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Identification of cryptic species of *Miniopterus* bats (Chiroptera: Miniopteridae) from Madagascar and the Comoros using bioacoustics overlaid on molecular genetic and morphological characters

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The number of *Miniopterus* bat species on Madagascar and the nearby Comoros islands (Malagasy region) has risen from four to 11. These recently described cryptic taxa have been differentiated primarily based on molecular markers and associated a posteriori morphological characters that corroborate the different clades. Members of this Old World genus are notably conservative in morphology across their range. Several sites on Madagascar hold up to four small-bodied taxa of this genus that are morphologically similar to one another, although they can be distinguished based on the tragus, an ear structure associated with echolocation. *Miniopterus* often emit species-specific calls. In the present study, we analyze the bioacoustics of the 11 species of *Miniopterus* currently recognized from the Malagasy region, with an initial identification of the 87 recorded and collected individuals based on molecular markers and certain morphological characters. In most cases, bioacoustic parameters differentiate species and have taxonomic utility. *Miniopterus griveaudi* populations, which occur on three islands (Madagascar, Anjouan, and Grande Comore), showed no significant differences in peak echolocation frequencies. After running a discriminant function analysis based on five bioacoustic parameters, some mismatched assignments of Malagasy species were found, which include allopatric sister-taxa and sympatric, phylogenetically not closely-related species of similar body size. Because the peak echolocation frequencies of two species (*Miniopterus sororculus* and *Miniopterus aelleni*) were independent of body size, they were acoustically distinguishable from cryptic sympatric congeners. The small variation around the allometric relationship between body size and peak echolocation frequency of Malagasy *Miniopterus* species suggests that intraspecific communication rather than competition or prey detection may be the driver for the acoustic divergence of these two species. Our well-defined echolocation data allow detailed ecological work to commence aiming to test predictions about the relative roles of competition, prey availability, and social communication on the evolution of echolocation in Malagasy *Miniopterus* species. *Biological Journal of the Linnean Society*, 2011, **104**, 284–302.

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INTRODUCTION

In recent years, different types of characters have been used to identify cryptic species of metazoan animals, ranging from reproductive and copulatory gland morphology (Simó, Seguí & Pérez-Miles, 2002), discrete karyological variation (Nakayama *et al*., 2001; Milhomem *et al*., 2008), communication signals used to attract mates (Narins, 1993), subtle differences in morphology (Klimov *et al*., 2004; Sztencel-Jabłonka, Jones & Bogdanowicz, 2009), and, with greater frequency, molecular markers to measure sequence divergence (Bickford *et al*., 2006; Blanquer & Uriz, 2008; Pagès *et al*., 2009). These studies have been important for elucidating previously unrecognized aspects of our planet's biological diversity and of the evolutionary history of the organisms concerned. In turn, such work also indicates that classical museum studies, based exclusively on morphology, are often unable to characterize and differentiate cryptic species.

The importance of non-morphological characters for providing insight into cladogenesis and speciation can be found amongst bats, particularly insectivorous species that use ultrasound for hunting and communication, as well as nonvisual aspects of mate choice and breeding. For such animals, differences in external morphology may be less important in species recognition compared to acoustic signalling, such as echolocation. A considerable number of subtly morphologically recognizable species have been identified and described, largely based on molecular sequence divergence (Goodman *et al*., 2007; Mayer, Dietz & Kiefer, 2007). In several cases, fixed bioacoustic distinctions between these hidden species have been recognized (Russo & Jones, 2000; Thabah *et al*., 2006), as well as fixed morphological differences in the external ear and structures associated with echolocation (Gannon *et al*., 2001). Within bats, differences in communication, species recognition, and hunting echolocation frequencies have little to do with gross external morphology. An excellent example is the widespread Old World genus *Miniopterus* Bonaparte, 1837 (Miniopteridae), which, across their range, contain numerous species complexes that are now known, based on genetic markers, to be paraphyletic using a phylogenetic species concept and these cryptic species demonstrate remarkable levels of morphological convergence (Miller-Butterworth *et al*., 2005; Juste *et al*., 2007; Goodman *et al*., 2009a). Members of this genus are known to have species-specific echolocation calls (Russ *et al*., 2003; Miller-Butterworth *et al*., 2005).

Recent molecular research based on a phylogenetic species concept, as employed in the present study, has disclosed previously unappreciated levels of species diversity within the *Miniopterus* bats of Madagascar. In a review monograph on the bats of Madagascar, Peterson, Eger & Mitchell (1995) recognized four species on the island based on pelage and craniodental characters. These included two endemics (*Miniopterus gleni* and *Miniopterus manavi*) and two species with broader distributions extending into sub-Saharan Africa or the nearby Comoro archipelago (*Miniopterus fraterculus* and *Miniopterus majori*). Based on recent molecular studies, 11 species of *Miniopterus* are currently recognized from Madagascar, nine of which are endemic and two are shared with the Comoros (= Malagasy region) (Goodman, 2011; Goodman *et al*., 2011). In several cases, the molecular data have provided the means to sort specimens in an a posteriori fashion to uncover fixed morphological characters that corroborate the genetic clades, such as size, pelage coloration, and, most importantly, the tragus (i.e. external portion of the ear), which is associated with echolocation (Lawrence & Simmons, 1982; Gannon *et al*., 2001).

For echolocating organisms, such as bats or even certain birds, bioacoustic information has been widely used to provide additional insight into taxonomic delimitations (Russo & Jones, 2000; Rydell *et al*., 2002; Kingston & Rossiter, 2004; Jacobs *et al*., 2006; Thomassen & Povel, 2006). Hence, bioacoustics can provide additional characters to support the delimitation of diagnosable differences between cryptic species and help provide coherent diagnoses in accordance with the International Code of Zoological Nomenclature. Furthermore, in many areas of the world, catalogues of the echolocation calls of local bats, made with bat detectors, provide a direct means for species-specific identifications, without the animal in hand. This has comprised an extraordinary tool for conducting bat surveys and providing insight into the ecology of these organisms (McCracken *et al*., 1997; Ochoa, O'Farrell & Miller, 2000; Russo & Jones, 2002, 2003), although, for aspects of the limitations of this technique, see Barclay (1999).

Several karst areas of Madagascar have multiple sympatric *Miniopterus* spp. of similar body size, living in and around the same cave systems (Goodman *et al*., 2009a, b). Furthermore, two species of the genus are shared in common between the Comoros and Madagascar, and these populations are separated by a minimum distance of 370 km of open sea. These different cases provide natural experiments for testing predictions on the relative roles of competition, adaptations to contrasting ecological factors, sexual selection or drift on the evolution of echolocation parameters (Guillén, Juste & Ibánez, 2000; Jones & Barlow, 2004). For example, a central albeit controversial tenet of community ecology proposes that, if resources are limiting, there is a limit to how similar

species can be and coexist (Hutchinson, 1959; for critiques on the idea of limiting similarity, see Roughgarden, 1979; Maynard Smith & Szathmáry, 1995), and hence predicts that sympatric species with similar body size and morphology can only coexist if they occupy different niches, mediated, for example, by different echolocation parameters (Siemers & Schnitzler, 2004).

The present study aimed to document the bioacoustics of 11 *Miniopterus* species in the Malagasy region based on the unambiguous identification of recorded and vouchered animals by molecular genetics and morphological characters. Previously assembled catalogues of the echolocation calls for *Miniopterus* spp. in the region came from now out-of-date species delimitations (Russ *et al*., 2003; Kofoky *et al*., 2009) or nonvouchered individuals, and ambiguity exists in the echolocation calls of regional members of this genus. We investigate two different aspects in this respect. (1) We examine intraspecific variation in a broadly distributed species, *Miniopterus griveaudi*, occurring on Madagascar and in the Comoros, to establish levels of geographical variation in echolocation calls. (2) We document and contrast the vocalizations of the 11 members of this genus known from Madagascar using a discriminant function analysis (DFA) of five well-known echolocation parameters and a regression of peak echolocation frequency against body size to investigate patterns of allopatric sister-taxa and sympatric cryptic species that are morphologically similar and not phylogenetically closely-related.

MATERIAL AND METHODS

Field sites, capture, and associated collections

In 2009 and 2010, 13 sites were visited in several habitats on Madagascar and three sites in the nearby Comoros Archipelago; specifically, the islands of Grande Comore and Anjouan (Fig. 1). In most cases, sites chosen were based on previous bat surveys that yielded members of the genus *Miniopterus*, specimens of which had been previously used in systematic revisions of this genus.

We used mist nets and harp traps to capture *Miniopterus* bats. At some sites, animals were removed by hand or with a long-handled butterfly net from cave day-roost sites. Soon after being captured, individual bats were recorded under constrained, free-flying conditions (see below). Animals were handled in accordance with the guidelines of American Society of Mammalogists (Sikes, Gannon & The Animal Care and Use Committee of the American Society of Mammalogists, 2011). Bats were then recaptured and prepared as voucher specimens, which were deposited in the Université d'Antananarivo, Département de Biologie Animale (UADBA), Antananarivo and the Field Museum of Natural History (FMNH), Chicago. Details on the specimens and localities are presented in the Supporting information (Appendix S1).

Recordings

We recorded only adult *Miniopterus* bats, which were distinguished from juveniles based on patterns of ossification of their finger bones (Anthony, 1988), when they were either flying in a flight cage or along a zip-line. These techniques ensured that, after recording, bats could be recaptured to serve as voucher specimens. Furthermore, these two methods have been shown to provide good quality recordings for clutter-edge and clutter bats (Szewczak, 2000, 2004; Siemers, 2004). A previous study of Malagasy bat bioacoustics showed little variation in the call characteristics of *Miniopterus* bats recorded using flight cages, zip-lines, and after hand release (Kofoky *et al*., 2009).

Individuals were released into a flight cage completely enclosed with thin cloth-mesh cage, measuring $3 \times 3 \times 3$ m. Search phase echolocation calls were recorded at ×10 time expansion with a Pettersson bat detector (D-240X; Pettersson Elektronik AB) connected to a minidisk recorder (Sony Net MD Walkman MZ-N505) and stored onto minidiscs for subsequent analysis. The exception was *Miniopterus egeri*, which were released in a flight cage, measuring $1.8 \times 1.4 \times 5.4$ m, and their echolocation calls were recorded directly onto an ASUS EEE 1005HA netbook (ASUSTek Computer Inc.) with an Avisoft UltraSound Gate 116 bat detector (Avisoft Bioacoustics). For the zip-line recordings (Szewczak, 2000), one end of a thin 1-m long elastic filament was carefully wrapped around the bat's neck and the other to a sliding fishing tackle snap swivel attached to a 25-m long taught fishing line suspended between two poles, approximately 1.5 m off the ground. Bats were recorded as they flew along the line. Individuals of rarely-captured species were only flown inside the flight cages to reduce risk of escape.

The recorded wave files were analyzed in BATSOUND PRO, version 3.2 (Pettersson Elektronik AB) at a sampling rate of 44.1 kHz (16 bits, mono) for Pettersson recordings, and 500 kHz (16 bits, mono) for Avisoft recordings, with a threshold of 16. To avoid pseudoreplication (Hurlbert, 1984), one search phase sequence per individual was analyzed and the choice was based on a high signal to noise ratio (Weller *et al*., 2007). In accordance with methods previously used to analyse echolocation calls of Malagasy bats (Russ *et al*., 2003; Kofoky *et al*., 2009; Goodman *et al*.,

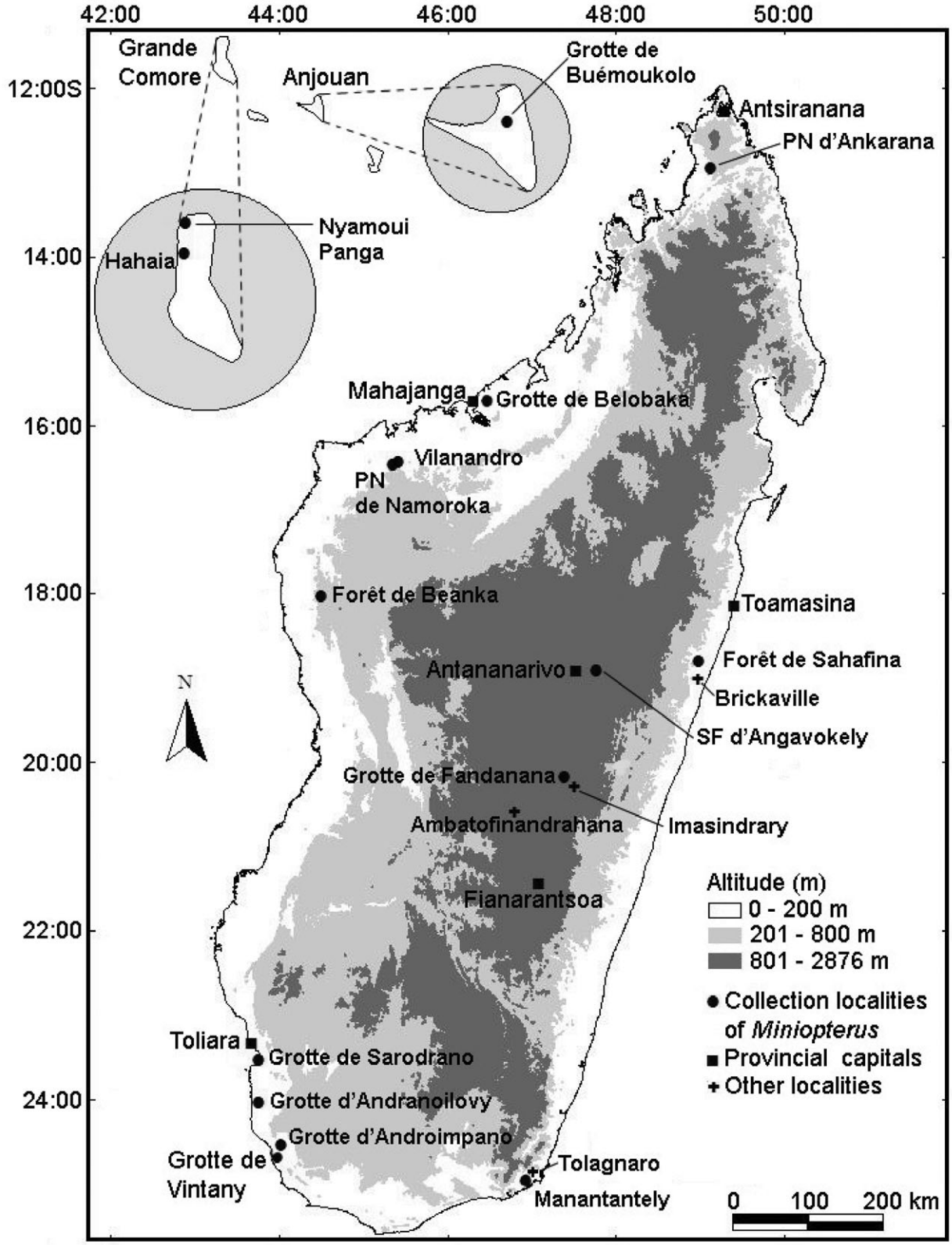

Figure 1. Map showing study sites on Madagascar and on Anjouan and Grande Comoro (Comoros Archipelago), where *Miniopterus* spp. were recorded and collected for the present study, as well as other localities mentioned in the text. Enlargements of Anjouan and Grande Comoro are presented to show the collection sites more clearly.

2011), the peak echolocation frequency in kHz (PF) was extracted from the power spectrum, and the maximum frequency in kHz (F_{max}), the minimum frequency in kHz (F_{min}), the duration in ms (Dur), and the interval between successive pulses in ms (IPI) were measured directly from the spectrogram. In several cases, we revisited type localities to record topotypic echolocation calls of *Miniopterus* spp. For *M. egeri*, recordings were from animals forming part of the original type series.

Molecular genetic analysis

The molecular analysis aimed primarily to establish the specific identity of the recorded individuals based on genetic markers used in recent phylogenetic work on Malagasy region *Miniopterus*. The entire mitochondrial cytochrome *b* gene was chosen as a result of its widespread use in similar studies (Cardinal & Christidis, 2000; Miller-Butterworth *et al*., 2005; Goodman *et al*., 2007, 2010a; Furman, Öztunc & Çoraman, 2010a). *Miniopterus fraterculus* from South Africa was included as the outgroup. After a given individual was recorded, the animal was collected as a voucher specimen for morphological comparisons (see below), and a muscle tissue sample was saved in lysis buffer for the molecular genetic work. Additional tissue samples were made available by the National Museum of Natural History, Smithsonian Institution, Washington, DC (formerly known as the United States National Museum). For all 11 species of Malagasy region *Miniopterus*, sequence data are available for animals used in the original description or at least topotypic or near topotypic material to positively confirm the specific clade identity of recorded animals. Specimens used in the genetic analysis and the locations of their capture are provided in the Supporting information (Appendix S1).

Genomic DNA was extracted using a lithium chloride and chloroform extraction method, *sensu* Gemmel & Akiyama (1996). The cytochrome *b* gene was amplified and sequenced using the primers L14724, H15506, L15171, and H15915 (Smith & Patton, 1991). All polymerase chain reaction and sequencing protocols were conducted *sensu* Goodman *et al*. (2011) All new sequences were deposited in GenBank (accession numbers JF440219–JF440287; see also the Supporting information, Appendix S1).

Sequence analysis

JMODELTEST (Guindon & Gascuel, 2003; Posada, 2008) was used to determine the most appropriate model of molecular evolution. The model, HKY+G, was estimated from the Bayesian information criterion. JMODELTEST estimated parameter settings with base frequencies = 0.3031, 0.2916, 0.1398, 0.2655, and –lnL = 5822.0042, and shape parameter of gamma distribution = 0.1700.

Maximum parsimony (MP) and minimum evolution (Neighbour-joining, NJ) phylogenetic analyses were conducted using PAUP* 4.0 (Swofford, 2003). Heuristic MP searches were conducted using the random addition option and the tree bisection–reconnection branch-swapping algorithm. The NJ method used pairwise sequence distances estimated by the HKY+G model, as reported in the Results. Nodal support of MP and NJ trees was estimated by 1000 bootstrap pseudoreplicates. Maximum likelihood analysis was conducted using the RAxML Blackbox (Stamatakis, Hoover & Rougemont, 2008) online interface (http://phylobench.vital-it.ch/raxml-bb/index.php) with 100 bootstrap replicates.

Bayesian analysis was conducted using MRBAYES, version 3.1.2 (Ronquist & Huelsenbeck, 2003). The HKY+G model was specified, flat priors were used, and starting trees were random. We ran four chains (three hot and one cold) for 2 000 000 generations, sampling trees every 100 generations. We ensured that our Bayesian runs achieved sufficient convergence by establishing that the average SD of split frequencies between chains had reached below 0.01 (0.008528) at the end of the run and the potential scale reduction factor (PSRF) of each parameter was within $1.000 < PSRF < 1.022$. Plots of generation versus the log probabilities of observing actual data did not reveal any trends for the last 75% of generations. We excluded the first 25% (5 000) of generations from the calculation of posterior probabilities.

Morphological comparisons

In recent taxonomic revisions of Malagasy region *Miniopterus*, a number of characters that differentiate species have been delineated. These include aspects of size (largely forearm length), pelage coloration, and, most importantly, the form and shape of the tragus (Goodman *et al*., 2007, 2008, 2009a, b, 2010a, 2011). We have used these characters, particularly the tragus, in the specific identification of the recorded and collected individuals used in the present study. To allow segregation of the different species into different size classes, they were divided into three separate groups based on mean forearm length: small body < 38.5 mm, medium bodied between 41 and 45.9 mm, and large body > 46 mm.

Statistical analysis

All five bioacoustic variables (PF, F_{max}, F_{min}, Dur, and IPI) were used in the analyses. Preliminary *t*-tests or Wilcoxon tests of echolocation parameters detected limited sexual dimorphism in *Miniopterus* spp.

Hence, we combined the data for analysis. After testing the normality of the data with Kolmogorov–Smirnov tests (Dytham, 2003), we used analysis of variance (ANOVA) tests and post-hoc Tukey's tests to identify differences in PF, F_{max}, Dur and IPI of *M. griveaudi* populations among the three islands (Madagascar, Anjouan, and Grande Comore). Because F_{min} data were not normally distributed, a Kruskal–Wallis test with post-hoc Wilcoxon rank sum tests was used.

To determine which echolocation parameters were most useful to identify species (Digby & Kempton, 1987), we used a discriminant function analysis (DFA) in SPSS, version 10.0.01 (SPSS Inc, 1999) on the five bioacoustic variables, with parameter 'all groups equal' within the prior probabilities to avoid the problem of unequal sample sizes, and 'within-groups' for the covariance matrix. The classification was based on species designations from the molecular genetic analysis and morphological comparisons. DFA has been widely used as a tool for classifying species based on predictive variables. In recent years, this method has been used to classify and identify bats based on different bioacoustic parameters (Russo & Jones, 2002; Fenton *et al*., 2004; Papadatou, Butlin & Altringham, 2008; Kofoky *et al*., 2009). The technique is particularly useful for assessing cases of mismatched designations.

Because there is typically a strong correlation between peak echolocation frequency and body size in insectivorous bats, species that deviate from this allometric relationship are important for investigating the influence of competition, diet, and social communication on the evolution of echolocation. Using COMPARE, version 4.2b; Martins, 2004), a phylogenetic general least squares regression (Martins & Hansen, 1997) of log-transformed mean forearm versus mean PF was utilized to ensure that species were statistically independent. The 95% confidence limits of the allometric relationships were used as the criterion for determining whether the PF of any given species deviated from its expected linear relationship with forearm length.

RESULTS

In total, 87 individuals, representing all 11 currently recognized species of *Miniopterus* spp. from the Malagasy region, were captured during the course of this study and their vocalizations recorded using the flight cage and zip-line methods (Table 1). These different taxa were divided into three different body size classes based on the mean length of the forearm (Table 1). All of these individuals were used in the morphological comparisons and 72 of them in the molecular analysis (see Supporting information,

Table 1. List of the different *Miniopterus* spp. employed in the bioacoustical portion of the present study, their body size based on mean forearm length (FA), and the methods used to make acoustic recordings

Species	Number of individuals recorded	Recording method	
		Zip-line	Flight cage
Miniopterus aelleni (FA = 38.3 mm, SB)	7	5	2
Miniopterus brachytragos (FA = 36.6 mm, SB)	8	4	4
Miniopterus gleni (FA = 48.4 mm, LB)	6	3	3
Miniopterus griffithsi (FA = 48.8 mm, LB)	2	0	2
Miniopterus griveaudi (Madagascar) (FA = 36.9 mm, SB)	17	6	11
Miniopterus griveaudi (Anjouan) (FA = 36.8 mm, SB)	8	2	6
Miniopterus griveaudi (Grande Comore) (FA = 36.3 mm, SB)	6	0	6
Miniopterus mahafaliensis (FA = 37.4 mm, SB)	9	0	9
Miniopterus majori (FA = 45.4 mm, MB)	12	6	6
Miniopterus manavi (FA = 38.5 mm, SB)	2*	0	2
Miniopterus petersoni (FA = 39.8 mm, MB)	1	0	1
Miniopterus sororculus (FA = 43.5 mm, MB)	7	1	6
Miniopterus egeri (FA = 38.5 mm, SB)	2	0	2
Total	87	27	60

After the scientific name, the mean FA length and the designation of the taxon as small-bodied (SB), medium-bodied (MB), and large-bodied (LB) are presented. These data are based on Goodman (2011) and Goodman *et al*. (2010b, 2011). The standard flight cage of $3 \times 3 \times 3$ m was used, with the exception of *M. egeri*, which measured $1.8 \times 1.4 \times 5.4$ m.
*Based on the genetic data, the two recorded individuals (FMNH 209178 and 209179) represent an unnamed species and are not referable to *M. manavi*.

Appendix S1). Individuals of *M. griveaudi* were recorded from Madagascar, Grande Comore, and Anjouan. These data were used to establish levels of intraspecific geographical variation in bioacoustic parameters. However, the small sample size for certain taxa did not allow for a detailed analysis of variation for all species known from Madagascar.

Genetic analysis

The phylogenetic tree (Fig. 2) shows strong support for clades using four different analyses: minimum evolution, maximum parsimony, maximum likelihood, and Bayesian approaches. All tree-building methods constructed the same tree topology. However, some internal branches in the minimum evolution and maximum parsimony trees (not shown) collapsed with bootstrapping, but remained robust using maximum likelihood and Bayesian methods. For most of the clades recovered in these analyses, sequences from previous analyses were also used (Goodman *et al*., 2007, 2008, 2009a, b, 2010a, 2011), which included holotypic or topotypic material, and these provided further assurance that the associated binomials for animals used in the bioacoustic study were correct.

Congruency of data sets

The identification of separate taxonomic units based on the bioacoustic and genetic data sets were congruent and when overlaid on the morphological characters, particularly tragus shape, provided consistent species identifications. The only exception was the cluster of closely-related clades including *M. manavi* and *M. petersoni*. The morphological aspects of body size and tragus shape, as well as recordings for specimens FMNH 209178 and 209179 were attributed to *M.* '*manavi*', whereas the genetic distance separating the *M. manavi* clade, based on other specimens, and the one formed by these two animals ('unnamed' in Fig. 2) is slightly less than 4% (Table 2). However, based on previous studies describing the morphological differences between *M. petersoni* and *M. manavi* (Goodman *et al*., 2008) and a subsequent study describing *M. egeri* (Goodman *et al*., 2011), it appears that the samples comprising the 'unnamed' clade form a previously unrecognized and equally divergent group. Within-clade divergences also exist, which closely approximate that seen within the *manavi/petersoni/egeri* clade. These variations have not yet been investigated to determine the taxonomic standing of the divergences compared to other recognized taxa.

Table 2. Genetic distances within and between all major clades of *Miniopterus* spp. represented in the phylogenetic tree (Fig. 2)

	aelleni	*brachytragos*	*gleni*	*griffithsi*	*griveaudi*	*mahafaliensis*	*majori*	Unnamed	*manavi*	*petersoni*	*sororculus*	*egeri*	*fraterculus*
aelleni	0.0115	0.0694	0.1008	0.1125	0.0996	0.1033	0.1012	0.0912	0.0889	0.091	0.0908	0.0831	0.1138
brachytragos		**0.0046**	0.1005	0.1084	0.1026	0.1051	0.1065	0.0836	0.082	0.0826	0.1114	0.0888	0.1253
gleni			**0.0075**	0.0749	0.1035	0.1146	0.0846	0.1001	0.0913	0.0892	0.1092	0.1083	0.1129
griffithsi				**0.009**	0.1193	0.1179	0.089	0.1143	0.1046	0.1076	0.1077	0.1139	0.1156
griveaudi					**0.0087**	0.1155	0.1135	0.1128	0.1088	0.1078	0.1082	0.1149	0.1066
mahafaliensis						**0.0148**	0.1213	0.1018	0.1055	0.1045	0.1089	0.1075	0.1447
majori							**0.0105**	0.0984	0.0939	0.101	0.1097	0.1008	0.116
Unnamed								**0.0337**	0.03799	0.04172	0.11562	0.04753	0.13203
manavi									**0.0054**	0.023	0.1119	0.0432	0.1175
petersoni										**0.0046**	0.1113	0.0413	0.1203
sororculus											**0.004**	0.1152	0.1212
egeri												**0.0126**	0.1317
fraterculus													**0**

Bold values on a diagonal indicate the within species HKY85 distances, whereas the values above the diagonal represent the mean HKY85 distances between species. Intraspecific comparison for the single extralimital taxon (*fraterculus*) is not shown.

ECHOLOCATION CALLS AND TYPE LOCALITY OF *MINIOPTERUS* SPP. FROM MADAGASCAR AND THE COMOROS

Miniopterus species from Madagascar and the Comoros emitted low-duty-cycle frequency modulated echolocation calls. PF calls were in the range 40.1–62.4 kHz, F_{max} was in the range 61.0–130.0 kHz, F_{min} was in the range 36.0–58.0 kHz, IPI was in the range 40.0–134.8 ms, and Dur was in the range 2.1–5.0 ms (Fig. 3, Table 3). In the present study, we took the forearm length as a predictor of bat size. The PF was inversely correlated with size ($r = 0.921$, $F_{1,11} = 61.87$, $P = 0.000008$; Fig. 4). Only *Miniopterus aelleni* and *Miniopterus sororculus* fell outside the 95% confidence limits. The call characteristics of low-duty-cycle bats, including miniopterids, may change in different habitats (Barclay, 1999; Schnitzler & Kalko, 2001); hence, it is possible that the echolocation parameters may be different in open habitats.

Large-size Miniopterus

Miniopterus gleni Peterson, Eger & Mitchell, 1995

The type locality of this widespread species is the Grotte de Sarodrano to the south of Toliara, from where we recorded the vocalizations of three individuals, as well as an additional three individuals from the Forêt de Beanka.

Miniopterus griffithsi Goodman, Maminirina, Bradman, Christidis & Appleton, 2010

Two individuals of this species were captured in the extreme southwest: one at the type locality of Grotte d'Androimpano and the other at the nearby Grotte de Vitany. In Figure 2, we also include sequence data from two paratypes associated with the description of this species (Goodman *et al*., 2010a).

Medium-sized Miniopterus

Miniopterus majori Thomas, 1906

Thirteen individuals of this species were recorded from the Grotte de Fandanana, in close proximity to the type locality of Imasindrary near Fandriana (Thomas, 1906; Jenkins & Carleton, 2005), as well as the Station Forestière d'Angavokely.

Miniopterus petersoni Goodman *et al*., 2008

The single individual of this species was captured and recorded at the type locality of Manantantely, near Tolagnaro. Additional samples are used in the molecular analysis (Fig. 2) associated with the description of this species (Goodman *et al*., 2008).

Miniopterus sororculus Goodman *et al*., 2007

Seven individuals of this species were obtained from the Grotte de Fandanana and the Station Forestière d'Angavokely, which are 75 km and 210 km, respectively, from the type locality of Ambatofinandrahana (Goodman *et al*., 2007). One animal from the original type series locality was used in the molecular analysis (Fig. 2).

Small-bodied Miniopterus

Miniopterus aelleni Goodman, Maminirina, Weyeneth, Bradman, Christidis, Ruedi & Appleton, 2009

The holotype of this species was obtained in the Parc National d'Ankarana (Goodman *et al*., 2009b). In the present study, four individuals were recorded from Ankarana and three individuals from the Forêt de Beanka. One of the paratypes associated with the description of this species was used in the molecular analysis (Fig. 2).

Miniopterus brachytragos Goodman, Maminirina, Bradman, Christidis & Appleton, 2009

The holotype of this species was collected in the Parc National de Namoroka, in the general vicinity of Ambovonomby Cave and near Vilanandro (Goodman *et al*., 2009b). We used eight animals obtained at this site in the current study. Sequence data from the holotype and paratype were included in the molecular analysis (Fig. 2).

Miniopterus griveaudi Harrison, 1959

The type series of this species comes from Grande Comore in an area along the western flank of Mont Karthala (Harrison, 1959; Goodman *et al*., 2010b). Animals captured on the western side of Mont Karthala, as well different sites on Anjouan and Madagascar, were recorded, and near topotypic sequence data was included in the molecular analysis (Fig. 2).

Miniopterus mahafaliensis Goodman, Maminirina, Bradman, Christidis & Appleton, 2009

This species was described based on the type series from the Grotte d'Andranoilovy in the Parc National de Tsimanampetsotsa (Goodman *et al*., 2009b). Three animals from the type locality and six individuals slightly further south at the Grotte d'Androimpano were recorded. Topotypic material was used in the molecular analysis (Fig. 2).

Miniopterus manavi Thomas, 1906

The holotype of this species was obtained in the Fandriana region in the Central Highlands (Thomas, 1906). The two individuals from Grotte de Fandanana (FMNH 209178 & 209179), in close proximity to the type locality, and attributed in the bioacoustic analysis to *M. manavi*, are genetically divergent ('unnamed' clade in Fig. 2) from another two individuals from the same site identified as *M. manavi* based on a separate molecular analysis (Goodman *et al*.,

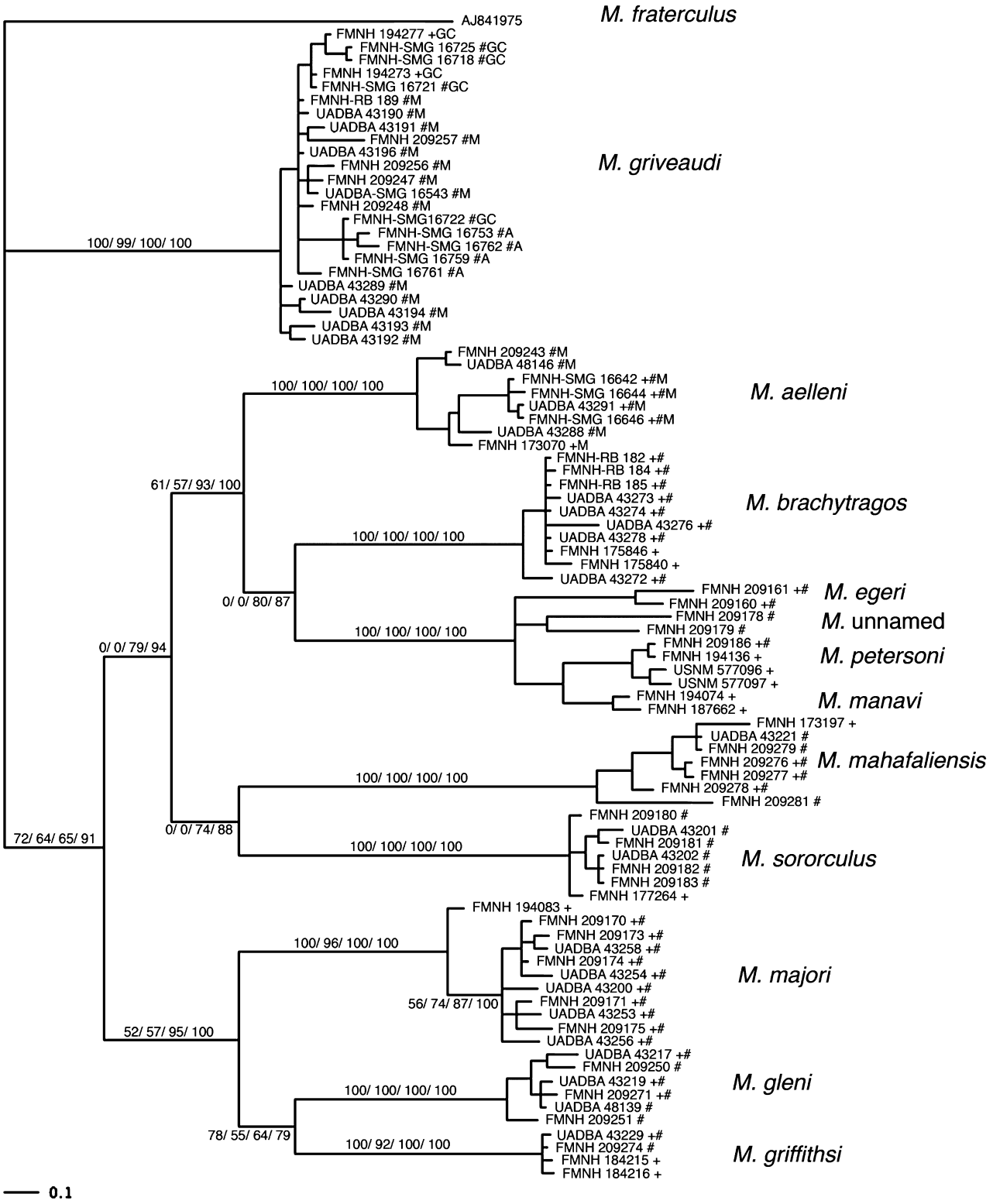
M. fraterculus
AJ841975
FMNH 194277 +GC
FMNH-SMG 16725 #GC
FMNH-SMG 16718 #GC
FMNH 194273 +GC
FMNH-SMG 16721 #GC
FMNH-RB 189 #M
UADBA 43190 #M
UADBA 43191 #M
FMNH 209257 #M
UADBA 43196 #M
M. griveaudi
FMNH 209256 #M
FMNH 209247 #M
UADBA-SMG 16543 #M
FMNH 209248 #M
FMNH-SMG16722 #GC
FMNH-SMG 16753 #A
FMNH-SMG 16762 #A
FMNH-SMG 16759 #A
FMNH-SMG 16761 #A
UADBA 43289 #M
UADBA 43290 #M
UADBA 43194 #M
UADBA 43193 #M
UADBA 43192 #M
100/ 99/ 100/ 100
FMNH 209243 #M
UADBA 48146 #M
FMNH-SMG 16642 +#M
FMNH-SMG 16644 +#M
UADBA 43291 +#M
FMNH-SMG 16646 +#M
UADBA 43288 #M
FMNH 173070 +M
100/ 100/ 100/ 100
M. aelleni
FMNH-RB 182 +#
FMNH-RB 184 +#
FMNH-RB 185 +#
UADBA 43273 +#
UADBA 43274 +#
UADBA 43276 +#
UADBA 43278 +#
FMNH 175846 +
FMNH 175840 +
UADBA 43272 +#
61/ 57/ 93/ 100
M. brachytragos
100/ 100/ 100/ 100
FMNH 209161 +#
FMNH 209160 +#
FMNH 209178 #
FMNH 209179 #
FMNH 209186 +#
FMNH 194136 +
USNM 577096 +
USNM 577097 +
FMNH 194074 +
FMNH 187662 +
0/ 0/ 80/ 87
M. egeri
M. unnamed
M. petersoni
M. manavi
100/ 100/ 100/ 100
0/ 0/ 79/ 94
FMNH 173197 +
UADBA 43221 #
FMNH 209279 #
FMNH 209276 +#
FMNH 209277 +#
FMNH 209278 +#
FMNH 209281 #
M. mahafaliensis
100/ 100/ 100/ 100
FMNH 209180 #
UADBA 43201 #
FMNH 209181 #
UADBA 43202 #
FMNH 209182 #
FMNH 209183 #
FMNH 177264 +
0/ 0/ 74/ 88
M. sororculus
100/ 100/ 100/ 100
72/ 64/ 65/ 91
FMNH 194083 +
FMNH 209170 +#
FMNH 209173 +#
UADBA 43258 +#
FMNH 209174 +#
UADBA 43254 +#
UADBA 43200 +#
FMNH 209171 +#
UADBA 43253 +#
FMNH 209175 +#
UADBA 43256 +#
100/ 96/ 100/ 100
56/ 74/ 87/ 100
M. majori
52/ 57/ 95/ 100
UADBA 43217 +#
FMNH 209250 #
UADBA 43219 +#
FMNH 209271 +#
UADBA 48139 #
FMNH 209251 #
100/ 100/ 100/ 100
M. gleni
UADBA 43229 +#
FMNH 209274 #
FMNH 184215 +
FMNH 184216 +
78/ 55/ 64/ 79
100/ 92/ 100/ 100
M. griffithsi
0.1

Figure 2. Phylogenetic position based on the mitochondrial cytochrome *b* gene of individual *Miniopterus* spp. used in the construction of the bioacoustic dictionary (designated with #), as well as other nonrecorded animals associated with the clade designation (type material or topotypic animals) (designated with +). In a few cases, individuals were both recorded and from sites associated with the original species description (designated + #). For *Miniopterus griveaudi* and *Miniopterus aelleni*, the island the specimen was obtained is also noted (M, Madagascar; A, Anjouan; GC, Grande Comore). The outgroup is the African species *Miniopterus fraterculus*. The tree presented was produced using Bayesian analysis. Neighbour-joining, maximum parsimony bootstrap, maximum likelihood, and Bayesian posterior probabilities are indicated on the major nodes (NJ/PARS/ML/BAYES). Labels include museum catalogue number and species identification is included to the right of each clade. In a few cases, the specimens have not been catalogued and the field number is given.

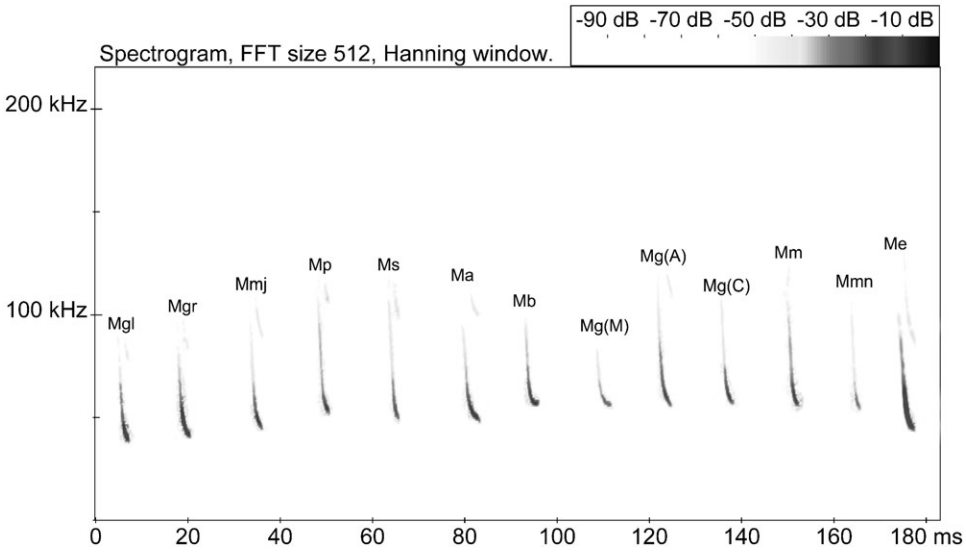

Figure 3. Summary of the echolocation calls of *Miniopterus* spp. from Madagascar and the Comoros. Ma, *Miniopterus aelleni*; Mb, *Miniopterus brachytragos*; Me, *Miniopterus egeri*; Mg(A), *Miniopterus griveaudi* from Anjouan; Mg(C), *Miniopterus griveaudi* from Grande Comore; Mgl, *Miniopterus gleni*; Mg(M), *Miniopterus griveaudi* from Madagascar; Mgr, *Miniopterus griffithsi*; Mm, *Miniopterus mahafaliensis*; Mmj, *Miniopterus majori*; Ms, *Miniopterus sororculus*; Mp, *Miniopterus petersoni*; Mmn, *Miniopterus* '*manavi*'.

2009a). Further research is needed to resolve the relationship between the 'unnamed' and *M. manavi* clades.

Miniopterus egeri Goodman *et al.*, 2011

The type locality of this species is the Forêt de Sahafina near Brickaville (Goodman *et al.*, 2011). Two individuals making up part of the original type series were recorded and used in the molecular analysis.

Intraspecific variation in *M. griveaudi* from Madagascar and the Comoros

On Madagascar, we captured, recorded inside a flight cage, and collected *M. griveaudi* at three different localities: Belobaka, Beanka and Namoroka (Fig. 1). Because only one individual was recorded at Namoroka, we were unable to statistically test for differences in call parameters among the three

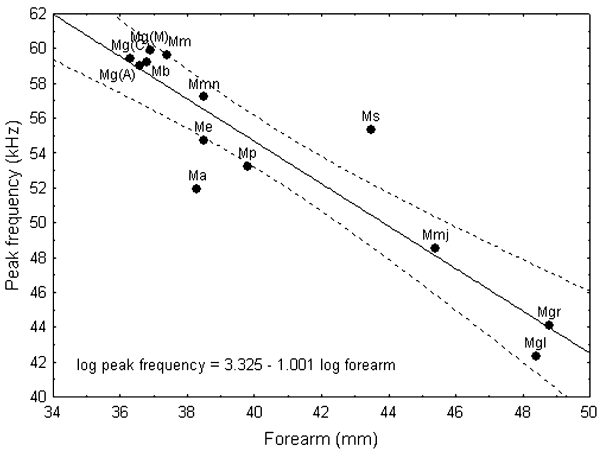

Figure 4. Regression plot of log-transformed mean forearm lengths against mean PF for 11 Malagasy region *Miniopterus* species. Data derived from Tables 1 and 3. For definitions of acronyms, see Fig. 3.

Table 3. Measurements of different bioacoustic parameters of *Miniopterus* spp. from Madagascar, Grande Comore and Anjouan

Species	*n/N*	PF (kHz)	F_{max} (kHz)	F_{min} (kHz)	Dur (ms)	IPI (ms)
Miniopterus aelleni	7/19	51.9 ± 1.57	99.8 ± 8.48	48.0 ± 1.43	4.1 ± 0.65	87.3 ± 16.75
		49.5–54.8	75.0–119.0	46.0–50.0	3.0–5.0	61.0–118.5
Miniopterus brachytragos	8/30	59.0 ± 1.06	105.8 ± 11.20	55.7 ± 0.84	3.4 ± 0.49	84.6 ± 18.99
		57.3–61.7	85.0–128.0	54.0–57.0	2.6–4.3	56.1–122.5
Miniopterus gleni	6/16	42.3 ± 1.29	82.6 ± 8.22	37.4 ± 0.94	3.7 ± 0.49	88.9 ± 17.75
		40.1–44.6	70.0–93.2	36.0–38.9	3.0–4.4	66.7–124.1
Miniopterus griffithsi	2/8	44.1 ± 0.67	80.4 ± 15.66	40.0 ± 0.0	3.2 ± 0.29	89.2 ± 27.42
		43.5–45.3	61.0–99.0	40.0–40.0	2.9–3.6	56.4–128.5
Miniopterus griveaudi (Madagascar)	17/49	59.9 ± 1.73	105.6 ± 12.05	55.9 ± 1.36	3.5 ± 0.54	85.7 ± 19.80
		56.4–62.4	82.0–130.0	53.0–58.0	2.7–4.4	40.0–123.6
Miniopterus griveaudi (Anjouan)	8/27	59.2 ± 0.73	104.3 ± 8.86	54.5 ± 1.22	3.2 ± 0.53	83.8 ± 15.60
		58.2–60.8	86.0–123.0	53.0–57.0	2.7–4.6	57.6–128.1
Miniopterus griveaudi (Grande Comore)	6/22	59.4 ± 0.78	110.0 ± 11.10	55.0 ± 0.76	3.1 ± 0.34	83.5 ± 13.18
		58.2–60.9	74.0–124.0	53.0–56.0	2.6–3.6	66.1–123.9
Miniopterus mahafaliensis	9/24	59.6 ± 1.46	113.7 ± 8.08	55.1 ± 1.47	3.3 ± 0.25	68.7 ± 14.04
		57.3–62.2	95.0–123.0	53.0–57.0	2.9–3.8	43.5–95.3
Miniopterus majori	12/39	48.5 ± 1.15	82.5 ± 11.78	44.4 ± 0.97	3.6 ± 0.42	85.0 ± 16.34
		46.1–52.0	64.0–102.0	43.0–46.0	2.8–4.5	59.5–134.8
Miniopterus 'manavi'	2/9	57.2 ± 0.77	98.4 ± 7.60	53.0 ± 0.0	2.5 ± 0.32	66.0 ± 8.70
		55.5–58.2	89.0–110.0	53.0–53.0	2.1–3.0	54.1–84.7
Miniopterus petersoni	1/6	53.2 ± 0.75	106.5 ± 6.66	49.0 ± 0.63	2.9 ± 0.32	71.0 ± 4.63
		52.0–53.9	95.0–115.0	48.0–50.0	2.5–3.3	63.8–76.7
Miniopterus sororculus	7/20	55.3 ± 0.92	103.2 ± 9.04	51.7 ± 1.08	3.3 ± 0.29	79.3 ± 13.06
		53.9–56.6	83.0–121.0	50.0–53.0	2.7–3.9	50.8–99.8
Miniopterus egeri	2/16	54.7 ± 1.02	113.8 ± 3.62	49.0 ± 0.52	2.9 ± 0.26	62.6 ± 12.57
		53.2–56.3	107.0–123.0	48.0–50.0	2.5–3.4	43.2–81.1

Data are presented as the mean ± SD, minimum – maximum. *n*, number of recorded individuals; *N*, number of utilized pulses; PF, frequency of maximum energy; F_{max}, maximum frequency; F_{min}, minimum frequency; Dur, call duration; IPI, interval between successive pulses.

populations. Nonetheless, the high overlap in call parameters among the Madagascar populations indicates that there was no significant intraspecific variation (Table 3).

We found no significant differences in PF, F_{max}, and IPI among *M. griveaudi* populations on Madagascar, Anjouan, and Grande Comore (ANOVA: $F = 2.866$, $P = 0.062$, $F = 1.761$, $P = 0.177$, $F = 0.166$, $P = 0.847$, respectively). However, Dur of *M. griveaudi* from Madagascar was significantly longer than Dur from Anjouan and Grande Comore populations (ANOVA: $F = 3.686$, $P = 0.029$; post-hoc Tukey's test: Madagascar and Anjouan, $P = 0.143$; Madagascar and Grande Comore, $P = 0.041$; Anjouan and Grande Comore, $P = 0.817$). In addition, F_{min} was significantly different between Madagascar, Anjouan, and Grand Comore populations (Kruskal-Wallis: $H = 20.275$, $P < 0.001$; post-hoc Wilcoxon rank sum test: Madagascar and Anjouan, $W = 681.5$, $P < 0.001$; Madagascar and Grande Comore, $W = 553.0$, $P = 0.002$; Anjouan and Grande Comore, $W = 596.5$, $P = 0.09$).

Species identification based on DFA

The DFA correctly identified 75.8% of the recorded calls to species. Discriminant function 1 explained 97.3% of the total variance and the remaining four functions explained an additional 2.7%. The Malagasy *Miniopterus* spp. were most significantly separated by DFA on three echolocation parameters: PF, Fmin and Dur (Table 4). All *M. griffithsi*, *M. majori*, *M. 'manavi'* (labelled 'unnamed' in Fig. 2), and *M. petersoni* individuals were correctly identified (100% in Table 5). For the remaining species, there were varying numbers of mismatched designations, and the percentages of correctly identified calls ranged between 49.0% (*M. griveaudi*) and 93.8% (*M. gleni*) (Table 5).

DISCUSSION

Recent work using molecular genetic and morphological datasets has shown that species diversity of Malagasy region *Miniopterus* is far greater than previously

Table 4. Results of a discriminant function analysis associated with the calls of *Miniopterus* spp. from Madagascar and the Comoros

Function	F_{min}	PF	Dur	F_{max}	IPI	Eigenvalues	Cumulative (%)	Wilks' lambda	χ^2	d.f.	*P*
1	0.821*	0.665*	–0.025	0.141	–0.017	43.764	97.3	0.008	1090.204	50	< 0.001
2	–0.294	–0.652	0.765*	–0.602	0.540	0.678	98.8	0.367	227.287	36	< 0.001
3	–0.427	–0.046	0.639	0.616*	0.064	0.331	99.5	0.616	109.824	24	< 0.001
4	0.133	–0.322	0.017	0.483	0.127	0.164	99.9	0.820	44.983	14	< 0.001
5	–0.200	0.165	–0.066	0.077	0.830*	0.048	100.0	0.954	10.603	6	0.101

PF, frequency of maximum energy; F_{max}, maximum frequency; F_{min}, minimum frequency; Dur, call duration; IPI, interval between successive pulses. Bioacoustic parameter values from the discriminant function analysis in bold have the heaviest loadings and the variables are ordered relatively to their importance. *P*-values are based on the χ^2 comparisons of the Wilks' lambda values for each compared function.

realized, and that Madagascar holds at least 11 species (Goodman, 2011; Goodman *et al*., 2011). One of the morphological characters that show fixed differences between these species is the shape and, in some cases, the length of the tragus. The tragus is an anatomical feature that has been shown to create a second path to the ear canal for returning echoes and contributes to vertical sound localization (Lawrence & Simmons, 1982; Chiu & Moss, 2007), and is correlated with differences in the frequency ranges of different species of echolocating bats (Gannon *et al*., 2001). Hence, by extrapolation, it would be expected that the Malagasy *Miniopterus* taxa, given differences in tragus shape and form, would show, at least in part, non-overlapping ranges in bioacoustic variables. This is what we found in the present study. Species were initially identified using molecular markers and tragus morphology, and the respective precise details of these were reported in previous studies (Goodman *et al*., 2007, 2008, 2009a, b, 2010a, 2011), thus eliminating the tautological problems encountered when only using bioacoustic aspects to define the different taxa.

Given the broad geographical disjunction across water barriers of *M. griveaudi* between the Comoros and Madagascar, the current dataset allows questions to be tested about the role of environmental factors versus phylogenetic history in the evolution of echolocation calls in the regional members of this genus. We examined this aspect in two different ways: (1) at the intraspecific level of disjunct populations of a widely-distributed species, *M. griveaudi*, on Madagascar and inter-island comparisons (Madagascar, Anjouan, and Grande Comore) and (2) with respect to interspecific differences across the different recognized species on Madagascar, contrasting patterns of allopatric sister taxa (e.g. *M. gleni*/*M. griffithsi*) and sympatric species of similar size but not sister taxa or falling within the same clade (e.g. former *M. manavi* group).

Intraspecific differences in echolocation calls of *M. griveaudi*

One important aspect that should be noted before making interspecific comparisons between Malagasy region *Miniopterus* spp. concerns establishing the level of intraspecific variation in echolocation parameters. Other extralimital species of *Miniopterus* have been shown to exhibit geographical variation in their calls (Conole, 2000). We compared, at the intraspecific level, differences in echolocation parameters of *M. griveaudi* from Madagascar, Anjouan, and Grande Comore. Among Malagasy individuals from Namoroka, Belobaka, and Beanka, comprising sites separated by a direct distance of 330 km, there was considerable overlap in the five bioacoustic variables. Hence, we conclude that, at least across this portion of the species' distribution, no measurable variation occurs with respect to echolocation calls. This is concordant with the genetic data (Fig. 2), which demonstrate no pronounced phylogeographical structure.

By contrast, statistically significant differences in the F_{min} and Dur were found between populations from Madagascar and Comores (Anjouan and Grande Comore). Anjouan is the closest known Comorian population of *M. griveaudi* to Madagascar, and a minimum distance of 370 km of open sea separates these islands. Using coalescent analysis, Weyeneth *et al*. (2011) have shown that *M. griveaudi* on the Comoros are derived from Madagascar populations. Currently, there is no measurable gene flow between Madagascar and the Comoros in either direction for mitochondrial or nuclear markers. It was estimated that the Malagasy and Comorian populations of *M. griveaudi* diverged approximately 183 000 years ago. The measured differences in the F_{min} and Dur of these separate populations, although statistically

Table 5. Percentage of calls of Malagasy *Miniopterus* spp. correctly classified by the discriminant function analysis

	Predicted group											
Species	Ma	Mb	Mgl	Mgr	Mg	Mmh	Mmj	Mmn	Mp	Ms	Me	Total
Ma												
N	14	0	0	0	0	0	0	0	2	3	0	19
%	73.7[1]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.5	15.8	0.0	
Mb												
N	0	19	0	0	8	3	0	0	0	0	0	30
%	0.0	63.3[2]	0.0	0.0	26.7	10.0	0.0	0.0	0.0	0.0	0.0	
Mgl												
N	0	0	15	1	0	0	0	0	0	0	0	16
%	0.0	0.0	93.8[3]	6.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Mgr												
N	0	0	0	8	0	0	0	0	0	0	0	8
%	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Mg												
N	0	17	0	0	24	7	0	1	0	0	0	49
%	0.0	34.7	0.0	0.0	49.0[4]	14.3	0.0	2.0	0.0	0.0	0.0	
Mmh												
N	0	4	0	0	4	14	0	0	0	2	0	24
%	0.0	16.7	0.0	0.0	16.7	58.3[5]	0.0	0.0	0.0	8.3	0.0	
Mmj												
N	0	0	0	0	0	0	39	0	0	0	0	39
%	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	
Mmn												
N	0	0	0	0	0	0	0	9	0	0	0	9
%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	
Mp												
N	0	0	0	0	0	0	0	0	6	0	0	6
%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	
Ms												
N	0	0	0	0	0	0	0	1	1	18	0	20
%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	5.0	90.0[6]	0.0	
Me												
N	0	0	0	0	0	0	0	0	3	0	13	16
%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	18.8	0.0	81.3[7]	

Ma, *Miniopterus aelleni*; Mb, *Miniopterus brachytragos*; Mgl, *Miniopterus gleni*; Mgr, *Miniopterus griffithsi*; Mg, *Miniopterus griveaudi* (Madagascar); Mmh, *Miniopterus mahafaliensis*; Mmj, *Miniopterus majori*; Mmn, *Miniopterus 'manavi'*; Mp, *Miniopterus petersoni*; Ms, *Miniopterus sororculus*; Me, *Miniopterus egeri.*

[1]*Miniopterus aelleni* does not occur in sympatry with either *M. petersoni* or *M. sororculus.*

[2]*Miniopterus brachytragos* occurs in sympatry with *M. griveaudi* across portions of its range but does not with *M. mahafaliensis*.

[3]*Miniopterus gleni* is the allopatric sister species to *M. griffithsi*.

[4]*Miniopterus griveaudi* occurs in sympatry with *M. brachytragos* across a portion of its range but not with *M. manavi* or *M. mahafaliensis*.

[5]*Miniopterus mahafaliensis* is found to occur allopatric to *M. brachytragos*, *M. griveaudi*, and the notably larger *M. sororculus*.

[6]*Miniopterus sororculus* overlaps in range with the distinctly smaller *M. manavi* and is completely allopatric to *M. petersoni.*

[7]*Miniopterus egeri* is the allopatric sister species to *M. petersoni*.

significant, are probably not important from an ecological or sensory perspective. For example, on Anjouan, *M. griveaudi* occurs in sympatry with the similarly-sized *M. aelleni*, and the two can be found foraging at the same sites. Competition theory would predict a greater level of divergence associated with selection between the Anjouan and mainland Madagascar populations of *M. griveaudi.* The lack of divergence can partly be explained by the strong allometric relationship between body size and echolocation frequencies (see below). Furthermore, *M. aelleni* is one of the two species whose peak echolocation frequency was independent of body size (see below).

Low-duty-cycle FM echolocating bats reduce call duration to reduce pulse-echo overlap (Kalko & Schnitzler, 1993). However, the 0.3-ms difference in Dur between the Anjouan and mainland Madagascar populations will add 5.1 cm to the minimum detection in the signal overlap zone for the Anjouan bats (Schnitzler & Kalko, 2001). Furthermore, a 1.5-kHz difference in F_{min} changes the resultant wavelength by 0.17 mm, which suggests that the minimum size of prey detectable by the populations should be similar. Even a 10-kHz difference in echolocation frequency is unlikely to equate to differences in insect detectability, if the consumed prey are small (Jones & Barlow, 2004). It is perhaps notable that acoustic divergence among cryptic species and populations usually involves PF and bandwidth rather than Dur and F_{min} (Heller & von Helversen, 1989; Russo & Jones, 2000; Kingston *et al*., 2001; Jacobs, *et al*., 2006; Jacobs, Barclay & Walker, 2007; Furman *et al*., 2010a). Similarly, although echolocation signals reflected niche partitioning in sympatric, morphologically similar *Myotis* spp., capture success was unrelated to Dur and terminal frequency (Siemers & Schnitzler, 2004). By contrast, Weyeneth *et al*. (2011) found gene flow in the mitochondrial markers from Grande Comore to Anjouan ($M = 0.033$) and in nuclear markers from Grande Comore to Anjouan ($M = 0.083$), as well as in the opposite direction ($M = 0.019$); this level of dispersal is concordant with no measurable differences in the bioacoustic variables between populations on these islands separated by 80 km of sea.

In a study of genetic divergence between *Miniopterus schreibersii schreibersii* from eastern Europe and *Miniopterus schreibersii pallidus* from Asia Minor, Furman, Öztunc & Çoraman (2010a) found that these populations were reciprocally monophyletic based on two mitochondrial DNA markers (ND2 and cytochrome *b*) and the average genetic divergence for the latter marker was 3.5%. Although differences in size, wing shape, and echolocation call parameters were sufficient to discriminate between the *M. schreibersii* lineages, they were not fully diagnostic to individuals (Furman *et al*., 2010b). Furthermore, based on the cytochrome *b* data, these populations are estimated to have diverged between 1.95 and 0.45 Myr BP. Although this is notably longer than the case of disjunct populations of *M. griveaudi* on Madagascar and the Comoros, when overlaid upon one another, the two studies provide a series of reference points in relatively recent geological time associated with divergence in molecular and echolocation evolution. Morphological similarity appears to characterize *Miniopterus* species complexes (Furman *et al*., 2010b). This suggests that the limited echolocation differences between *M. griveaudi* populations with no gene flow may indicate intrinsic deficiency in phenotypic or echolocation call plasticity.

Interspecific differences in echolocation calls of Malagasy *Miniopterus*

Given that previously published bioacoustic catalogues of Malagasy *Miniopterus* spp. were incomplete and based on out-of-date taxonomy (Russ *et al*., 2003; Kofoky *et al*., 2009), the new data reported in the present study on members of the genus *Miniopterus* fill a gap. This is particularly important with the use of bat detectors during acoustic field inventories and ecological research, during which it is necessary to specifically identify bat taxa at a site and often without capture (Parsons & Jones, 2000). The DFA correctly identified certain species 100% in accordance between the molecular/morphometric and bioacoustic data, whereas, for others, more than half the individuals were mismatched. The mismatched cases are discussed below in detail.

In the case of the large-bodied species, all of the individuals of *M. griffithsi* were correctly identified and 6.3% of *M. gleni* were assigned to *M. griffithsi.* These two taxa are allopatric sister species (Fig. 2) (Goodman *et al*., 2010a) and are separated by 7.5% sequence divergence. Hence, the period of speciation between these taxa is not a recent event in their evolutionary history. Furthermore, the former species occurs in a variety of different forest types on Madagascar across an elevational range from sea-level to 1200 m, including spiny bush, and the latter species occurs across an elevational range from 25 to 110 m only in spiny bush habitat and adjacent habitats (Goodman, 2011). Hence, it is assumed that these species live largely under different ecological conditions.

Amongst the medium-sized species, all calls of *M. petersoni* were correctly identified. *Miniopterus egeri* and *M. petersoni* are allopatric sister taxa that, based on current distributional information, have relatively small distributional ranges separated by approximately 300 km. These two species show 4% divergence from one another and occur in eastern

portion of the island where the natural vegetation cover is humid forest. *Miniopterus egeri* occurs across an elevational range from 50 to 550 m and *M. petersoni* from 10 to 550 m. The other two medium-sized not closely-related species, *M. majori* and *M. sororculus*, live in broad sympatry and are known to use the same caves and rock shelters as day roost sites (Goodman *et al.*, 2007). With the exception of tragus shape and some subtle differences in size and pelage coloration, these two species are difficult to tell apart in the hand. However, there was no confusion regarding the bioacoustic parameters in separating them. *Miniopterus sororculus* was one of the two species deviating from the allometric relationship between PF and body size (Fig. 4); its PF was higher than expected from its body size. Five percent of *M. sororculus* calls were mismatched for allopatric *M. petersoni*, and 5% were mismatched for the distinctly smaller and sympatric '*M. manavi*' (unnamed clade in Fig. 2), which has a PF (57.2 kHz) and notably greater than *M. sororculus*.

The situation with small-bodied *Miniopterus* is distinctly more complex, and this group contains at least six different species (Table 1) that, in several cases, are not phylogenetically closely-related to one another (Fig. 2) and demonstrate remarkable convergence in morphology and size (Goodman *et al.*, 2009a, b, 2011). More specifically, in the karst area of Namoroka in the west of Madagascar, four small-bodied *Miniopterus* species occur in sympatry (*M. aelleni*, *M. brachytragos*, *M. griveaudi*, and another taxa that has yet to be specifically identified; Goodman *et al.*, 2009b). These species are known to occur in the same day roosts and are presumed to forage in similar habitats. *Miniopterus aelleni* was the second species deviating from the allometric relationship between PF and body size, with its PF being notably lower than the PFs of other similarly small-bodied Malagasy taxa (Fig. 4). On the basis of the DFA, 73.7% of the *M. aelleni* calls were correctly identified, with the mismatches designating 10.5% as *M. petersoni* and 15.8% as *M. sororculus*; the latter two species have allopatric distributions with *M. aelleni* and are distinctly larger. Only 63.3% of the calls of *M. brachytragos* were correctly identified, with 26.7% being assigned to *M. griveaudi*, which at least in part occurs in sympatry, and 10.9% to *M. mahafaliensis*, which is completely allopatric. *Miniopterus griveaudi* shows the greatest level of mismatched designation, with less than 50% of the calls being correctly identified, with almost 35% being assigned to *M. brachytragos* and the balance of the mismatched designations for the nonsympatric taxa *M. manavi* (labelled 'unnamed' in the tree) and *M. mahafaliensis*. *Miniopterus brachytragos* is known from both dry deciduous forest and eastern humid forest and appears to have a rather patchwork distribution across an elevational range from sea-level to 600 m and *M. griveaudi* is found in a variety of dry deciduous forest formations across the same elevation range. For *M.* 'unnamed', all of the calls were correctly identified. For *M. mahafaliensis*, 58.3% of the calls were correctly identified. The close association between echolocation frequency and body size made identification difficult for many small Malagasy miniopterids based on their echolocation parameters.

The significant correlation between peak echolocation frequency and body size of *Miniopterus* species from Madagascar and the Comoros is found in several families of aerial animalivorous bats (Jones, 1996; Fenton & Bogdanowicz, 2002; Jacobs *et al.*, 2007) and can be attributed to the physics of sound (i.e. large bats have thicker vocal cords and larger resonant chambers than small bats, hence the former produce lower frequency sounds; Hartley & Suthers, 1990) and ecological factors (i.e. large bats are less manoeuvrable than small bats; hence, they must use lower frequency calls to increase the range at which they detect objects in space, allowing them time to manoeuvre to catch prey or avoid obstacles; Barclay & Brigham, 1991). Thus, if the peak echolocation frequency of a species is relatively independent of body size, it is probably ecologically adaptive or socially informative.

At least three non-mutually exclusive hypotheses (Jacobs *et al.*, 2007) may explain why the echolocation frequencies of *M. sororculus* and *M. aelleni* species deviate from the allometric relationship with body size. There is little evidence in this case for the foraging habitat (Jones & Barlow, 2004) and prey detection (Houston, Boonman & Jones, 2004) hypotheses because the variation around the relationship between echolocation frequency and body size of Malagasy *Miniopterus* species was not equal or greater than for North American *Myotis* spp. ($r = 0.31$, $F_{1,13} = 1.4$, $P > 0.2$), whose evolution has been driven by ecological partitioning of their niches (Fenton & Bogdanowicz, 2002). There was evidence for the acoustic communication hypothesis because the variation for Malagasy miniopterids was smaller than for African rhinolophids ($r = 0.72$, $F_{1,8} = 8.5$, $P < 0.02$) whose evolution of frequency differences has been associated with the partitioning of bioacoustic frequency bands to allow effective communication in a social context (Jacobs *et al.*, 2007). If echolocation calls are used for communication, social information about important resources that are patchily distributed, such as roosts, feeding areas or mating sites, would have to be encoded in small intraspecific variation in calls because individuals that use calls that are very different from those used by conspecifics would not be effective for communication purposes.

Indeed, facilitation of intraspecific communication may be the most reasonable hypothesis for acoustic divergence among cryptic low-duty-cycle echolocation bats (Jones & Barlow, 2004). This might partially explain why we found small intraspecific differences in echolocation calls among *M. griveaudi* populations despite limited gene flow and potential competition from congenerics.

It is possible that the echolocation calls of *M. sororculus* and *M. aelleni* diverged from allometry for reasons other than communication. For example, small echolocation differences may allow sympatric bats to exploit different microhabitats, resulting in the availability and consumption of different prey types and resource partitioning (Saunders & Barclay, 1992). Alternatively, higher echolocation frequencies may enable *M. sororculus* to detect smaller-sized prey than species that use lower frequencies (Barclay & Brigham, 1991; Jones, 1995), and therefore take a larger range of insects because it can detect both small and large prey. On the other hand, animalivorous bats foraging in similar habitats often consume the same types of prey (Fenton, 1982; Aldridge & Rautenbach, 1987), even if their peak echolocation frequencies differ by as much as 10 kHz (Jacobs & Barclay, 2009).

Now that the echolocation calls of most of Malagasy region *Miniopterus* spp. are well defined, detailed ecological work on the social behaviour, microhabitat use, and dietary regime can commence in zones where taxa with similar body sizes and echolocation frequencies occur sympatrically and allopatrically, aiming to test predictions on the relative roles of competition, adaptations to contrasting ecological factors, and social communication on the evolution of echolocation parameters.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. List of specimens used in the dictionary and genetic portions of the present study, including species identification, field and museum catalogue numbers, locality, sex, techniques used for bioacoustic recordings, and Genbank numbers. The specimens are deposited in the Université d'Antananarivo, Département de Biologie Animale (UADBA), Antananarivo and the Field Museum of Natural History (FMNH), Chicago. Some specimens have yet to be catalogued and we use field numbers for reference (SMG, Steven M. Goodman; RB, Ramasindrazana Beza). Other tissue samples were obtained from National Museum of Natural History, Smithsonian Institution, Washington, DC (formerly known as the United States National Museum).

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