tree was generated by heuristic search, using the HKY85 model of substitution. The tree was rooted using *Rhinolophus monoceros* as an outgroup. Node support was assessed by bootstrapping (1000 replicates). Pairwise divergence values among all sequences were calculated according to the HKY85 model. Sequences from Indian bats were deposited in GenBank (accession numbers DQ257451–58).

RESULTS

ECHOLOCATION CALLS OF INDIAN BATS

The echolocation calls of 86 *H. larvatus* individuals captured at Tem-dibai cave showed a bimodal distribution in call frequency (Fig. 2). One group called at around a median of 84.7 kHz ($n = 34$; range 80.7–85.9 kHz), and the other group at around 97.5 kHz ($n = 52$; range 92–102 kHz). A silent band of 6.1 kHz separated the two peaks. The bimodality could not be explained by differences in either age (all bats were adults) or sex (each group included both sexes). We refer to the two phonic types of *H. larvatus* as 85-kHz bats and 98-kHz bats in the subsequent analyses. Call frequency differences are given in Table 1.

MORPHOLOGICAL DIFFERENCES BETWEEN PHONIC TYPES

Forearm length and ear length measurements were taken from a sample of bats of each phonic type

Table 1. Morphological and echolocation call frequency (frequency of most energy, FMAXE) measurements from *Hipposideros larvatus* in India, Myanmar, Malaysia, and China

Locality	Sex	Forearm length (mm)	Body mass (g)	FMAXE (kHz)	Ear length (mm)
India					
85-kHz	Male	62.77 ± 1.16 (12)	17.04 ± 1.95 (12)	83.70 ± 1.76 (12)	19.25 ± 4.15 (23)
	Female	64.18 ± 1.57 (10)	20.40 ± 3.08 (10)	85.10 ± 0.72 (10)	21.19 ± 1.12 (7)
98-kHz	Male	60.48 ± 1.45 (22)	18.29 ± 2.23 (22)	96.70 ± 2.05 (22)	17.55 ± 1.20 (10)
	Female	61.07 ± 1.72 (15)	17.60 ± 2.15 (15)	97.96 ± 2.12 (15)	18.19 ± 1.15 (9)
Myanmar					
	Male	61.14 ± 1.43 (30)	17.38 ± 1.41 (30)	92.55 ± 1.54 (30)	
	Female	61.77 ± 1.98 (21)	17.58 ± 2.05 (21)	93.00 ± 1.02 (21)	
Malaysia					
	Male	57.76 ± 1.91 (12)	17.59 ± 1.61 (12)	100.65 ± 0.82 (12)	
	Female	57.71 ± 1.17 (7)	17.14 ± 1.11 (7)	100.63 ± 0.64 (7)	
China					
	Male	60.28 ± 1.74 (20)	21.04 + 3.46 (18)	82.64 ± 2.62 (20)	19.87 + 2.13 (9)
	Female	60.79 ± 2.01 (19)	20.12 + 2.36 (25)	82.32 ± 1.71 (19)	18.50 + 1.53 (12)

Data are mean ± SD (sample size).
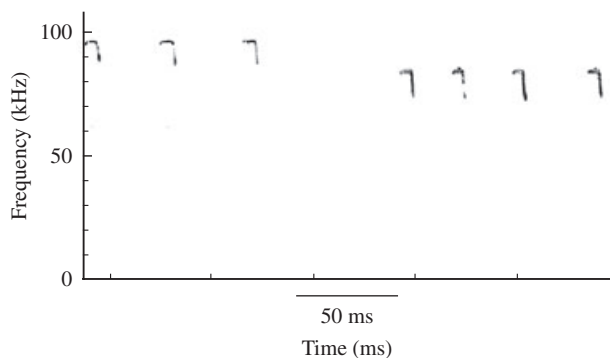
Figure 2. A sequence of calls from individuals of the 98-kHz and 85-kHz phonic types of *Hipposideros larvatus* from Tem-dibai Cave, Meghalaya, north-east India. The spectrogram was made with a 512-point Fast Fourier transform and a Hanning Window.

Table 2. Cranial and dental measurements from skulls of female 85-kHz and 98-kHz *Hipposideros larvatus*

Character	85-kHz bat	98-kHz bat
Greatest length of skull (mm)	24.70	23.10
Condylo-canine length (mm)	20.50	19.60
Zygomatic breadth (mm)	13.35	13.10
Breadth of braincase (mm)	9.30	9.00
Postorbital constriction (mm)	1.60	1.30
Maxillary toothrow length (mm)	8.90	8.50
Mandibular toothrow length (mm)	9.40	9.20
Mandible length (mm)	16.00	15.90

Definitions follow those given in Bates & Harrison (1997).

(Table 1). We used analysis of covariance to investigate whether morphological features varied according to phonic type, forearm length, and sex. Mean forearm length differed in relation to phonic type ($F_{1,55} = 13.98$, $P < 0.001$) (85-kHz bats had longer forearms than 98-kHz bats) and sex ($F_{1,55} = 25.89$ $P < 0.001$). Investigation of the interaction ($F_{1,55} = 13.51$ $P < 0.001$) between phonic type and sex revealed that sex differences were only apparent for 85-kHz bats, where females were significantly ($P < 0.001$) larger than males. However, there was considerable overlap in the forearm length of bats of the two phonic types. Mean ear length differed between phonic types, with 85-kHz bats having longer ears than 98-kHz bats ($F_{1,46} = 5.68$, $P < 0.05$). Ear length was not different between the sexes ($F_{1,46} = 1.01$, NS). Skull measurements from two female specimens (Fig. 3, Table 2) suggested a slightly larger skull in the 85-kHz phonic type.

WING MORPHOLOGY

Analyses of wing morphology were restricted to males because females differed greatly in body mass (and hence wing loading) according to reproductive status. Bats of the 85-kHz phonic type had slightly, but significantly larger mean values of wingspan and wing area than the 98-kHz bats (Table 3). Wing loading and aspect ratio did not differ significantly between phonic types (Table 3). Wing span, wing area, and forearm length were log-transformed then entered into a quadratic discriminant function analysis with cross validation to determine if the two phonic types could be

Table 3. Wing morphology measurements (means ± SD) from male bats of the two phonic types of *Hipposideros larvatus* from Tem-dibai cave, Meghalaya, north-east India
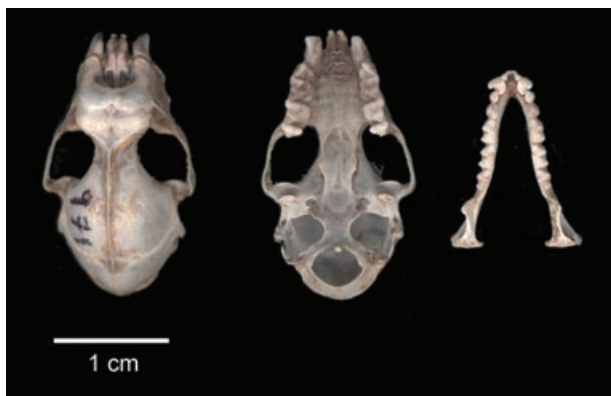
	85-kHz males	98-kHz males	t	P
Wingspan, B (m)	0.3829 ± 0.0195	0.3570 ± 0.0200	2.78	< 0.05
Wing area, S (m²)	0.0238 ± 0.0019	0.0214 ± 0.0016	2.38	< 0.05
Wing loading, L (Nm⁻²)	7.38 ± 1.08	8.28 ± 0.91	1.95	NS
Aspect ratio, A	6.28 ± 0.34	5.96 ± 0.37	1.88	NS

Data are for twelve 85-kHz males and seven 98-kHz males. Definitions follow those given in Norberg & Rayner (1987). NS, Not significant.

A

B

Figure 3. Skulls of female (A) 85-kHz and (B) 98-kHz *Hipposideros larvatus*.

discriminated by multivariate analysis of wingshape parameters. The discriminant analysis was only able to classify 11 of 19 (58%) bats to phonic type correctly, and the discrimination was not statistically significant (Wilk's $\lambda_{3,15} = 2.92$, $P > 0.05$). Therefore, despite clear and consistent differences in echolocation call frequency, the two phonic types showed extremely similar wing shape.

Table 4. Dietary composition, determined by faecal analysis, for 85-kHz and 98-kHz *Hipposideros larvatus* from Tem-dibai cave, Meghalaya, north-east India

	85-kHz bats		98-kHz bats	
	March	April	March	April
Coleoptera	93.2	81.5	83.9	82.0
Lepidoptera	4.6	5.0	11.4	9.4
Diptera	0.5	0	0.7	0.3
Culicidae	0	0	0	0.3
Isoptera	0	6.5	1.8	1.4
Hemiptera	0	2.5	0	0
Trichoptera	0.3	0	0	3.8
Hymenoptera				
Symphata	0.5	0	0	0
Fomicidae	0.3	4.0	2.1	2.8
Ichneumonidae	0.4	0	0	0
Arachnida	0	0.5	0	0
Acari	0.2	0	0.1	0

Data are percentage volume summed over single pellets selected at random from 14 bats of the 85-kHz phonic type, 20 bats of the 98-kHz phonic type in April, and ten pellets from different individual 85-kHz phonic type bats and 16 pellets from 98-kHz bats in April. Acari were represented in the form of ingested ectoparasites.

DIET

Analyses of diet for the period March to April 2003 revealed considerable overlap between both phonic types, with no obvious resource partitioning. For both types, faecal material consisted mainly of coleopterans (Table 4), with lepidopterans representing the second most important dietary component.

CALL FREQUENCIES AND BODY SIZES OF OTHER INDOMALAYAN *H. LARVATUS*

Call frequencies showed considerable regional variation, ranging from ~82 kHz in China to ~100 kHz in

Malaysia. Body size, as measured by forearm length, was smallest in Malaysia (perhaps reflecting the high frequencies emitted by bats there) and largest in the 85-kHz bats from India. All echolocation call frequencies and morphological measurements from *H. larvatus* in Myanmar, Malaysia, and China are summarized in Table 1. Interestingly, although a bat from Myanmar formed a monophyletic group with 98-kHz bats from India (see below), the Myanmar individuals used lower call frequencies than the Indian bats (Fig. 4). We found no evidence of 85-kHz bats in Myanmar, so it is possible that the 98-kHz bats there are showing a form of acoustic character release, and are able to use lower frequencies than in India where the presence of 85-kHz bats selects for the use of higher frequencies to facilitate intraspecific communication.
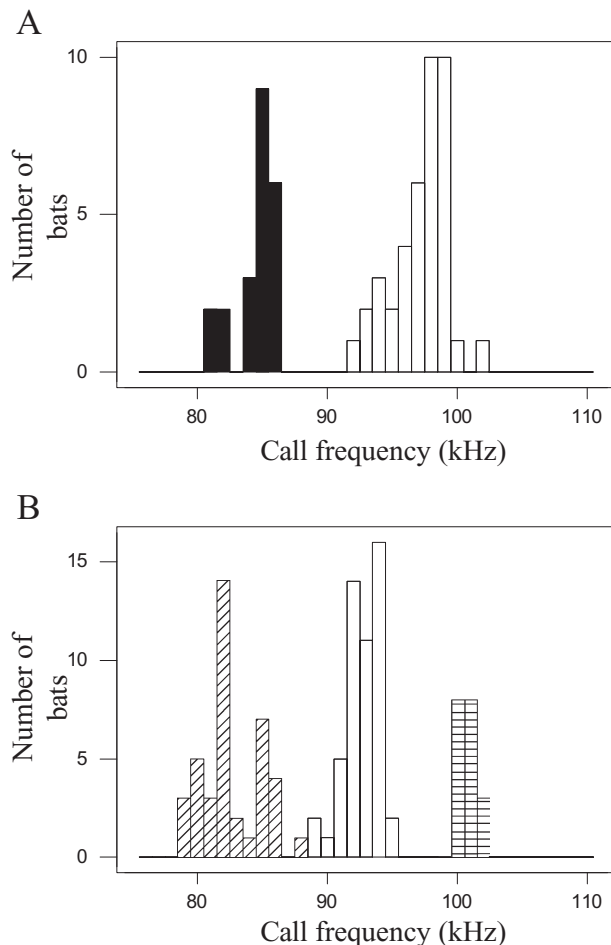
Figure 4. Distributions of call frequencies in bats: (A) from India (85-kHz *Hipposideros larvatus* shown in black, 98-kHz *H. larvatus* in white) and (B) from China (diagonal hatching), Myanmar (white) and Malaysia (horizontal hatching). The bats from Myanmar form a monophyletic group with the 98-kHz bats from India.

PHYLOGENETIC ANALYSES

A phylogram based on maximum likelihood analysis (Fig. 5) revealed that the Indian 98-kHz phonic bats formed a monophyletic clade with bats from all other regional populations sampled, to the exclusion of the Indian 85-kHz bats. Within the main *H. larvatus* clade, the Myanmar individual formed a monophyletic group with the 98-kHz bats from India. Malaysian and Chinese bats formed monophyletic groups within the main *H. larvatus* clade. Overall, echolocation call frequency was a poor measure of phylogenetic distinctiveness; although Chinese *H. larvatus* emitted call frequencies similar to 85-kHz bats from India, the bats from these areas were phylogenetically distant. Bats calling between 100–102 kHz from Malaysia were phylogenetically closer to bats from China (79–88 kHz) than they were to bats from India and Myanmar, calling at 92–102 kHz and 89–95 kHz, respectively.

Sequence divergence measurements (Table 5) confirmed the distinctiveness of the Indian 85-kHz bats. Of the three individuals of this type that were sequenced, two shared a haplotype (one is shown in Fig. 5), which demonstrated 1.77% sequence divergence with the third individual. By contrast, sequence divergence between the two phonic types from India was in the range 12–13.4%. The monophyletic origin of the 85-kHz bats, and the extent of sequence divergence between the two phonic types, supports our hypothesis that the 85-kHz bats from India represent a separate cryptic species, and should therefore be reclassified. Bats within the 98-kHz phonic type showed sequence divergences of between 0.1% and 5.5%, and were genetically close to the bat from Myanmar. Bats from China showed close genetic similarity (divergence 0–1.6%) despite being sampled over a large geographical area (Fig. 1). Indeed, four individuals from Guangdong shared the same haplotype, as did two from Yunnan, although only single representatives of both haplotypes were used in the phylogenetic analysis.

The taxonomic affinities among populations of the main *H. larvatus* clade (excluding the Indian 85-kHz bats) remain unclear, with bats from each geographical region forming distinct clades, and showing substantial genetic divergence from other clades. Further sampling and comparisons with other taxa in the genus *Hipposideros* are needed to determine whether these clades represent subspecies, or even distinct species.

DISCUSSION

CRYPTIC SPECIES AND THE EVOLUTION OF ECHOLOCATION CALL DIFFERENCES

The existence of two sympatric but genetically divergent phonic types of *H. larvatus* at Tem-dibai caves in
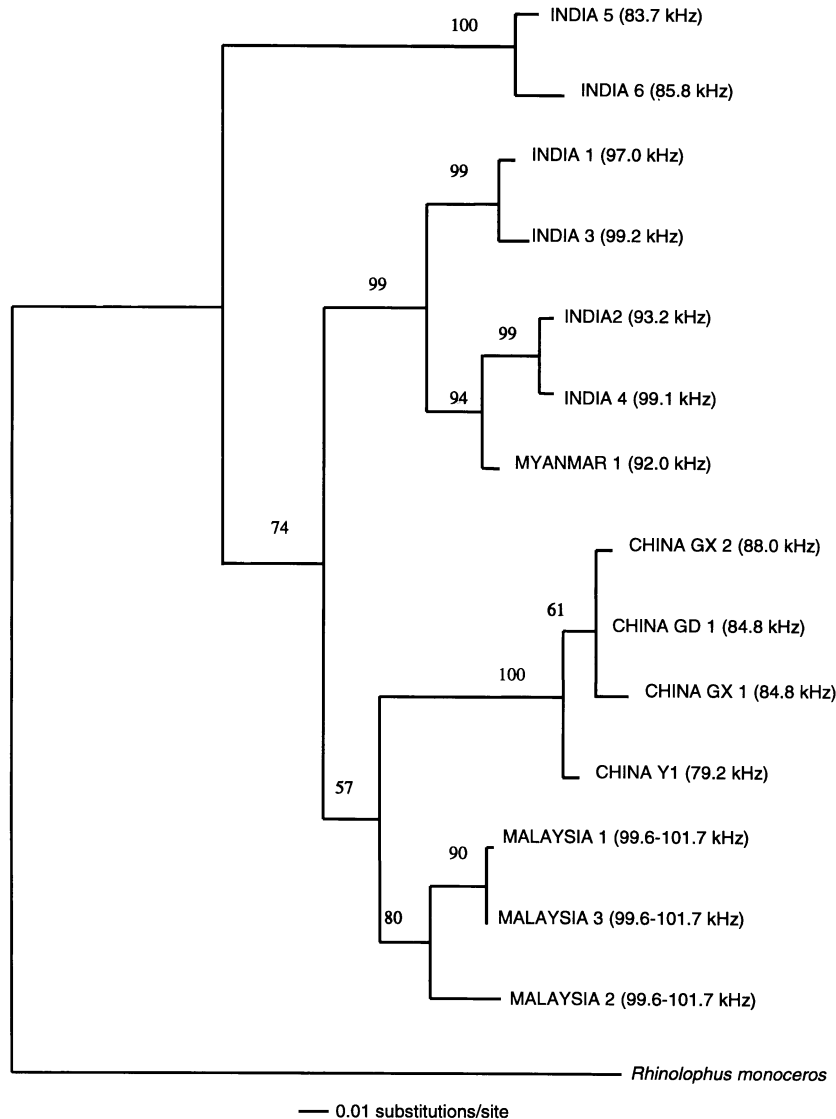
Figure 5. Phylogram showing branch lengths, generated by maximum likelihood analysis with heuristic search. Bootstrap values (% values based on 1000 replicates) are given on the nodes. DNA from five other bats not shown in the tree was also sequenced: one bat from India (85.2 kHz) shared a haplotype with INDIA 5, an additional bat from Yunnan, China (80.8 kHz) shared a haplotype with CHINA Y 1, and three bats from Guangdong, China (86.4 kHz, 83.2 kHz and 85.6 kHz) shared haplotypes with CHINA GD 1. INDIA 5 and 6 are bats of the 85-kHz phonic type from Tem-Dibai cave, Meghalaya, India. INDIA 1–4 are bats of the 98-kHz phonic type from the same site. MYANMAR 1 is from Nan Dan Ya Ma cave, Sagaing Division, Myanmar. CHINA GX bats are from Guilin, Guangxi Province, China. CHINA GD bats are from Longmen, Guangdong province China, and CHINA Y bats are from Yunnan Province. The MALAYSIA bats are from Peninsular Malaysia. Echolocation call frequencies of individuals are given in parentheses. Sequenced bats from Malaysia were not recorded and therefore call frequency ranges for Malaysian bats are based on the full sample.

north-east India is strongly indicative that cryptic species are present. This discovery adds to a growing number of reported cryptic species of hipposiderid bat that differ in call frequency. Pye (1972) discovered two phonic types of *Hipposideros commersoni* (56 and 66 kHz call frequencies) in a cave in Kenya, likely to be cryptic species, and the species *Hipposideros caffer* and *Hipposideros ruber*, which are sympatric in West Africa, are similar morphologically but are readily separated by echolocation call frequency (Jones *et al.*, 1993). More recently, *Hipposideros ridleyi* and *Hipposideros orbiculus* sp. nov. (which show a 19-kHz

Table 5. Sequence divergence measures between bats shown in the phylogenetic tree (Fig. 5)

	I5	I6	I1	I3	I2	I4	Mya	CGX2	CGD1	CGX1	CY1	Mal1	Mal2	Mal3	Rhinolophus monoceros
I5															
I6	1.77														
I1	11.99	12.50													
I3	12.38	13.00	1.37												
I2	12.58	13.19	4.51	5.49											
I4	12.77	13.39	4.90	5.49	0.08										
Mya	11.79	12.40	3.92	4.11	2.16	2.16									
CGX2	13.61	14.02	9.30	10.49	9.50	9.68	9.30								
CGD1	13.38	13.80	9.27	10.46	9.50	9.65	9.27	0.20							
CGX1	13.72	14.13	9.81	10.60	10.06	9.61	9.42	0.98	0.78						
CY1	13.53	13.94	9.03	10.20	9.22	9.22	9.02	0.99	0.79	1.56					
Mal1	11.76	12.37	7.26	7.07	7.26	7.26	6.67	6.70	6.88	7.05	6.65				
Mal3	11.96	12.56	7.46	7.26	7.06	7.46	6.87	6.90	7.08	7.24	6.85	0.20			
Mal2	12.35	12.76	8.24	8.05	7.46	7.85	6.87	7.30	7.08	6.85	7.24	3.33	3.13		
Rhinolophus monoceros	22.53	22.16	21.52	21.29	21.73	21.52	20.52	20.83	20.80	21.10	20.29	19.90	20.07	20.48	

The use of a prefix refers to bats from: I, India; Mya, Myanmar; C, China (GX, Guangxi; GD, Guangdong; Y, Yunnan); and Mal, Malaysia, respectively.

difference in call frequency) were separated in south-east Asia (Francis *et al*., 1999), while Kingston *et al*. (2001) described two cryptic species of *Hipposideros bicolor* (call frequencies 131 and 142 kHz) in Malaysia, which showed ~7% sequence divergence at the cytochrome *b* gene.

Despite the differences in the call frequency, we detected little morphological divergence between the two forms of *H. larvatus*. Although ear length was slightly greater in the 85-kHz bats, as predicted by allometric analyses of rhinolophid and hipposiderid bats (Zhao *et al*., 2003), our multivariate analyses failed to separate the two forms based on wing shape. Such close similarities in wing morphology indicate that both phonic types are likely to exhibit similar foraging habits, and this is also supported by our analysis of diet between March and April. Therefore, the two sympatric cryptic forms of *H. larvatus* appear to show little resource partitioning, although we cannot exclude the possibility that partitioning occurs at other times of the year, or during periods of food shortage when interspecific competition may be more severe.

A probable lack of resource partitioning is also supported by the observed differences in call frequency. Although Kingston & Rossiter (2004) recently proposed that divergence among sympatric size morphs of the horseshoe bat *Rhinolophus philippinensis* might be facilitated by disruptive selection on diet, resulting from large-scale harmonic shifts at low frequencies, Jones & Barlow (2004) suggested that the differences in the higher call frequencies reported for cryptic *Hipposideros* species are likely to have little impact on prey detection. This is because, although wavelength influences echo strength in relation to target size (Pye, 1993; Houston, Boonman & Jones, 2004), the wavelength differences at such high frequencies are small and unlikely to influence the bats' sensory performance (wavelengths of calls are 3.5 and 4.0 mm for 98 kHz and 85 kHz, respectively).

Guillén, Juste & Ibáñez (2000) suggested that variation in echolocation call frequency in *H. ruber* was correlated with body size, body condition, the presence of ecologically similar species, and with environmental factors (e.g. humidity). Lower frequencies were associated with higher humidity. Although such a correlation may be adaptive because humidity reduces the transmission of ultrasound in the atmosphere, a similar trend was not apparent in the present study because bats in conditions of highest humidity (Malaysia) showed highest call frequencies. In the present study, body size differences among populations were not related to call frequency: bats from China showed lowest frequencies, but were intermediate in size between bats from Myanmar & Malaysia. Robinson (1996) recorded call frequencies of around 85 kHz

from *H. larvatus* in western Thailand, and thus the phylogenetic position of Thai bats would be interesting to ascertain, given their closer resemblance in call frequency to bats from China compared with Malaysian individuals.

Therefore, we suggest that, in the case of *H. larvatus*, the formation of cryptic forms in north-east India is unlikely to have involved ecological divergence but, instead, probably arose because two populations of bats became isolated in the past. Drifts in call frequency resulting from geographical separation may theoretically lead to divergence in acoustic communication without concomitant changes in morphology. Jones & Barlow (2004) concluded that the major factor promoting acoustic divergence in cryptic pipistrelle species was associated with facilitating communication with conspecifics, rather than resource partitioning. Therefore, where frequency drift is sufficient to alter communication, reproductive isolation may be maintained on secondary contact. Furthermore, following secondary contact, differences in call frequency may be maintained and even further promoted via character displacement, so that each cryptic species has a 'private bandwidth', facilitating species recognition. This scenario appears to be supported by our comparison of call frequencies and phylogenetic relationships among *H. larvatus* across south-east Asia.

Character displacement of echolocation call frequencies may be supported by the observation that Myanmar bats, which are closely related to 98-kHz bats in India, use frequencies that often fall within the silent band at Tem-dibai caves. This could be because 85-kHz bats are absent in Myanmar, thus allowing *H. larvatus* there to use lower frequencies, or simply because the Myanmar bats are larger, and hence use lower frequency calls (Table 1). Similar social selection for acoustic divergence was suggested for cryptic species of *H. bicolor* (Kingston *et al*., 2001), although some resource partitioning in these species may be possible because they show clearer morphological divergence than the *H. larvatus* in the present study.

Apart from *H. ridleyi/orbiculus*, all cryptic hipposiderids recognized to date are highly dependent on caves for roosting. Cave roosting might make geographical isolation more likely, but a shortage of caves may also lead sibling taxa, which have diverged in allopatry, to share roosts following secondary contact, further necessitating and even perhaps accelerating the need for clear species recognition and character displacement. The theoretical models of social selection suggest that, if the various requisites are met (phenotypic covariation and developmental linkage between signal and receptor systems), divergence in the communication system may even occur in sympatry (Kingston *et al*., 2001).

RELATIONSHIPS WITH OTHER ASIAN POPULATIONS OF *H. LARVATUS*

Genetic divergence within Chinese bats was very small, despite sampling over a range of almost 1000 km. However, Chinese bats showed considerable genetic divergence (9–14%) with Indian bats of both phonic types, suggesting that the Chinese bats may represent a distinct species from the Indian ones. Interestingly, the 98-kHz Indian bats were closer genetically to Chinese bats, despite the latter calling at around 82–83 kHz on average. This emphasizes that genetic divergence and differences in echolocation call frequencies are not linked. Indeed, call frequency may be influenced more by the frequencies used by sympatric conspecifics, and is not therefore a reliable phylogenetic signal.

NOMENCLATURE

Differences in call frequency, coupled with marked genetic divergence, support the hypothesis that bats identified initially as *H. larvatus* in Tem-dibai cave, India comprises two sympatric, cryptic species. The taxonomy and nomenclature of *H. larvatus* has long been in a state of confusion. Several synonyms and subspecific names have been applied to *H. larvatus*, and Kitchener & Maryanto (1993) argued that *H. larvatus* in the Greater and Lesser Sunda Islands, Indonesia, comprise at least five species on the basis of skull, baculum, and penis morphology. Hill (1963) had previously recognized eight subspecies of *H. larvatus* in an earlier revision of the genus.

The holotype of *Rhinolophus* (= *Hipposideros*) *larvatus* Horsfield, 1823 originates from Java (Horsfield, 1821–24), but we have not been able to establish the affinities of bats from the type locality with our sample. Of the eight subspecies recognized by Hill (1963), Kitchener & Maryanto (1993) considered *H. l. larvatus* and *H. l. neglectus* to be synonymous, whereas *H. grandis*, described as *H. l. grandis* by Allen (1936), was elevated to species status on the basis of distinctive morphology of its skull, glans penis, and baculum. *Hipposideros grandis* is documented from India, Myanmar, Thailand, and Vietnam (Allen, 1936; Shamel, 1942; Topál, 1975; Kitchener & Maryanto, 1993). Specimens from Thailand and Vietnam were reliably identified from baculum morphology, whereas bats from Myanmar have been ascribed to this taxon on the basis of size (*H. grandis* is typically larger than *H. larvatus*, although some *H. grandis* can be as small as typical *H. larvatus* (Hill, 1963; Kitchener & Maryanto, 1993).

Kitchener & Maryanto (1993) also examined specimens assigned to *H. larvatus* from Malaysia, taken from Pahang, where the present studies were based. On baculum and skull morphology, these were considered similar to *H. l. larvatus* from Java (the type locality). A baculum from a bat from Peninsular Malaysia described by Zubaid & Davison (1987) also resembles that of *H. l. larvatus* from Java.

Clearly, current descriptions of subspecies of *H. larvatus* are inadequate and a full understanding of the taxonomy of these bats requires investigations of morphology, echolocation calls, and DNA sequences. On the basis of previous studies, the most likely explanation of our tree is as follows. The Indian 85-kHz bats are phylogenetically distinct, and should be recognized as a new species. Neither the morphology of the glans penis, nor the baculum resemble those of *H. grandis* described by Kitchener & Maryanto (1993) (authors' unpubl. data). Dobson (1874) described *Phyllorhina* (= *Hipposideros*) *leptophylla* (subsequently *H. l. leptophyllus*; Hill, 1963) from the Khasi Hills in Meghalaya, an area close to the present study site in India. Apparently, no type material exists for this taxon (Tate, 1941). Tate (1941) considered bats of this taxon as synonymous with *H. larvatus grandis,* fitting with a similarity in forearm lengths for the two taxa (Hill, 1963). It is unclear whether bats identified as *H. l. leptophyllus* would be 85-kHz or 98-kHz phonic types, or even a mixture of both. Given the lack of type material for *H. leptophylla* and noting that this taxon has been suggested as being synonymous with *H. l. grandis* by Tate (1941), and that the likelihood that museum specimens of this subspecies may not all have been ascribed to the correct taxon, we feel that this synonym is inadequate as a name for the 85-kHz bats from Meghalaya. We propose using the name *Hipposideros khasiana* sp. nov. for the 85-kHz bats from Meghalaya, known at present only from India. The name *khasiana* refers to the Khasi hills from where the taxon is described. Material from a female deposited at the Harrison Zoological Museum, Sevenoaks, Kent (http://www.harrison-institute.org/) can serve as the holotype for this taxon, with a second female as a paratype.

The 98-kHz bats from India and the closely related bat from Myanmar are likely to be *H. grandis* on the basis of their large body size (Hill, 1963), accepting the convincing argument of Kitchener & Maryanto (1993) that this taxon is a distinct species. A specimen from Tem-dibai cave has also been lodged at the Harrison Zoological Museum. Bats from Myanmar bats in the same clade as the 98-kHz bats from India, and echolocating at frequencies similar to those recorded in the present study, have bacula resembling those described for *H. grandis,* although variation in baculum shape may make this character not as reliable as initially assumed (P. J. Bates and I. J. Mackie, pers. comm.). The Malaysian bats are almost certainly *H. l. larvatus* given their relatively short forearm lengths, and because they came from sites where this taxon had

been captured previously. The Chinese bats form a clade with little genetic divergence amongst its representatives, but with considerable divergence from other clades. The bats in China also used different echolocation calls from those in Malaysia. The Chinese bats should therefore be at least considered a distinct subspecies of *H. larvatus* (*H. l. poutensis* has been applied to Chinese *H. larvatus*; Allen, 1906), although so far only to bats from Hainan Island), and may even deserve raising to specific status given that the extent of genetic divergence of this clade from its closest relatives is similar to that of *H. l. larvatus* from *H. grandis*.

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