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Acoustic divergence in two cryptic *Hipposideros* species: a role for social selection?

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We present evidence that a relatively widespread and common bat from South East Asia comprises two morphologically cryptic but acoustically divergent species. A population of the bicoloured leaf-nosed bat (*Hipposideros bicolor*) from Peninsular Malaysia exhibits a bimodal distribution of echolocation call frequencies, with peaks in the frequency of maximum energy at *ca.* 131 and 142 kHz. The two phonic types are genetically distinct, with a cytochrome *b* sequence divergence of just under 7%. We consider the mechanisms by which acoustic divergence in these species might arise. Differences in call frequency are not likely to effect resource partitioning by detectable prey size or functional range. However, ecological segregation may be achieved by differences in microhabitat use; the 131 kHz *H. bicolor* is characterized by significantly longer forearms, lower wing loading, a lower aspect ratio and a more rounded wingtip, features that are associated with greater manoeuvrability in flight that may enable it to forage in more cluttered environments relative to the 142 kHz phonic type. We suggest that acoustic divergence in these species is a consequence of social selection for a clear communication channel, which is mediated by the close link between the acoustic signal and receptor systems imposed by the highly specialized nature of the hipposiderid and rhinolophid echolocation system.

Keywords: acoustic resource partitioning; echolocation; wing morphology; speciation

1. INTRODUCTION

Operationally, most species are defined by their morphology. The problem posed to this morphospecies approach by cryptic species has long been recognized (Mayr 1942) and the advent of molecular genetic techniques has further revealed how extensive this problem can be; morphologically cohesive populations may harbour several genetically distinct species (e.g. Baker *et al.* 1995; Bruna *et al.* 1996; Trewick 1998). Cryptic species frequently go undetected because their diagnostic features are in sensory modalities very different from our own, e.g. 'silent' insect songs (Henry 1994), sex pheromones (Foster *et al.* 1991) and toxicity resistance (Sturmbauer *et al.* 1999) or because of a poor understanding of the functional and ecological significance of the differences that are observed. Microchiropteran bats are a classic example of this situation. They operate in an acoustic world that is largely beyond the range of human hearing (>20 kHz) and are the only truly volant mammals.

Morphologically cryptic but acoustically divergent bat species may be widespread (Jones 1997; Jones & Barlow 2001). At least 13 pairs of cryptic bat species have been described (see Jones & Barlow (2001), for a review), primarily from the temperate zone where most work has been conducted. Acoustic differences in tropical bat faunas may be greatest in the Old World families Hipposideridae and Rhinolophidae (superfamily Rhinolophoidea) (Jones 1997; e.g. Francis *et al.* 1999). The structure of the echolocation calls in these families is itself very simple and greatly constrained within individuals, but its function is highly sophisticated and involves a number of auditory specializations. All species of the two families so far studied emit a composite sound consisting of a pure tone (constant frequency, CF) of relatively long duration (5–100 ms) terminated by a brief frequency-modulated (FM) sweep (for a review, see Neuweiler 1990). A narrowly tuned receiving filter—an acoustic fovea—ensures that the pure tone frequencies used in echolocation are greatly over-represented in both the basilar membrane of the cochlea and the auditory neural centres of the inferior colliculus (Schuller & Pollak 1979; Neuweiler 1990). The extreme sensitivity of these auditory filters enables them to detect minute, repetitive frequency and amplitude modulations (acoustic glints) in the returning echo generated by insect wing beats (Neuweiler 1981; Schnitzler 1987). Thus, hipposiderids and rhinolophids are capable of foraging for insects in acoustically cluttered space and are able to distinguish the fluttering of insect wings from the background echoes of surrounding vegetation (flutter detection) (Schnitzler & Flieger 1983; Bell & Fenton 1984; Link *et al.* 1986). The returning echoes must fall within the frequency range of the acoustic fovea for such fine-tuned frequency discrimination. Flying bats therefore compensate for the Doppler shifts resulting from their own velocity by altering the frequency at which the pulse is emitted, although such Doppler shift compensation may be incomplete in the Hipposideridae (Schnitzler 1968, 1973; Habersetzer *et al.* 1984).

Hipposideros bicolor is known in southern Thailand, Peninsular Malaysia, Sumatra, Java, Borneo and the Philippines. In the course of a study of rhinolophoid bats at Kuala Lompat, Krau Wildlife Reserve, Malaysia, it became apparent that bats identified as *H. bicolor* exhibited a bimodal distribution of echolocation call frequencies,

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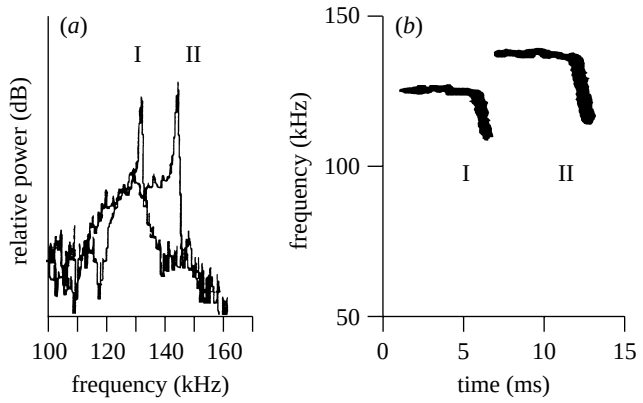

Figure 1. Echolocation calls of two phonic types of *H. bicolor*. (*a*) Power spectra and (*b*) sonagrams. Call I had a frequency of maximum energy of 131 kHz and call II a frequency of maximum energy of 142 kHz.

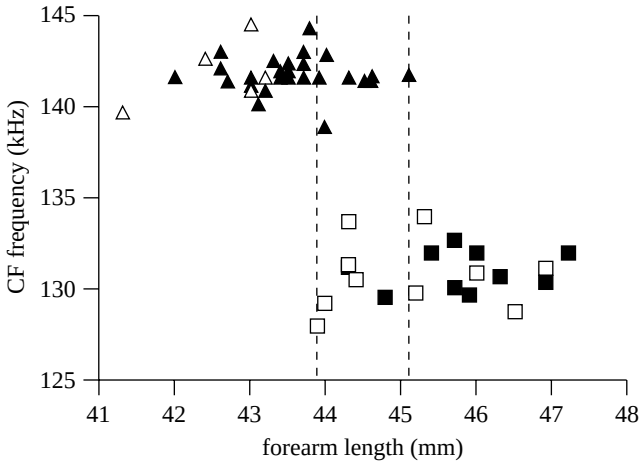

Figure 2. The relationship between constant frequency component of calls and forearm length for 20 131 kHz *H. bicolor* and 32 142 kHz *H. bicolor*. Squares denote 131 kHz bats and triangles 142 kHz bats. Filled symbols are females and open symbols males. Dotted lines demarcate the zone of overlap in forearm length, which includes seven 131 kHz bats and eight 142 kHz bats.

with some individuals echolocating at *ca.* 131 kHz (hereafter referred to as the 131 kHz *H. bicolor*) and others at 142 kHz (hereafter referred to as the 142 kHz *H. bicolor*) (figure 1). The aim of the present study was to determine whether the two call types represent distinct phylogenetic lineages and perhaps cryptic species and to establish the degree of morphological difference: are the two phonic types truly morphologically cryptic or are there differences that might be of functional significance?

2. MATERIAL AND METHODS

Two of the authors (T.K. and Z.A.) sampled bats between January 1996 and March 1997 and during August 1999 at Kuala Lompat Research Station, an area of primary lowland evergreen dipterocarp forest on the eastern edge of the Krau Wildlife Reserve, Pahang, Peninsular Malaysia (3°43′ N, 102°10′ E).

Bats were captured in the forest understorey using four-bank harp traps (Francis 1989) positioned across trails. Individuals were identified as *H. bicolor* following Medway (1982) and Payne & Francis (1985). All bats were marked with wing bands (rings) for individual identification in order to avoid resampling. Adult individuals were weighed and the length of their forearms measured with dial calipers (±0.1 mm). A ruler was used to measure the lengths of their ears, hind feet and tails. Pregnant females were excluded in order to avoid skewing the wing loading estimates based on body mass.

Echolocation calls were recorded and analysed following Kingston *et al.* (2000). Power spectra were used for deriving the frequency (kHz) of the CF component of the resting frequency for each of six calls chosen randomly from each individual and the means used in subsequent analyses. Wing parameters were derived from tracings after Norberg & Rayner (1987).

Differences between the phonic types were investigated using both univariate and multivariate analyses of log-transformed data. Univariate *t*-tests were carried out in order to evaluate morphological differences between the phonic types and between the sexes. In order to determine the relative importance of variables in distinguishing between the phonic types, we used stepwise discriminant analysis. Quadratic discriminant analysis was conducted in order to establish whether individuals could be identified to the correct phonic type from external morphology and classification success was estimated by cross-validation. Canonical discriminant analysis was used for describing the relationship between the two phonic types in multivariate space. All statistical analyses were conducted using SAS for Unix v. 6.12 (SAS Institute, Inc. 1994).

Wing biopsies were taken and stored in 95% ethanol. A 402 bp portion of the mitochondrial cytochrome *b* gene was amplified and sequenced from 18 individuals representing both call types (five 131 kHz and 13 142 kHz) using the primers MVZ05 and MVZ04 (Patton & Smith 1992) for both a polymerase chain reaction and sequencing. Both strands were sequenced completely using standard dye termination protocols. Sequences were aligned by eye using the translated amino acid sequences and analysed using PAUP* 4.0b2 (Swofford 2000). Sequences are deposited in GenBank (accession numbers AF358115–AF358132).

3. RESULTS

A total of 53 individuals were captured. Twenty individuals (11 females and nine males) were identified as 131 kHz *H. bicolor* and 33 individuals (28 females and five males) were identified as 142 kHz *H. bicolor.*

Parsimony analysis with all characters unordered revealed that each call type of *H. bicolor* contained an exclusive group of mtDNA sequences. The differences within call type were minimal (zero to one nucleotide substitutions) but the differences between call types were substantial, with 26–27 nucleotide substitutions occurring between the two groups (6.5–6.8% observed sequence difference). The divergence between phonic types was consistent with species level differences in bats in general (Jones 1997) and hipposiderids in particular (A. Guillén, unpublished data). The two phonic types of *H. bicolor* are sister groups that formed a well-supported monophyletic group in a larger phylogenetic analysis of species relationships within *Hipposideros* (A. Guillén, unpublished data).

There was no evidence of sexual dimorphism in forearm length or call frequency in the 131 kHz *H. bicolor*

Table 1. *Contribution of the morphological variables to discrimination between the phonic types from stepwise and canonical discriminant analysis*

(The partial R^2 describes the variability attributable to each variable when controlling for those already in the model. The λ-values indicate the cumulative strength of the discrimination as each variable is added. The variables are presented in the order of entry into the stepwise discriminant analysis with significance level for entry = 0.15 ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Wilks' λ for forearm length $p = 0.0003$. Arm-wing area = hand-wing area + arm-wing area (Norberg & Rayner 1987).)

	stepwise discriminant analysis		canonical discriminant analysis
variable	partial R^2	Wilks' λ	standardized canonical coefficient
forearm length (m)	0.5270^{***}	0.4730	0.7978
hand-wing area (m^2)	0.0773	0.4364	0.4668
hand-wing length (m)	0.0042	0.3943	−0.1228
arm-wing area (m^2)	0.0004	0.3938	0.0358

(forearm length, $t_{18} = -1.13$ and $p = 0.2734$ and call frequency, $t_{18} = -1.24$ and $p = 0.2310$), but females were the larger sex in the 142 kHz *H. bicolor* with longer forearms (mean ± s.d., male 0.04258 ± 0.0008 m and female 0.04358 ± 0.0007 m) ($t_{31} = -2.95$ and $p = 0.0060$) (figure 2), although body mass was not dimorphic. The small sample sizes for the other variables precluded further testing. Based on the lack of sexual dimorphism in the 131 kHz *H. bicolor* and the small male sample of the 142 kHz *H. bicolor*, samples from males and females were pooled in all subsequent analyses.

The mean forearm length of the 131 kHz *H. bicolor* was significantly greater than that of the 142 kHz *H. bicolor* (0.0455 versus 0.0434 m), but substantial overlap was evident (see electronic Appendix A available on The Royal Society's Web site, and figure 2). The 142 kHz *H. bicolor* was also characterized by significantly smaller hand- and arm-wing areas resulting in a smaller overall wing area (see electronic Appendix A). Since wingspan and body mass did not vary significantly between the two phonic types, the smaller wing area in the 142 kHz *H. bicolor* resulted in a higher aspect ratio (5.72 versus 5.45) and a higher wing loading (6.46 versus 5.64 N m^{-2}). The length of the hand wing in the 142 kHz *H. bicolor* was also shorter, which, in combination with the smaller hand-wing area, describes a smaller wingtip.

Since the sample size was small, we restricted the stepwise discriminant analysis to the four measured parameters that the *t*-tests had identified as differing between the phonic types: forearm length, hand-wing area, arm-wing area and hand-wing length. Forearm length was the most useful variable for distinguishing between the phonic types and was in fact the only significant variable (table 1), although we retained all four variables for the discriminant analysis. The quadratic discriminant function analysis showed a significant difference in external morphology between the two phonic types (Wilks' $\lambda_{4,15} = 0.4344$ and $p = 0.0101$) and correctly classified 16 out of the 20 individuals. Two individuals from each phonic type were misclassified. The canonical variable that maximized the multivariate distance between the two phonic types was primarily a function of forearm length and hand-wing area (table 1). The slightly negative value for the hand-wing length, in combination with the large hand-wing area, suggests a rounded wingtip. The 131 kHz *H. bicolor* had a mean canonical variable value of 1.326, whereas that for the 142 kHz *H. bicolor* was −0.884. Thus, in both the univariate and multivariate analyses the 131 kHz *H. bicolor* had a longer forearm and a large rounded wingtip. Figure 3 illustrates the difference between the phonic types in the morphological space described by the canonical variable, with two 142 kHz *H. bicolor* and three 131 kHz *H. bicolor* in an overlap zone. Despite the morphological overlap (figures 2 and 3) between the two phonic types, there was no acoustic overlap; the two phonic types were separated by a 'silent band' of just under 5 kHz and the means differed by 11 kHz (figure 2).

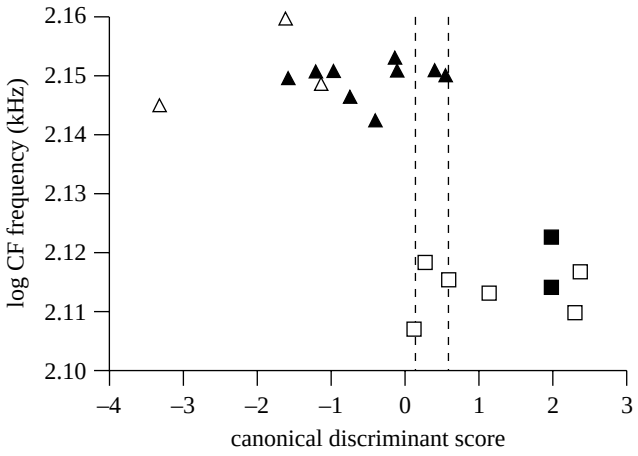

Figure 3. Relationship between log CF component of calls and canonical discriminant scores. Symbols as in figure 2.

4. DISCUSSION

The two phonic types represent distinct phylogenetic lineages that are characterized by substantial acoustic divergence but only limited morphological divergence. Although we have described significant morphological differences between the two phonic types, these were not sufficient for confident discrimination between species, as has been the case in other studies of cryptic bat species (Herd & Fenton 1983; Arlettaz *et al*. 1997; Barlow *et al*. 1997). In addition, the mean separation in call frequency was twice that predicted from morphological differences. The negative relationship between forearm length and

call frequency described for 24 species of *Hipposideros* predicts a call frequency of 123.4 kHz for the 131 kHz *H. bicolor* and 128.8 kHz for the 142 kHz *H. bicolor*, a difference of only 5 kHz (regression equation, log frequency (kHz) = 3.60 − 0.91 log forearm (mm)) ($r = -0.77$ and $p < 0.001$) (Kingston *et al.* 2000). The question therefore arises as to the functional and evolutionary significance of both the acoustic and morphological differences and to what extent these differences allow for coexistence.

We now consider the applicability of some of the hypotheses proposed by Jones (1997) and Jones & Barlow (2001) in explaining the evolution of acoustic divergence in morphologically conserved bats. As these authors pointed out, non-adaptive hypotheses cannot be discounted; genetic drift and/or founder effects in isolated populations could result in call frequency divergence. However, the acoustic specializations of flutter-detecting hipposiderids and rhinolophids add a number of intriguing elements to the discussion of adaptive hypotheses. Foremost is the dual role that call frequency plays in both resource acquisition and in intraspecific communication. In many bat families, echolocation calls have a limited role in intraspecific communication, primarily effected by information leakage or 'eavesdropping' (Fenton 1995). Bats often have a rich repertoire of communication vocalizations in addition to echolocation calls (Fenton 1985). However, in hipposiderid and rhinolophid bats, the tuning of constant frequency to the acoustic fovea necessary for flutter detection greatly constrains the production and reception of sound for communication purposes. Consequently, adult rhinolophid and hipposiderid bats emit sound consisting of a predominantly constant frequency part regardless of a communicative or an echolocation context (Möhres 1966; Matsumura 1979; Habersetzer & Marimuthu 1986) and it may be the temporal or phasic arrangement of calls that facilitates communication, as in mother–infant interactions in *Rhinolophus ferrumiquinum* (Matsumura 1981). Call frequency is therefore likely to be an essential part of the mate recognition system, raising the possibility that divergent ecological selection on call frequency could result in reproductive divergence and speciation in rhinolophoid bats. This model for speciation is plausible if resource partitioning between species is mediated by differences in echolocation call frequency (acoustic resource partitioning); thereby disruptive ecological selection on call frequency could lead to reproductive isolation.

Central to the acoustic resource partitioning hypothesis is the relationship between call frequency and the size of prey that can be detected (Barclay 1986; Barclay & Brigham 1991; Jones 1997). Calls of higher frequency are predicted to reflect more strongly from small prey items than calls of lower frequencies (Pye 1993; Houston *et al.* 2001). Therefore, individuals using higher frequencies should be more efficient at catching smaller insects and those using lower frequencies should be more efficient at taking larger insects. Under disruptive selection, where intermediates are at a competitive disadvantage, it is expected that populations will diverge in call frequency (Jones & Van Parijs 1993). However, experimental work has suggested that the 10 kHz (1.4 mm wavelength) difference in call frequency between the two cryptic species of pipistrelles (45 kHz for *Pipistrellus pipistrellus* and 55 kHz for *Pipistrellus pygmaeus*) is not sufficient to influence target strengths from the major prey types eaten (Jones & Barlow 2001). Differences in target strength are even less likely to distinguish the two phonic types of *H. bicolor* as their wavelengths differ by only 0.2 mm (2.6 mm wavelength for the 131 kHz *H. bicolor* and 2.4 mm wavelength for the 142 kHz *H. bicolor*). We might suggest that differences in echolocation frequency be related to foraging efficiency in cluttered versus uncluttered environments: bats that forage in less-cluttered microhabitats should use lower echolocation call frequencies in order to increase their detection distances. However, in opposition to this expectation, the 131 kHz *H. bicolor* has a wing morphology that suggests that it is more manoeuvrable and, therefore, more likely to forage in cluttered microhabitats than the 142 kHz *H. bicolor* (see below). In fact, it does not seem likely that the effect of atmospheric attenuation will differ sufficiently between the two phonic types to influence the functional range of the two calls; assuming the calls are produced at equal intensity, attenuation at the two frequencies will differ by less than 0.5 dB m^{-1} (25 °C and 50% relative humidity) (Lawrence & Simmons 1982). Thus, it seems unlikely that the two phonic types of *H. bicolor* use their different echolocation call frequencies for partitioning prey by size or microhabitat and this means that speciation models based on disruptive ecological selection (e.g. Rice & Hostert 1993) may not apply.

As an alternative hypothesis, we suggest that selection on call frequency for intraspecific communication may drive acoustic divergence (Heller & Von Helversen 1989; Guillén *et al.* 2000). This divergence may be initiated by the need for a clear frequency band; individuals that use call frequencies that are too similar to those of their cryptic counterparts may be at a social disadvantage, resulting in a form of 'social character displacement' when populations are sympatric. Sampling populations throughout their range in order to determine whether call frequency differences persist in allopatry might go some way toward answering this. However, caution would be warranted because acoustic divergence might persist in allopatry or even arise in sympatry as a result of social selection. Social selection, i.e. selection in which an individual's fitness is determined in part by the phenotype of its social partners (Crook 1972; West-Eberhard 1979, 1983; Wolf *et al.* 1999), is an attractive hypothesis in the present context because the matching of call frequency to the acoustic fovea in rhinolophoid bats has consequences that fulfil two requisites of social selection models. First, there is phenotypic covariation among interacting individuals. Individuals should be most receptive to communication signals from conspecifics that are closest to their own call frequency as these will fall within their acoustic fovea and elicit the greatest auditory neurological response. The selection of social partners based on shared phenotypic attributes results in phenotypic covariance among individuals (Wolf *et al.* 1999). Second, there is a developmental linkage between the signal and receptor systems. During postnatal development the tuning of the auditory fovea increases innately and the vocalization system tracks the frequency shifts by a system of auditory feedback control (Rübsamen & Shäfer 1990). The tight linkage between the signal and receptor systems means that pleiotropic

effects of changes in the genes controlling the social signal or the auditory system may result in the rapid evolution of the social communication system, even in the absence of population subdivision (Tanaka 1991, 1996). Thus, the phenotypic covariance between individuals combined with the developmental linkage of the signal and receptor systems within individuals could facilitate rapid changes in the species or mate recognition systems (Butlin & Ritchie 1989; Boake 1991) and provides ample opportunity for the evolution of both the signal and receptor systems due to sensory drive (Endler & Basolo 1998). Furthermore, since the call and receptor systems are fine-tuned during early ontogeny, small changes in the morphology of either the call or receptor organs could result in substantial shifts in call frequency. Divergence in the receptor systems of either sex could thereby drive the evolution of call frequency.

If differences in call frequency are unlikely to result in resource partitioning, are there other mechanisms by which these species may coexist? Slight differences in wing morphology can have a major impact on flight performance and, consequently, influence the microhabitat used or the type of prey that can be effectively pursued (e.g. Aldridge 1986; Saunders & Barclay 1992). The 131 kHz *H. bicolor* is characterized by a lower wing loading, lower aspect ratio and a more rounded wingtip, which are all features that enhance manoeuvrability (Norberg & Rayner 1987). Thus, it is possible that the two phonic types are separated in their microhabitat use, with the 131 kHz *H. bicolor* foraging in more densely cluttered situations than 142 kHz *H. bicolor*, although, as noted above, call frequency is not correlated with wing morphology. Alternatively, the more manoeuvrable 131 kHz *H. bicolor* may be more efficient at capturing prey that have unpredictable flight paths. Both possibilities could lead to interspecific differences in diet. Thus, analysis of diet and habitat use for the two phonic types would clearly be a profitable focus for future studies.

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