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# GENETIC AND PHENOTYPIC DIFFERENCES BETWEEN SOUTH AFRICAN LONG-FINGERED BATS, WITH A GLOBAL MINIOPTERINE PHYLOGENY

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The Natal long-fingered bat (*Miniopterus natalensis*) and lesser long-fingered bat (*M. fraterculus*) are morphologically almost indistinguishable and occur sympatrically over much of their southern African range. This raises the possibility that they are sister taxa. We employed a multidisciplinary approach to examine their taxonomic relationship to one another and to other *Miniopterus* species, whose global phylogeny requires review. We examined echolocation, morphological, and dietary differences between *M. natalensis* and *M. fraterculus*, as well as both nuclear and mitochondrial DNA variation between them in the context of a phylogeny incorporating 13 *Miniopterus* species and subspecies. Despite similarities in their morphology and distribution, *M. natalensis* and *M. fraterculus* echolocate at peak frequencies separated by 12 kHz, and both nuclear and mitochondrial DNA markers confirm they are distinct species. Analysis of cytochrome-*b* (*Cytb*) sequences further indicates that *M. fraterculus* and *M. natalensis* are not sister taxa; *M. fraterculus* appears to be more closely related to the greater long-fingered bat (*M. inflatus*). Examination of the global taxonomy of *Miniopterus* confirms that Schreibers's long-fingered bat (*M. schreibersii*) forms a paraphyletic species complex. Furthermore, the miniopterine bats are divided into 2 geographically isolated monophyletic groups, one containing African and European species, and the other taxa from Australasia and Asia. *Cytb* sequence divergence also suggests that *M. natalensis* is distinct from the European *M. schreibersii*. These results support the elevation of *M. natalensis* to full species rank.

Key words: Chiroptera, cytochrome *b*, echolocation, microsatellite, *Miniopterus*, morphology, phylogeny

The phylogeny and taxonomy of the long-winged (or bent-winged) bats of the genus *Miniopterus* Bonaparte 1837, remain unresolved. This genus is the sole member of the subfamily Miniopterinae, which traditionally has been classified within the family Vespertilionidae. However, this taxonomic assignment has been questioned because of distinctive dental, embryological, reproductive, morphological, immunological, and genetic differences between the miniopterines and other vespertilionid bats and corresponding similarities between the miniopterines and the molossids or free-tailed bats (Agrawal and Sinha 1973; Bernard et al. 1996; Cooper and Bhatnagar 1976; Dwyer 1963; Gopalakrishna and Chari 1983; Hoofer and Van Den Bussche 1999, 2003; Krutzsch and Crichton 1990; Mein and Tupinier 1977; Pierson 1986; Racey and Entwistle 2000; Richardson 1977; Stadelmann et al. 2004; van der Merwe 1986). Furthermore, the number of species recognized across the global distribution of *Miniopterus* varies greatly, with recent studies generally describing 11–18 species worldwide (Nowak 1999; Simmons 2005; Skinner and Smithers 1990), although more than 20 have been named in the past (Maeda 1982). Superficially, most members of this genus resemble one another morphologically, and many species currently are designated solely on the basis of size differences (Hayman and Hill 1971; Skinner and Smithers 1990).

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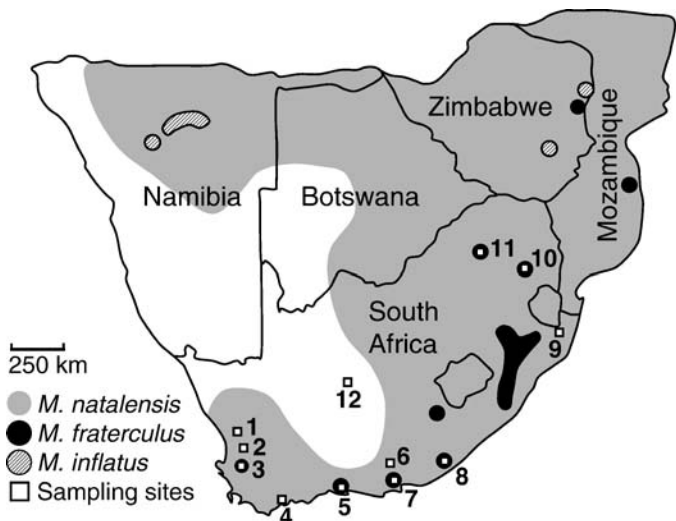


FIG. 1.—Known distribution ranges of *Miniopterus natalensis*, *M. fraterculus*, and *M. inflatus* in southern Africa (modified from Taylor [2000] to include our sampling localities). Sites in South Africa at which samples were collected are numbered as follows: 1, Steenkampskraal; 2, Algeria Forestry Station; 3, Die Hel; 4, De Hoop Nature Reserve; 5, Knysna; 6, Grahamstown; 7, Maitland Mines; 8, Shongweni Dam; 9, Jozini Dam and Pongola River bridge; 10, Sudwala; 11, Peppercorn Cave; 12, Koegelbeen. Geographic locations and details of which species were collected at each locality are given in Appendix I.

In Africa, up to 18 *Miniopterus* species have been named historically (Hayman and Hill 1971), but 6 are recognized at present (Simmons 2005): *M. schreibersii*, *M. africanus*, *M. minor*, *M. inflatus*, *M. fraterculus*, and *M. natalensis*. The Natal long-fingered bat (*M. natalensis* Smith 1834) was formerly considered a subspecies of Schreibers' long-fingered bat (*M. schreibersii*), but recently has been recognized as distinct, and has been reclassified as *M. natalensis* (Simmons 2005). In addition to *M. natalensis*, the lesser long-fingered bat (*M. fraterculus* Thomas and Schwann 1906) and the greater long-fingered bat (*M. inflatus* Thomas 1903) are found in southern Africa (Fig. 1). *M. natalensis* is found throughout sub-Saharan Africa, with its range extending from South Africa to the Sudan and southwestern Arabia (Simmons 2005). *M. fraterculus* has been recorded only in the eastern regions of South Africa, in Zambia, Angola, Mozambique, Malawi, and Madagascar, whereas *M. inflatus* occurs predominantly in Central and East Africa, but also has been identified in a few localities in Namibia, Zimbabwe, and Mozambique (Simmons 2005; Taylor 2000). However, geographic ranges for these species are uncertain, largely because of difficulty in distinguishing among these morphologically similar species (Stoffberg et al. 2004).

All 3 species occupy a variety of vegetation types, ranging from moist mistbelt forests to dry savanna bushveld (Taylor 2000), but are restricted to areas that offer suitable roosting sites such as caves, abandoned mines, and tunnels (Mills and Hess 1997). *M. natalensis* migrates seasonally between wintering roosts where both sexes hibernate, and summer maternity roosts where the females give birth and raise their young (Mills and Hess 1997). These migrations take place over hundreds of kilometers (Miller-Butterworth et al. 2003), and individuals at maternity colonies may number more than 100,000 (Mills and Hess 1997). *M. natalensis* is known to use both open and cluttered habitats (Jacobs 1999), feeding on a range of insect prey, including dipterans, hemipterans, isopterans, and to a lesser extent, lepidopterans and coleopterans (Jacobs 2000; Schoeman and Jacobs 2003). Little is known about the ecology of *M. fraterculus* and *M. inflatus*, although they also are presumed to make seasonal migrations (Taylor 2000). Their distribution ranges overlap with *M. natalensis* (Fig. 1) in South Africa, Namibia, Zimbabwe, and Mozambique (Skinner and Smithers 1990; Taylor 2000), and they frequently roost side by side in the same caves, although they generally occur in much lower numbers than *M. natalensis* (Mills and Hess 1997; Taylor 2000).

The 3 species resemble one another closely in overall appearance and pelage coloration and currently are distinguished primarily based on size differences, despite considerable morphological overlap, particularly between *M. natalensis* and *M. fraterculus*. The latter has a forearm length range of 41–44 mm and mass range of 6–11 g, whereas *M. natalensis* has a forearm range of 42–48 mm and mass range of 9–17 g (Herselman and Norton 1985; Mills and Hess 1997; Taylor 2000). Consequently, in the field, individual *M. fraterculus* may be mistaken for small or juvenile *M. natalensis*, and their classification as separate species has been questioned in the past (Ellerman et al. 1953).

Traditionally, *M. natalensis* and *M. fraterculus* were distinguished by skull length differences (Hayman and Hill 1971; Stoffberg et al. 2004) or a bivariate plot of forearm length against mass (Bernard 1980; Herselman and Norton 1985; Taylor 2000). However, the species overlap significantly in their ratios of forearm to mass (Herselman and Norton 1985; Miller-Butterworth 2001; Stoffberg et al. 2004). Furthermore, Stoffberg et al. (2004) found that the skull lengths of the largest *M. fraterculus* and smallest *M. natalensis* in their collection differed by only 0.4 mm. The lack of overlap in skull length of these species may thus be an artifact of the small sample sizes in their study (16 *M. fraterculus* and 20 *M. natalensis*). The most reliable means of distinguishing between *M. natalensis* and *M. fraterculus* in the field appears to be a discriminant function derived from total body length and hind-foot length (Stoffberg et al. 2004).

In light of the morphological similarities between *M. natalensis* and *M. fraterculus*, their sympatric occurrence over much of their southern African range, and the continued debate over their classification, we employed a multidisciplinary approach to examine their relationship to one another and to other miniopterine bats. We used both nuclear (microsatellite) and mitochondrial (cytochrome-*b* [*Cytb*]) DNA markers to determine whether they are distinct species, and, if so, whether they are sister species, as their co-occurrence and morphological similarity might suggest. Sympatry of closely related sister species raises the question of how intense competition for resources is avoided. We therefore also examined morphological, echolocation, and dietary data collected from both species in sympatry to investigate the extent of ecological similarity

between them. Finally, we used DNA sequences from the *Cytb* gene to examine the taxonomy of the southern African long-fingered bats in the context of other species in the genus, the phylogeny of which is in need of review.

## Materials and Methods

*Sample collection and DNA extraction.*—Species nomenclature and subspecies designations are based on Simmons (2005). Tail membrane biopsies (Worthington Wilmer and Barratt 1996) were collected from 309 *M. natalensis* and 14 *M. fraterculus* between 1998 and 2003 during field expeditions in South Africa (Fig. 1) and Zambia, as described previously (Miller-Butterworth et al. 2003). Samples were collected humanely, in accordance with the guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998), and this research was approved by the University of Cape Town Animal Experimentation Committee. Additional ethanol-preserved tissue samples from *Miniopterus* species and outgroup taxa for the *Cytb* analysis (see below) were obtained from various institutions and researchers (Appendix I). Total genomic DNA was extracted from tail biopsies and ethanol-preserved tissue according to a standard phenol–chloroform protocol (Sambrook et al. 1989). Resulting DNA pellets were resuspended in 150 μl of 1× Tris-ethylenediaminetetraacetic acid or molecular-grade water.

*Microsatellite analysis.*—All samples of *M. natalensis* and *M. fraterculus* (Appendix I) were genotyped at 6 highly polymorphic microsatellite loci, as described previously (Miller-Butterworth et al. 2002, 2003; Moore et al. 1998). A likelihood-based assignment test (Paetkau et al. 2004) was implemented in the software program AGARst (Harley 2001) to determine whether each species has a diagnostic set of multilocus allele frequencies. An unrooted neighbor-joining tree was constructed from the microsatellite data of *M. natalensis* and *M. fraterculus* using the modified Cavalli-Sforza distance (Da—Nei et al. 1983), with 1,000 bootstrap replicates, implemented in the program POPTREE (N. Takezaki, Max-Planck Institut für Biologie, Tuebingen: Germany).

*Cytochrome b.*—A 604-base pair (bp) fragment of the mitochondrial *Cytb* gene was amplified and sequenced in representative accessions or voucher specimens of 33 *Miniopterus* and 2 outgroup taxa (Appendix I) using primers L14724 and H15275 (Irwin et al. 1991). Individual *M. natalensis* included in this analysis were selected to represent the 3 genetically distinct South African subpopulations identified previously (Miller-Butterworth et al. 2003). Polymerase chain reactions (PCRs) were performed in 25 μl reaction volumes containing 0.2 U of BIOTAQ DNA polymerase (Bioline, supplied by Whitehead Scientific, Cape Town, South Africa), 1× Bioline reaction buffer (16 mM ammonium sulfate, 67 mM Tris-HCl pH 8.8, 0.01% Tween 20), 0.2 mM of deoxynucleoside triphosphates, 3 mM of $MgCl_2$, 0.4 μM of each primer, and approximately 100 ng of template DNA. Reaction mixtures were covered with a layer of mineral oil before thermal cycling on a thermal cycler (PTC-100, MJ Research Inc., Johannesburg, South Africa). The cycling profile consisted of an initial denaturation step at 94°C for 5 min, followed by 5 cycles at 94°C for 30 s, 42°C for 45 s, 72°C for 45 s, a further 30 cycles at 94°C for 30 s, 47°C for 45 s, 72°C for 45 s, and a final extension step at 72°C for 10 min. Amplified PCR products were subjected to electrophoresis through a 1% agarose gel. Bands of the correct size were purified from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega, supplied by Whitehead Scientific). Purified products were cycle sequenced using the above primers, with the ABI Prism BigDye Cycle Sequencing kit (Applied Biosystems, Cape Town, South Africa), according to the manufacturer's instructions. Sequenced products were purified by ethanol precipitation and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). In addition to sequences obtained as above, 4 *Cytb* sequences from *Miniopterus* were downloaded from GenBank (Appendix I).

All *Cytb* sequences were trimmed to 525 bp to facilitate aligment by CLUSTAL X (Thompson et al. 1997). Phylogenetic analyses were executed in PAUP* version 4 beta10 (Swofford 2003) using maximum-parsimony (MP), minimum-evolution, and maximum-likelihood (ML) optimality criteria. Bayesian phylogenetic inference was performed in MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001). *Cistugo lesueuri* (family Vespertilionidae) and *Cynomops paranus* (family Molossidae) were designated as outgroup taxa because of the uncertain phylogenetic placement of *Miniopterus* (Hoofer 2003; Hoofer and Van Den Bussche 1999). The high sequence divergence between these outgroup taxa and *Miniopterus* (average 20.4%) raises concerns of data randomization. Phylogenetic analyses therefore also were performed with these 2 taxa excluded, and the Asian–Australasian individuals designated as outgroups. Where multiple individuals were found to have identical sequences, only 1 representative of each haplotype was included in the subsequent phylogenetic analyses.

Base frequency stationarity was evaluated using a chi-square test implemented in PAUP* with uninformative characters excluded (Waddell et al. 1999). The remaining parsimony-informative characters were given equal weights in the analysis. Trees were generated using the heuristic search option with tree-bisection-reconnection branch swapping and stepwise addition using 1,000 random sequence addition replicates. For the ML analysis, the most appropriate nucleotide substitution model was estimated with a hierarchical likelihood-ratio test, implemented in Modeltest version 3.06 (Posada and Crandall 1998). The general time reversible model (GTR+Γ model) with variable sites assumed to follow a discrete gamma distribution (Yang 1994) was accordingly selected as the best-fit model for the sequence data. A heuristic ML analysis with 10 random addition replicates and tree-bisection-reconnection branch swapping was implemented in PAUP* with the following parameters: base frequencies $A = 0.32$, $C = 0.31$, $G = 0.11$, $T = 0.26$; rate matrix $R_{[A\text{-}C]} = 0.52$, $R_{[A\text{-}G]} = 22.62$, $R_{[A\text{-}T]} = 0.24$, $R_{[C\text{-}G]} = 1.39$, $R_{[C\text{-}T]} = 14.91$, $R_{[G\text{-}T]} = 1.00$, gamma correction $\alpha = 0.11$. Nodal support for MP and minimum evolution analyses was assessed from 1,000 nonparametric bootstrap replicates, whereas ML bootstrap support was based on 100 pseudoreplicates (full heuristic search; starting tree obtained by neighbor-joining).

Bayesian analysis was implemented using the default settings in MrBayes. A random tree generated by MrBayes was used as a starting tree for each Markov chain. Four Markov chains were run for 1 million generations, comprising 1 cold chain and 3 incrementally heated chains. Tree sampling was performed every 50 generations, thereby generating 20,000 sample points. Five hundred trees were discarded as burn-in after ensuring that likelihood values reached convergence after this point, using the sump command (Huelsenbeck 2002). A 50% majority-rule consensus of the remaining 19,500 trees was then used to generate posterior probability approximations for each clade. To ensure that the analyses were not trapped in local optima, 3 independent Bayesian runs with different starting trees were performed to ensure convergence on the same topology.

*Hypothesis testing.*—Alternative tree topologies were compared with the best ML tree topology using the approximately unbiased test (Shimodaira 2002). These tests were implemented in CONSEL v1.0g (Shimodaira and Hasegawa 2001).

*Dating species divergences: testing for a molecular clock.*—It is not possible to calculate divergence times statistically with the present taxa because such dating techniques require deeper phylogenetic nodes with

corresponding, accurate fossil data to calibrate the tree. Furthermore, doubt recently has been cast on the widely accepted molecular timescales generally proposed for deep evolutionary divergence events (Graur and Martin 2004). Given the lack of available fossil calibration points for the data set at hand, our best estimate on dating miniopterine divergences would be to establish whether our *Cytb* sequences evolve in a clocklike manner, and if so, to apply a *Cytb* molecular clock with rates of divergence of approximately 2% per million years. Although some rate heterogeneity may exist across taxa (Gissi et al. 2000), this molecular clock is generally accepted for mammalian *Cytb* (Brown et al. 1979; Irwin et al. 1991) and has previously been assumed for bats (Cardinal and Christidis 2000; Ditchfield 2000; Hoffmann et al. 2003).

The existence of a molecular clock for the present *Cytb* sequences was evaluated by using a likelihood-ratio test (Felsenstein 1981). This is satisfied if DNA substitutions follow a Poisson process and the mean rate of substitution has remained constant in different lineages. An ML heuristic search was performed in PAUP* with and without the "molecular clock enforced" option activated, using model parameters estimated by Modeltest. The significance of the difference in log likelihoods of the 2 trees was evaluated using the likelihood-ratio test statistic $\delta = 2(\ln L1 - \ln L2)$, with $s - 2$ degrees of freedom where $s$ is the number of terminal taxa. This statistic is evaluated against critical values of the chi-square distribution (Huelsenbeck and Rannala 1997).

*Morphology, dietary, and echolocation analyses.*—Morphological, dietary, and echolocation data were obtained for a total of 25 specimens of *Miniopterus* captured at Knysna, South Africa (Fig. 1, site 5). They were caught over a period of 14 days with hand nets in unused mines in which both species roosted together, or with mist nets placed across tracks in the forest. Both species were caught in these nets. Seven individuals were conclusively identified as either *M. fraterculus* ($n = 5$) or *M. natalensis* ($n = 2$) by their *Cytb* sequences (see above). We used morphology and echolocation (see below) for these 7 known bats, in conjunction with the morphological ranges specified for the 2 species by Stoffberg et al. (2004), to classify the remaining 18 bats provisionally. Fourteen of the 25 bats were provisionally classified as *M. fraterculus* and 11 as *M. natalensis*. Based on this classification, morphological data were available for all 25 bats, echolocation calls for 12 *M. fraterculus* and 10 *M. natalensis*, and dietary data for 13 *M. fraterculus* and 10 *M. natalensis*.

Body mass (to nearest 0.5 g) and forearm length (to nearest 0.1 mm) were measured for each captured bat. Juvenile bats and pregnant females were excluded from the morphological analyses. Juvenile bats were identified by the presence of cartilaginous epiphyseal plates between the metacarpal and phalangeal joints (Anthony 1988). The reproductive status of females was assessed by means of gentle palpation of the abdomen to determine if they were pregnant (Racey 1988). The extended right wing of each individual (after Saunders and Barclay 1992) was photographed with an Olympus C-730 digital camera, ensuring that the camera was positioned at 90° above the wing. These images were used to measure wingspan (in cm) and wing area (in $cm^2$) including body area without the head, and the area of the uropatagium (Norberg and Rayner 1987) using SigmaScan Pro 5 software (version 5.0.0, SPSS Inc., Chicago, Illinois).

Echolocation calls were recorded from hand-released *M. fraterculus* and *M. natalensis* by using a Pettersson D980 bat detector (Pettersson Electronik AB, Uppsala, Sweden) on a Compaq Presario 1400 personal computer using a DAQ 6062E sound card (National Instruments, Austin, Texas) via an anti-aliasing filter (F2000, Pettersson Electronik AB), with a sampling rate of 44,100 Hz (16 bits, mono) and a threshold of 15. Bats were followed for as long as possible after release to ensure that search-phase calls were recorded (O'Farrell et al. 1999). Recordings were analyzed by using BatSound Pro software (version 3.20, Pettersson Electronik AB). For each bat, we selected 1 call with the best signal-to-noise ratio and measured peak frequency (frequency of maximum intensity), lowest call frequency (measured from the spectrogram), call duration, interpulse interval (from the end of one call to the beginning of the next call), and bandwidth (measured from the power spectrum) at 18 dB below peak frequency (Barclay et al. 1999). Peak frequency of the dominant harmonic was measured from the fast Fourier transformed power spectrum (size 512) using a Hanning window to eliminate the effects of background noise.

Echolocation and morphological parameters of the 2 species were compared using a 2-factor analysis of variance (ANOVA) with species and sex as the factors with an interactive term. Echolocation parameters found to be significantly different were used, in conjunction with genetic identification of the 7 known bats discussed above, to classify provisionally those individuals for which we had morphological data but no DNA sequences. A forward stepwise discriminant function analysis was performed on morphological parameters to test our provisional classifications and to identify those variables that best discriminated between *M. natalensis* and *M. fraterculus*. The discriminant function analysis was performed on all morphological variables except wingspan and wing area because they were strongly correlated with forearm length and wing loading, respectively. The data were log-transformed (Zar 1999) and all analyses were performed using Statistica version 6.1 (Statsoft, Inc., Tulsa, Oklahoma).

The diets of bats captured at Knysna (Fig. 1, site 5) were determined from fecal pellets collected from bats kept individually in cotton bags overnight. Between 3 and 10 pellets were analyzed per bat, producing 71 pellets for *M. natalensis* ($n = 10$) and 72 for *M. fraterculus* ($n = 13$). Each pellet was softened in 70% ethanol before being teased apart under a dissecting microscope. Fragments of exoskeleton were isolated from each pellet and identified to order using a reference collection of insects and the work of Scholtz and Holm (1996). The percentage volume of each insect order in each pellet was calculated according to the method of Whitaker (1988). Dietary overlap between the 2 species was calculated using Pianka's (1973) overlap index. However, all niche overlap indices are sensitive to the number of species and niche categories compared, and may give spurious results as different species or resources are added or deleted from the data (Feinsinger et al. 1981). In addition, even in the absence of competition, species will differ in their use of resources (Connel 1980). We therefore constructed a null model using EcoSim version 7.0 software (Gotelli and Entsminger 2001) and compared the observed niche overlap between the 2 species with 1,000 simulated niche overlap matrices. The RA3 randomization algorithm was employed, meaning that for each simulation the niche breadth of each species was retained, but the particular resource state used was randomized (Gotelli and Entsminger 2001). Winemiller and Pianka (1990) have shown that the RA3 algorithm is superior to other randomization algorithms in detecting nonrandom niche-overlap patterns. The different resource states were treated as "equiprobable," which assumes that they are equally abundant or usable by both species (Gotelli and Entsminger 2001). We also compared the proportions of each arthropod order eaten by the 2 species using a Kruskal–Wallis test followed by multiple comparison tests in Statistica version 6.1 (StatSoft, Inc.).

## RESULTS

*Microsatellites.*—An assignment test on *M. natalensis* and *M. fraterculus* correctly identified 99% of individual *M. natalensis* (mean likelihood value $1.25 \times 10^5$ with range 2.73–$1.71 \times 10^{10}$) and 100% of individual *M. fraterculus* (mean likelihood value $1.55 \times 10^7$ with range $2.36 \times 10^6$–$2.76 \times 10^9$), based on their characteristic microsatellite allele frequencies

(Figs. 2 and 3). Two individuals of *M. natalensis* were incorrectly identified as *M. fraterculus*, but with low likelihood values (1.90 and 5.10). The unrooted neighbor-joining tree (Fig. 4) constructed from microsatellite data from *M. natalensis* and *M. fraterculus* indicates strong (95%) bootstrap support for 2 distinct species clades. This tree also clearly resolves the 3 genetically distinct South African subpopulations of *M. natalensis* identified by Miller-Butterworth et al. (2003), with very strong bootstrap support (95–100%). Similar population substructure is not evident for *M. fraterculus*; however, this may be a consequence of the small sample size available for this species ($n = 14$) or the fact that 13 of these samples were collected from a relatively limited geographical area (Fig. 1, sites 3, 5, 7, 8, 10, and 11), corresponding to a single genetic subpopulation for *M. natalensis* (Miller-Butterworth et al. 2003).

*Cytochrome b*.—When all taxa were examined, the 525 bp of *Cytb* sequence contained 193 variable characters, of which 152 were parsimony informative. No frameshift or stop codon mutations were identified, and only 1 band was amplified by PCR; therefore, these sequences were assumed to be of mitochondrial origin and not nuclear pseudogenes (Bensasson et al. 2001). Plots of the total numbers of transitions and transversions versus sequence divergence for all nucleotide positions resulted in linear plots (data not shown), indicating that the *Cytb* fragment sequenced is not saturated for the taxa (including outgroups) included in this study. Base frequencies did not deviate from stationarity across all lineages ($\chi^2 = 84.91$, $d.f. = 81$, $P = 0.36$).

A single tree (Fig. 5) was recovered from ML analysis of the *Cytb* sequence data (lnL = −2,843.02). The single tree recovered from MP analysis of the sequences (length = 468, consistency index [CI] = 0.48, retention index [RI] = 0.76) differed from the ML tree with respect to phylogenetic relationships within the African–European clade. In the ML tree, *M. s. pallidus* and *M. schreibersii* (Spain) are progressively basal to a clade comprising the African samples, with the Zambian and Madagascan samples more closely related to *M. fraterculus* and *M. inflatus* rather than to *M. natalensis*. In the MP tree, *M. schreibersii* (Spain) is the sister taxon to a clade comprising *M. inflatus* and *M. fraterculus*, with *M. s. pallidus* the sister taxon to *M. natalensis* and the miniopterids from Zambia and Madagascar. However, these conflicting nodes were not strongly supported in either topology. All 3 independent Bayesian runs converged on the same consensus topology, which was identical to the ML topology, with the exception that the position of *M. s. pallidus* was unresolved within the African–European clade, and the grouping of *M. macrocneme* and *M. australis* with Asian *Miniopterus* species was not recovered. The Asian representatives of *M. schreibersii* downloaded from GenBank clustered together with the sample of *M. s. fuliginosus* from Japan.

The MP analysis was repeated with the Australasian–Asian individuals as outgroups rather than *Cynomops* and *Cistugo*. Although support increased slightly for the association between *M. macrocneme* and *M. australis*, the position of *M. schreibersii* (Spain) was unresolved, and the branching order of the remaining taxa was identical to the MP topology recovered

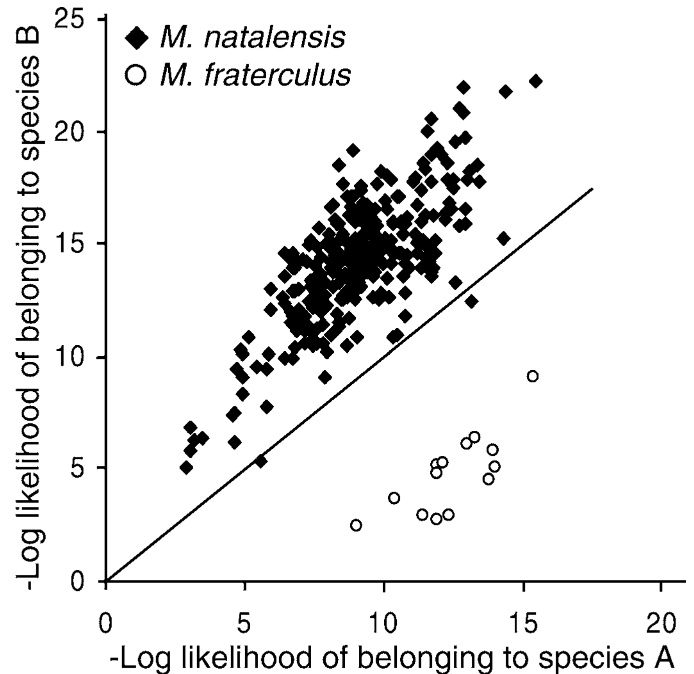


**FIG. 2.**—Results of an assignment test for *Miniopterus natalensis* and *M. fraterculus*, based on microsatellite allele frequencies. The negative log likelihood of each individual belonging to its own species is plotted against the negative log likelihood of it belonging to the other species. The clouds of data points for each species are clearly separated and fall predominantly above or below the diagonal (along which an individual is equally likely to belong to either species).

when using *Cynomops* and *Cistugo* as outgroups. Similarly, although bootstrap support increased slightly for the sister-taxon relationship between *M. macrocneme* and *M. australis* and for the association between *M. inflatus* and *M. fraterculus*, the branching order remained identical to that of the ML analyses using *Cynomops* and *Cistugo* as outgroups (data not shown).

When all *Miniopterus* taxa were examined, 2 monophyletic clades were generally recovered: 1 consisting of African and European species, and the 2nd containing the Australasian and Asian species, in agreement with Appleton et al. (2004). Average *Cytb* sequence divergences between the 2 major geographic clades range between 9.5% and 16.4% (Table 1; individual pairwise distances between individuals available from author). In contrast, divergences between species or subspecies within each geographic clade range between 4.4% and 11.5% within Africa–Europe and between 2.9% and 11.2% within Australasia–Asia.

Interspecific divergences between nonidentical haplotypes for *M. natalensis* and *M. fraterculus* averaged 1.0% (range 0.2–1.7%) and 0.4% (range 0.2–0.6%), respectively. All samples of *M. natalensis* fell within a single, strongly supported clade. Similarly, all samples of *M. fraterculus* formed a strongly supported monophyletic group. Despite the morphological similarity between these 2 species, they are not sister taxa. Instead, *M. fraterculus* and *M. inflatus* appear to be sister taxa, with this association supported by MP, minimum-evolution, and Bayesian analyses. On average, haplotypes of *M. natalensis* and *M. fraterculus* differed by 11.0% (range 10.5–

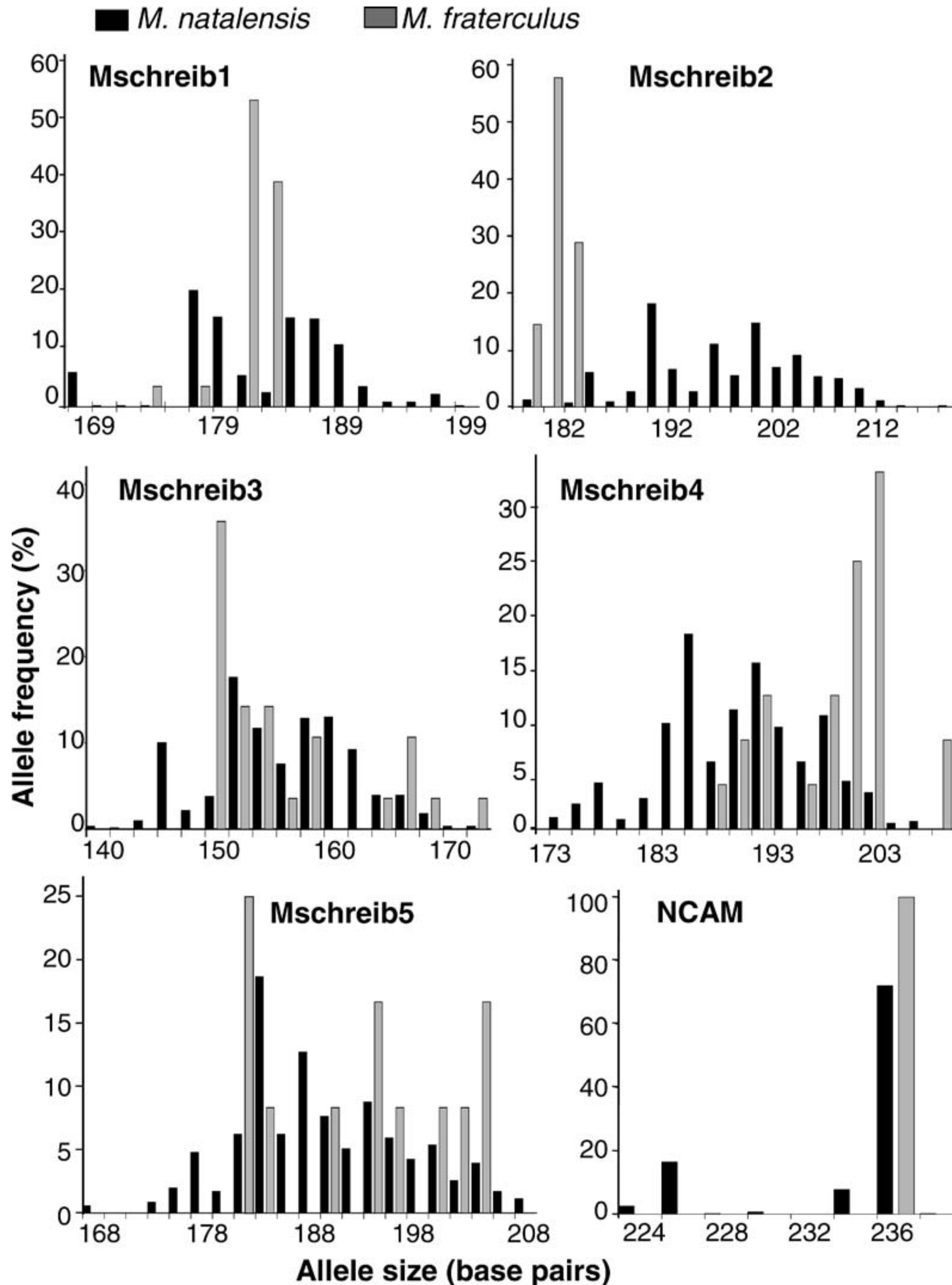


**Fig. 3.**—Allele frequency distributions for 6 microsatellite loci genotyped in 309 *Miniopterus natalensis* and 14 *M. fraterculus*. Loci Mschreib1–Mschreib5 were designed specifically for *M. natalensis* (Miller-Butterworth et al. 2002); NCAM is a general mammalian microsatellite locus located in the gene for the neural cell adhesion molecule (Moore et al. 1998).

11.4%), *M. fraterculus* and *M. inflatus* by 8.5% (range 8.2–8.8%), and *M. natalensis* and *M. inflatus* by 11.3% (range 10.7–12.0%). *M. natalensis* shows phylogenetic affinity with the Israeli subspecies of *M. schreibersii*, *M. s. pallidus* (average divergence 4.4%, range 4.2–4.6%). This latter clade forms the sister group to 2 *Miniopterus* species from Zambia and Madagascar in the MP topology, although there was no significant support for this node. The mean *Cytb* distances between *M. natalensis* and the Zambian and Madagascar samples were 10.7% and 8.5%, respectively. The phylogenetic position of the specimen of *M. schreibersii* from Spain was unresolved in most analyses.

The low level of resolution provided by the *Cytb* sequence data is reflected by the results of hypothesis testing (Table 2). The *Cytb* data could not reject the null hypothesis that the African–European or Australasian–Asian clades are not monophyletic. Similarly, the null hypothesis that *M. inflatus* and *M. fraterculus* are not sister taxa to one another could not be rejected. However,

the *Cytb* data did not support the hypothesis that *M. natalensis* and *M. fraterculus* are more closely related to each other than to other species in the genus, because a sister-taxon relationship between these 2 species was rejected (Table 2).

*Molecular clock.*—The hypothesis that rates of substitution across branches were homogenous was rejected at the 95% confidence level for *Cytb* for the taxa included in this study ($\chi^2 = 47.16$, $d.f. = 26$, $P < 0.01$). It is therefore not appropriate to apply a molecular clock to these *Cytb* sequences. Furthermore, the rejection of a molecular clock hypothesis indicates that sequence divergence estimates may not accurately reflect time since divergence of various lineages, and should be treated with caution. Nevertheless, they are included in this study for purposes of comparison with previous studies.

*Morphology, echolocation, and diet.*—The echolocation calls of the 2 species differed significantly (2-factor ANOVA, $F = 71.2$, $d.f. = 5, 14$, $P < 0.0001$; Table 3; Fig. 6). The mean peak frequency of calls of *M. fraterculus* was higher (Tukey test $q = 0.0006$) and the duration lower (Tukey test $q = 0.0002$) than those of *M. natalensis*. None of the other echolocation parameters differed significantly between the species (all Tukey tests $q > 0.1$). Echolocation also did not differ between the sexes of each species nor was the interaction between species and sex significant ($F = 0.4$, $d.f. = 5, 14$, $P > 0.8$; $F = 0.6$, $d.f. = 5, 14$, $P > 0.7$, respectively).

The 2 species differed significantly in all morphological parameters (2-factor ANOVA, $F = 147.3$, $d.f. = 7, 15$, $P < 0.001$; Table 3) but no significant differences were found between sexes ($F = 1.6$, $d.f. = 7, 15$, $P > 0.2$), nor was the interaction term significant ($F = 2.6$, $d.f. = 7, 15$, $P > 0.05$). We thus included data for males and females in the discriminant function analysis. This analysis supported all our provisional classifications of *M. natalensis* and *M. fraterculus* based on echolocation differences, and extracted 1 function (Table 4). Although mass, wing loading, and aspect ratio were included in the discriminant model, the standardized coefficients indicated that only mass and wing loading contributed significantly to discrimination between the bats (Table 4). This is supported by the comparison of these variables between the 2 species. *M. fraterculus* was smaller ($t = 14.0$, $d.f. = 23$, $P < 0.0001$; Table 3) than *M. natalensis* and had a lower wing loading ($t = 5.3$, $d.f. = 23$, $P < 0.0001$). However, although it also had a lower aspect ratio, aspect ratios were not significantly different ($t = 1.3$, $d.f. = 23$, $P > 0.2$; Table 4). Forearm length, total length, and wingtip shape index were excluded from the discrimination model (partial Wilks' $\lambda > 0.95$; Table 4).

Little difference was found in the diets of *M. natalensis* and *M. fraterculus* (Table 3). The observed niche overlap between the 2 species was 0.872, whereas the mean of the 1,000 simulated indices was 0.654. Out of 1,000 simulated matrices, 88 simulated niche overlaps were larger than the observed niche overlap ($P = 0.912$), and 912 overlaps were smaller ($P = 0.08$). Thus, the observed niche overlap between *M. natalensis* and *M. fraterculus* was high. These results remained the same when the analyses were repeated to include other species that occur in the sampled area. However, there were slight but significant differences in the proportions of each insect order taken (Kruskal–Wallis, $H = 4.71$, $d.f. = 1, 23$, $P < 0.05$). Although both species ate the same orders of arthropods in roughly the same proportion (multiple comparisons, $P > 0.05$), the larger *M. natalensis* consumed more beetles than the smaller *M. fraterculus* (multiple comparison, $P < 0.05$).

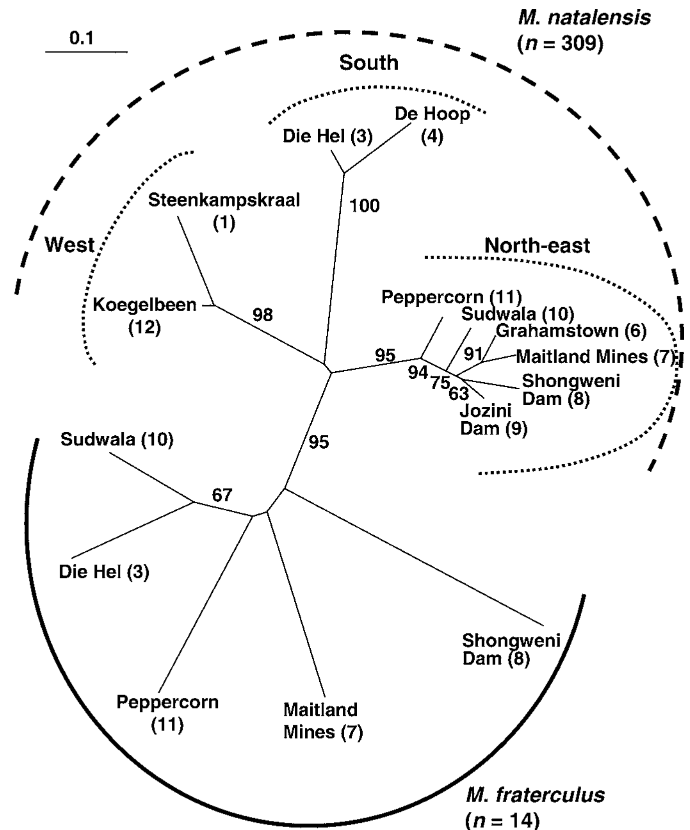


FIG. 4.—Unrooted neighbor-joining tree generated from microsatellite allele frequencies for *Miniopterus natalensis* and *M. fraterculus*, using the modified Cavalli–Sforza distance (Da—Nei et al. 1983). Bootstrap values (1,000 replicates) above 50% are given on the branches. Branch lengths are proportional to the genetic distance indicated by the scale bar. Source localities of individuals from both species are shown (site numbers from Fig. 1 are in parentheses after locality names), as are the 3 South African subpopulations previously identified for *M. natalensis* (Miller-Butterworth et al. 2003).

## DISCUSSION

*Miniopterus natalensis versus M. fraterculus.*—Multiple, independent methods confirm that *M. natalensis* and *M. fraterculus* are sufficiently different to justify their classification as distinct species, despite their morphological similarities and overlap in distribution. Both nuclear and mitochondrial DNA markers indicate that they are genetically distinct, and the *Cytb* phylogeny further indicates that they are not sister species. *M. fraterculus* is clearly more closely related to *M. inflatus* (mean 8.5% divergence) than either species is to *M. natalensis* (approximately 11% divergence). In addition, both *M. natalensis* and *M. fraterculus* have diagnostic microsatellite allele frequencies, forming 2 strongly supported clades on a neighbor-joining tree. Although the morphological differences between

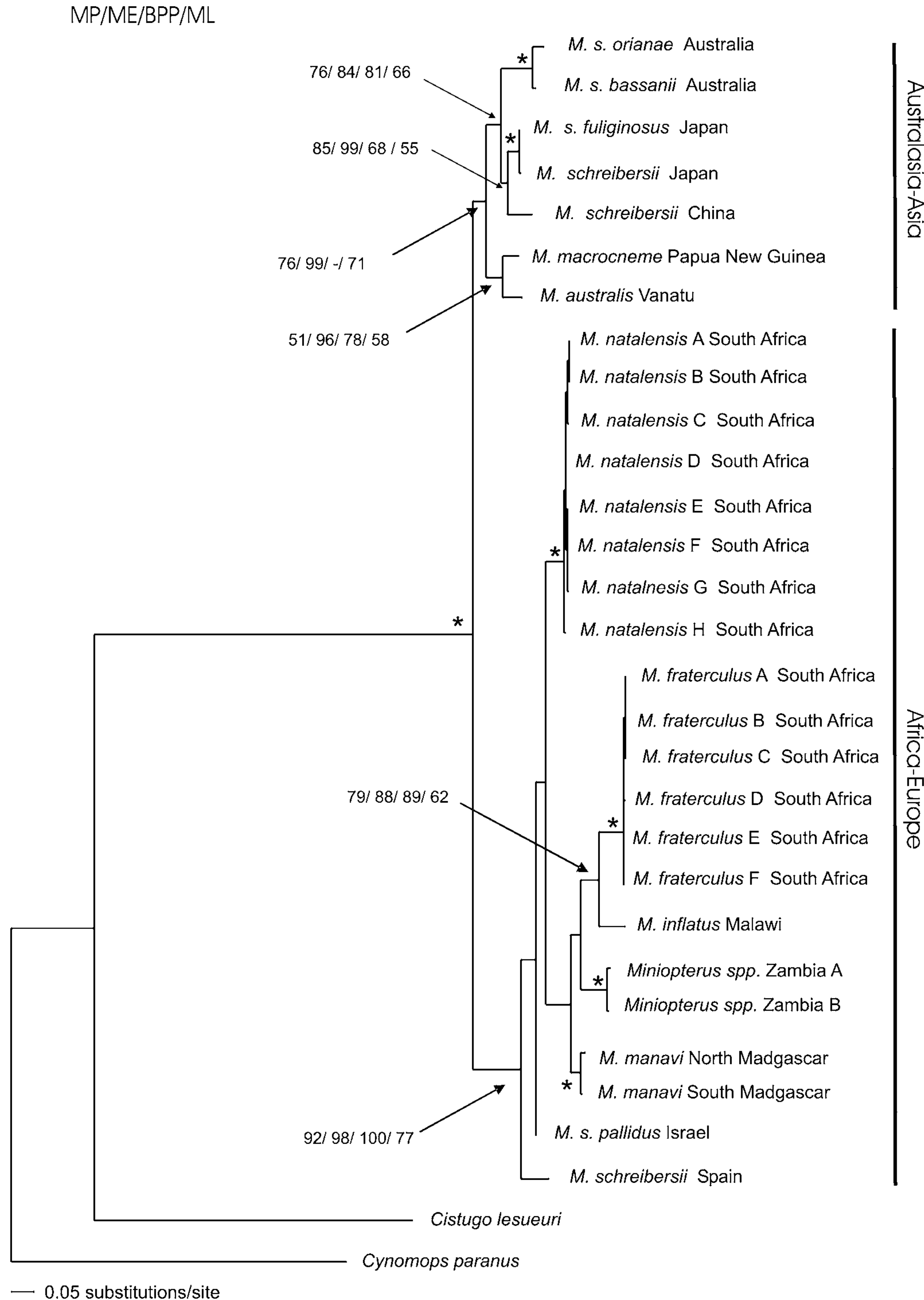


**Fig. 5.**—Single tree recovered from maximum-likelihood analysis of cytochrome-*b* sequences (lnL = −2,843.02). Nodes that received 100% support from all 4 methods of analysis (parsimony, minimum evolution, Bayesian analysis, and maximum likelihood) are indicated by an asterisk (*). Nodal support values for clades that received support ≥50% from at least 3 of the 4 methods of analyses are provided. Branch lengths are proportional to the number of nucleotide substitutions per site as indicated by the scale bar. Institutional or GenBank accession numbers of specimens included in this analysis are given in Appendix I.

*M. natalensis* and *M. fraterculus* were small, the species differ in their echolocation characteristics, and can be distinguished on the basis of peak frequency and call duration.

The *Cytb* divergences identified here within and among species are in agreement with previous studies. Mean intra-specific *Cytb* divergences for *M. natalensis* and *M. fraterculus* are ≤1.7% (Table 1), in accordance with reports for other conspecific bats sampled within a limited geographical area (Barratt et al. 1997; Bradley and Baker 2001; Cardinal and Christidis 2000; Ditchfield 2000; Hoffmann and Baker 2003;

**TABLE 1.**—Average cytochrome-*b* sequence divergences between species or subspecies of *Miniopterus* and outgroups. Where available, average intraspecific distances are given in bold text on the diagonal. Source localities of specimens are given in Appendix I.

| | Taxon | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Taxon | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Africa–Europe | | | | | | | | | | | | | | | |
| 1 *M. fraterculus* | **0.004** | | | | | | | | | | | | | | |
| 2 *M. inflatus* | 0.085 | — | | | | | | | | | | | | | |
| 3 *M. manavi* | 0.095 | 0.093 | **0.010** | | | | | | | | | | | | |
| 4 *M. natalensis* | 0.110 | 0.113 | 0.085 | **0.010** | | | | | | | | | | | |
| 5 *M. schreibersii*[a] | 0.097 | 0.112 | 0.099 | 0.100 | — | | | | | | | | | | |
| 6 *M. s. pallidus* | 0.090 | 0.084 | 0.062 | 0.044 | 0.063 | — | | | | | | | | | |
| 7 *Miniopterus*[b] | 0.107 | 0.110 | 0.074 | 0.107 | 0.115 | 0.084 | **0.010** | | | | | | | | |
| Australasia–Asia | | | | | | | | | | | | | | | |
| 8 *M. australis* | 0.141 | 0.131 | 0.121 | 0.126 | 0.128 | 0.108 | 0.144 | — | | | | | | | |
| 9 *M. macrocneme* | 0.132 | 0.125 | 0.123 | 0.120 | 0.104 | 0.095 | 0.137 | 0.062 | — | | | | | | |
| 10 *M. s. bassanii* | 0.157 | 0.139 | 0.128 | 0.135 | 0.135 | 0.096 | 0.149 | 0.112 | 0.100 | — | | | | | |
| 11 *M. s. fuliginosus* | 0.164 | 0.143 | 0.125 | 0.130 | 0.135 | 0.106 | 0.151 | 0.091 | 0.095 | 0.080 | — | | | | |
| 12 *M. s. orianae* | 0.151 | 0.145 | 0.127 | 0.134 | 0.133 | 0.096 | 0.139 | 0.110 | 0.104 | 0.029 | 0.093 | — | | | |
| 13 *M. schreibersii*[c] | 0.168 | 0.147 | 0.137 | 0.131 | 0.139 | 0.110 | 0.152 | 0.093 | 0.096 | 0.084 | 0.004 | 0.097 | — | | |
| 14 *M. schreibersii*[d] | 0.150 | 0.130 | 0.145 | 0.145 | 0.139 | 0.115 | 0.152 | 0.109 | 0.100 | 0.103 | 0.065 | 0.116 | 0.069 | — | |
| Outgroups | | | | | | | | | | | | | | | |
| 15 *Cistugo lesueuri* | 0.210 | 0.221 | 0.204 | 0.198 | 0.202 | 0.186 | 0.206 | 0.213 | 0.197 | 0.192 | 0.204 | 0.184 | 0.208 | 0.204 | — |
| 16 *Cynomops paranus* | 0.211 | 0.217 | 0.212 | 0.202 | 0.217 | 0.190 | 0.216 | 0.219 | 0.170 | 0.223 | 0.208 | 0.221 | 0.210 | 0.216 | 0.200 |

[a] *Miniopterus schreibersii* from Spain (Ruedi and Mayer 2001).
[b] *Miniopterus* specimen collected in Zambia, specific identity unknown.
[c] *M. schreibersii* from Japan (GenBank).
[d] *M. schreibersii* from China (GenBank).

Ruedi and Mayer 2001; Wright et al. 1999). In contrast, the distances identified here between *M. natalensis* and *M. fraterculus* (10.5–11.4%) clearly lie within the range typical of genetically distinct species (Appleton et al. 2004; Bradley and Baker 2001), supporting their current taxonomic designations. These values are similar to levels of *Cytb* divergence (approximately 13%) identified in Australia between *M. schreibersii* and *M. australis* (Cardinal and Christidis 2000), which are accepted as distinct species. Comparable levels of *Cytb* sequence differentiation also have been reported for other congeneric bats, for example, *Artibeus lituratus* and *A. obscurus* (6.2%—Ditchfield 2000), species of *Dermanura* (4.1–4.9%—Van Den Bussche et al. 1993), *Phyllostomus* (7.0–13.4%—Van Den Bussche and Baker 1993), *Glossophaga* (12.4%—Hoffmann and Baker 2001), and *Myotis mystacinus* and *M. brandtii* (16.0%—Ruedi and Mayer 2001).

Despite the morphological similarity between *M. natalensis* and *M. fraterculus*, there are pronounced differences in their echolocation, with their mean peak frequencies differing by 12 kHz. Similar differences have been reported for 2 morphologically similar phonic clades of pipistrelles (*Pipistrellus pipistrellus*), which also differ at *Cytb* by >11% and have a 10-kHz difference in their peak echolocation frequencies (Barlow and Jones 1999; Barratt et al. 1997; Jones and van Parijs 1993; Mayer and von Helversen 2001). Further examination of this species in the Mediterranean has revealed an additional cryptic North African form, which differs from the European types by about 7% at *Cytb* (Hulva et al. 2004). Each of the 2 phonic types of the European pipistrelles (Jones and Barratt 1999) and the North African form (Hulva et al. 2004) have been proposed as distinct species, based on the same criteria examined here between *M. natalensis* and *M. fraterculus*. Similarly, 2 cryptic phonic types of *Hipposideros bicolor* in Malaysia have been found to differ acoustically by 11 kHz and at *Cytb* by 7%, and are considered to represent 2 distinct phylogenetic lineages (Kingston et al. 2001). A new species of European whiskered bat (*Myotis alcathoe*) also has been described on the basis of significant mitochondrial sequence divergence (13% at the *ND1* gene) in addition to acoustic differences (von Helversen et al. 2001).

The southern African long-fingered bats also differ in the timing of their reproduction (Bernard 1980). *M. fraterculus* copulates 2 months later than *M. natalensis* from the same colonies, and the period of delayed implantation is correspondingly shorter in the former species. Bernard (1980) proposes that the reproductive strategy of *M. fraterculus* represents an intermediate form between that of *M. natalensis* from the same latitude

**TABLE 2.**—Approximate unbiased (AU) $P$ values for the best maximum-likelihood tree and alternative a priori and a posteriori phylogenetic hypotheses. Asterisk(*) indicates significance at $P < 0.05$.

| Hypothesis | $P$ (AU) |
|---|---|
| Best ML tree | 0.894 |
| Best MP tree | 0.263 |
| Monophyletic African–European *Miniopterus* clade | 0.250 |
| Monophyletic Asian–Australasian *Miniopterus* clade | 0.561 |
| *M. inflatus* and *M. fraterculus* not sister taxa | 0.517 |
| *M. fraterculus* and *M. natalensis* sister taxa | 0.045* |

**TABLE 3.**—Comparison between *Miniopterus natalensis* and *M. fraterculus* for morphological, echolocation, and dietary variables for bats captured in Knysna Forest, South Africa (Fig. 1, site 5).

| | *M. natalensis* (morphology: $n = 11$; echolocation: $n = 10$; diet: $n = 10$) | | *M. fraterculus* (morphology: $n = 14$; echolocation: $n = 12$; diet: $n = 13$) | |
|---|---|---|---|---|
| | Mean ± *SD* | Range | Mean ± *SD* | Range |
| Morphology and echolocation | | | | |
| Mass (g) | 12.5 ± 0.8 | 11.5–14.0 | 8.6 ± 0.6 | 8.0–9.5 |
| Forearm length (cm) | 4.7 ± 0.1 | 4.5–4.8 | 4.4 ± 0.1 | 4.3–4.6 |
| Total body length (cm)[a] | 11.6 ± 0.5 | 10.7–12.3 | 10.4 ± 0.3 | 9.7–10.9 |
| Wingspan (cm) | 32.1 ± 1.6 | 29.2–35.3 | 29.6 ± 1.1 | 27.4–31.6 |
| Wing area ($cm^2$) | 166.2 ± 10.4 | 144.5–184.1 | 138.5 ± 9.0 | 122.6–156.1 |
| Wing loading ($Nm^{-2}$) | 7.4 ± 0.6 | 6.8–8.8 | 6.1 ± 0.6 | 5.2–7.2 |
| Aspect ratio | 6.2 ± 0.3 | 5.8–6.7 | 6.3 ± 0.2 | 6.0–6.7 |
| Wingtip shape index | 0.8 ± 0.1 | 0.6–0.9 | 0.8 ± 0.1 | 0.1–0.8 |
| Peak frequency (kHz) | 49.7 ± 1.0 | 47.6–50.9 | 62.3 ± 1.9 | 59.8–65.8 |
| Lowest frequency (kHz) | 43.9 ± 1.2 | 43.0–47.0 | 54.4 ± 4.0 | 44.0–57.0 |
| Bandwidth (kHz)[b] | 34.5 ± 9.7 | 17.0–50.0 | 41.8 ± 10.1 | 28.0–54.0 |
| Interpulse interval (ms) | 93.1 ± 26.1 | 66.0–157.8 | 84.2 ± 39.9 | 40.0–160.0 |
| Duration (ms) | 5.3 ± 0.8 | 3.9–6.1 | 3.7 ± 0.7 | 2.6–4.5 |
| Prey categories | | | | |
| Lepidoptera | 19 ± 17.4 | | 33.8 ± 22.9 | |
| Hemiptera | 23.9 ± 30 | | 29.5 ± 22.4 | |
| Diptera | 27.6 ± 29.8 | | 27.8 ± 23.8 | |
| Coleoptera | 23 ± 29.1 | | 2.7 ± 7.8 | |
| Hymenoptera | 2.7 ± 9.1 | | 0 | |
| Unknown | 3.6 ± 9.2 | | 6.3 ± 10.3 | |

[a] Total body length was measured from tip of snout to tip of tail.
[b] Bandwidth was taken at 18 dB below peak frequency (Barclay et al. 1999).

and the typical reproductive pattern of tropical *Miniopterus*, in which mating occurs in late winter rather than late summer, and which lacks a period of delayed implantation. Assuming these species have a tropical origin, Bernard (1980) suggests that the strategy of *M. fraterculus* may be an intermediate form in the evolution of reproductive cycles, and may indicate that this species colonized southern latitudes after *M. natalensis*, thus having a shorter history in southern Africa. If so, then in light of the fact that *M. fraterculus* and *M. natalensis* are not sister species, it is unlikely that differences between them evolved in sympatry through a process of character displacement to minimize competition. Instead, one may assume, in the absence of evidence to the contrary, that each species evolved its echolocation strategies and timing of reproduction in isolation from the other, with reproductive isolation being achieved before the 2 species came together in sympatry. In this case, the shorter period of delayed implantation in *M. fraterculus* may be the result of adaptation to higher latitudes from a different starting point to that of *M. natalensis*. It might not therefore represent an intermediate stage, as suggested by Bernard (1980). Differences in reproduction and echolocation are thus probably not a consequence of their sympatric co-existence, but merely facilitate such co-existence.

Differences in echolocation and in mass and wing loading suggest that the 2 species might occupy different niches, at least in terms of foraging habitat, despite both species being captured in forests (this study; Jacobs 1999). The shorter-duration calls and higher peak frequency of *M. fraterculus* may be more clutter resistant (Schnitzler and Kalko 1998) and its smaller body size and lower wing loading may allow it to fly more maneuverably than *M. natalensis* (Norberg and Rayner 1987). If so, *M. fraterculus* may use more densely vegetated foraging habitats than *M. natalensis*, thus minimizing competition between them. Furthermore, the larger *M. natalensis* did consume slightly more beetles than the smaller *M. fraterculus*, suggesting some dietary partitioning. However, we did find overlap in their diets, although this may be because our analysis was course grained or because their feeding niches differ in terms of microhabitats and sizes of insects eaten. *Myotis lucifugus* and *M. volans*, for example, are 2 morphologically similar bats that occur in sympatry but use their habitat differently. The former forages mainly over open water whereas the latter primarily forages high above ground (Saunders and Barclay 1992). The 2 cryptic species of European pipistrelles also reportedly show differences in resource use, by foraging in different habitats (Vaughan 1997), which is reflected in dietary differences between them (Barlow 1997). The 55-kHz phonic type, *P. pygmaeus*, feeds primarily in aquatic and riparian habitats, and its diet consists predominantly of aquatic insect species, such as Chironomidae and Ceratopogonidae. The 45-kHz form, *P. pipistrellus*, forages mainly in riparian habitats but also occupies other habitats including grasslands, conifer plantations, and mixed woodlands, where it preys predominantly on flies. Future studies may yet reveal greater differences in microhabitats and prey between *M. fraterculus* and *M. natalensis*.

*Global phylogeny of miniopterines.*—Gene trees may differ from population or species trees (Maddison 1997), and therefore caution should be exercised when drawing species-level conclusions from a single gene tree. However, population- and species-level relationships may be inferred from gene trees when the populations or species under examination are effectively independent genetically and experience little or no immigration (Hillis et al. 1996). Although studies of many migratory bat species have indicated that extensive gene flow occurs across large geographic distances (Burland et al. 1999; McCracken et al. 1994; Petit and Mayer 1999), *Miniopterus* is known to exhibit significant mitochondrial and nuclear genetic differentiation even within comparatively small geographic regions (Cardinal and Christidis 2000; Miller-Butterworth et al. 2003). It is likely therefore that the species included in our study are effectively independent, given the large physical distances between them. Furthermore, multiple markers indicate that even sympatric populations of *M. natalensis* and *M. fraterculus* are distinct, and in particular the clustering of individuals from these species into separate monophyletic clades in the *Cytb* phylogeny (Fig. 5) indicates that lineage sorting between them is complete and thus they also are genetically independent.

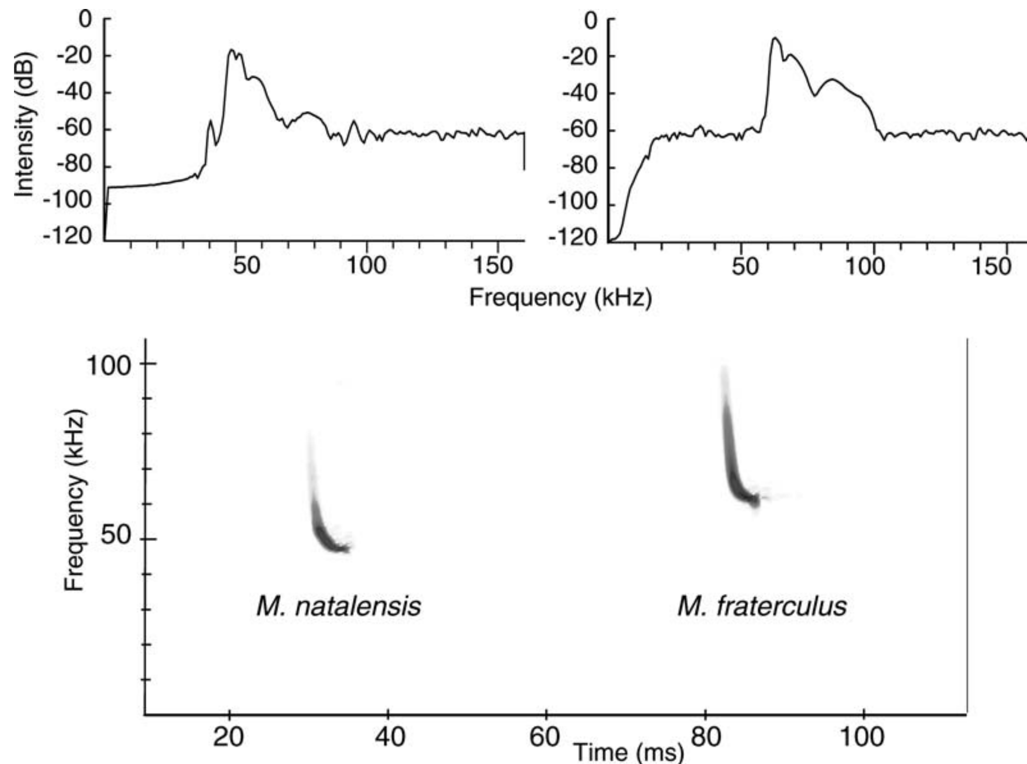


**FIG. 6.**—Power spectra and spectrograms for search-phase echolocation calls of *Miniopterus natalensis* (left) and *M. fraterculus* (right). Call durations are 7.8 ms and 5.8 ms, respectively. Call intensity is measured in reference to the average threshold for human hearing, which is set at zero. Calls were recorded from hand-released bats (to ensure specific identity was known) and were selected as typical search-phase calls of good quality, with high signal to noise ratio. They were chosen toward the end of the sequence to ensure they represent search-phase calls.

In addition to clarifying the taxonomic relationship between *M. fraterculus* and *M. natalensis*, the *Cytb* analysis (Fig. 5) also confirms that, rather than being a single species with a global distribution, *M. schreibersii* forms a paraphyletic species complex, with at least 7 different species and subspecies occurring worldwide. This supports similar findings by Appleton et al. (2004) based on the mitochondrial marker *ND2*. Furthermore, examination of our *Cytb* data confirms the presence of 2 reciprocally monophyletic geographic clades of *Miniopterus* originating from Africa–Europe and from Australasia–Asia, also reported by Appleton et al. (2004). *Cytb* sequence divergences between individuals from these regions are large (9.5–16.4%).

The strong support for a monophyletic *M. natalensis* group to the exclusion of the *M. schreibersii* from Spain (Fig. 5), together with an average of 10.0% divergence at *Cytb* between *M. schreibersii* and *M. natalensis*, supports the recent elevation of the Natal long-fingered bat from a subspecies of *M. schreibersii* to full species rank (Simmons 2005). It also supports the findings of Appleton et al. (2004) of >14.8% *ND2* divergence between African and European specimens of *M. schreibersii*. We thus support their recommendation that the designation *M. schreibersii* should be restricted to European and North African populations and that the remaining African individuals should be considered separate species. Similarly, the presence of 2 monophyletic miniopterine groups corresponding to an African–European group and Australasian–Asian group, which are separated by large sequence divergences, support the recommendation of Appleton et al. (2004) that species within the latter clade be recognized as distinct from the European *M. schreibersii*.

Surprisingly, despite the large geographic distance between them, *M. natalensis* and the Israeli subspecies of Schreibers' bat (*M. s. pallidus*) differ by only 4.4% at *Cytb*, whereas the Spanish *M. schreibersii* and *M. s. pallidus* differ by 6.3%. Simmons (2005) notes that the distribution of *M. natalensis* extends from South Africa to Sudan and southwestern Arabia. These sequence divergence values therefore suggest that *M. s. pallidus* and *M. natalensis* share a more recent common ancestor than do *M.*

**TABLE 4.**—The results of discriminant function analysis on morphological parameters for *Miniopterus natalensis* and *M. fraterculus* captured in Knysna Forest, South Africa (Fig. 1, site 5).

| | Root 1 | Wilks' $\lambda$ | *F*-remove (*d.f.* = 1, 21) | *P* |
|---|---|---|---|---|
| Mass | 1.37 | 0.444 | 94.11 | <0.0001 |
| Wing loading | −0.67 | 0.100 | 5.00 | <0.04 |
| Aspect ratio | −0.36 | 0.090 | 2.48 | >0.1 |
| Eigenvalue | 11.34 | | | |
| Cumulative % | 100 | | | |
| Wilks' $\lambda$ | 0.08 | | | |
| $\chi^2$ | 54.03 | | | |
| Degrees of freedom | 3 | | | |
| *P* | <0.0001 | | | |

*natalensis* and the European form of *M. schreibersii*. To our knowledge, this is the 1st time that the taxonomic position of *M. s. pallidus* has been examined within the context of a global phylogeny of *Miniopterus*. The relationship of the specimen of *M. schreibersii* from Spain to the African miniopterines was not resolved. This may be because *M. schreibersii* from Spain previously has been found to align more closely with European than southern African samples (Appleton et al. 2004), but it was not possible to obtain additional European representatives for the present study to confirm this. Overall, *Cytb* does not appear to contain sufficient phylogenetic signal to resolve the relationships between miniopterines. Increased taxonomic sampling, together with the use of a more slowly evolving marker than *Cytb*, such as a nuclear intronic region (Matthee et al. 2001), may provide increased resolution within the African–European clade, as well as strengthening support for deeper nodes in the miniopterine phylogeny.

Appleton et al. (2004) found levels of *ND2* divergence between *M. schreibersii natalensis* from South Africa (i.e., *M. natalensis*) and both *M. inflatus* and *M. manavi* (8.8–12.2%) that are comparable to those identified here when using *Cytb*. However, they found that *M. manavi* and *M. inflatus* clustered together, to the exclusion of *M. natalensis*. In contrast, our *Cytb* phylogeny places *M. manavi* within a sister clade to *M. natalensis* (although with <50% support), whereas *M. inflatus* clusters strongly with *M. fraterculus*. This difference simply may be the result of using genetic markers with slightly different evolutionary rates and therefore levels of resolution. However, Appleton et al. (2004) note that their sample of *M. manavi* originally was labeled as *M. minor*, but subsequently was renamed *M. manavi*, despite morphological measurements that more closely resembled *M. minor*. They also note that the morphology of their specimens of *M. schreibersii* from Tanzania more closely resembled *M. fraterculus*. The present *Cytb* phylogeny suggests that their Tanzanian individual may indeed be *M. fraterculus*, because it clusters with *M. inflatus* in both the *Cytb* and *ND2* phylogenies. Unfortunately, neither study was able to obtain a definitive specimen of *M. minor*, and therefore it is not possible to determine where it would be located within the African *Miniopterus* clade. However, given that *Cytb* groups *M. manavi* with *M. natalensis* rather than *M. inflatus*, it is possible that the sample of Appleton et al. (2004) that was relabeled *M. manavi* is in fact *M. minor*, as their morphological measurements suggest.

The specific identity of our specimens of *Miniopterus* from Zambia is unknown. Comparison of skull measurements from our voucher specimens of the Zambian form with those reported in the literature did not allow us to determine which of the African species it represented because of the considerable overlap in measurements reported for the different species. These individuals clearly are phylogenetically distinct from both *M. natalensis* and *M. fraterculus* (Fig. 5). They also differ substantially (11.5% divergence) from the specimen from Spain, and so are unlikely to be *M. schreibersii*. Simmons (2005) recognizes 2 other *Miniopterus* species in Africa, namely *M. minor* and *M. africanus*. Without definitive samples from either it is impossible to determine the exact identity of the Zambian specimens. However, if one assumes that the specimen of Appleton et al. (2004) that groups with *M. inflatus* is in fact *M. minor* rather than *M. manavi*, as discussed above, then these Zambian specimens may be the remaining African species, namely *M. africanus*.

In conclusion, *M. natalensis* and *M. fraterculus* echolocate at different frequencies, and both nuclear and mitochondrial DNA markers confirm that they are distinct species. Although morphologically they are very similar, examination of *Cytb* sequence data indicates that they are not sister species. Globally, *M. schreibersii* forms a paraphyletic species complex. Furthermore, the miniopterines are divided primarily into 2 reciprocally monophyletic groups, which are geographically separated, one containing African and European species, and the other including taxa from Australasia and Asia. The Natal long-fingered bat (*M. natalensis*) from southern Africa also was found to be genetically distinct from the European *M. schreibersii*. Our results therefore support the recent elevation of *M. natalensis* from a subspecies of *M. schreibersii* to full species rank.

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## APPENDIX I

*Specimens examined.*—The following are listed for each species included in this study: collection locality, source of material, the number of samples included in the microsatellite (M) and cytochrome *b* (*Cytb*) components, and, where applicable, identification of *Cytb* haplotypes (A–H) and institution or GenBank accession number of samples used for *Cytb* sequencing. Geographic locations of South African collection localities (sites 1–12) are shown in Fig. 1. Specimens for which vouchers were taken are indicated by an asterisk (*). DSJ indicates specimen was contributed by D. S. Jacobs. GenBank accession numbers for microsatellite primer sequences of *Miniopterus* are AY056588–AY056592, and for the *Cytb* sequences generated in this study are AY614732–AY614755 and AY675219–AY675220.

*Miniopterus natalensis.*—SOUTH AFRICA: Steenkampskraal (31°36′S, 18°45′E; site 1), DSJ (M: $n = 20$ [*2 voucher specimens], *Cytb*: $n = 1$), E, SKKF1; Die Hel (33°05′S, 19°05′E; site 3), DSJ (M: $n = 19$, *Cytb*: $n = 1$), F, DHLM12; De Hoop Nature Reserve (34°25′S, 20°21′E; site 4), DSJ (M: $n = 40$, *Cytb*: $n = 1$), H, DHPF1; Knysna (33°53′S, 22°59′E; site 5), DSJ (M: $n = 2$, *Cytb*: $n = 2$), D, G, DSJ147, DSJ197; Grahamstown (33°17′S, 26°31′E; site 6), DSJ (M: $n = 37$, *Cytb*: $n = 1$), A, GF6; Maitland Mines (33°59′S, 25°17′E; site 7), DSJ (M: $n = 37$, *Cytb*: $n = 0$); Shongweni Dam (29°52′S, 30°43′E; site 8), DSJ (M: $n = 29$, *Cytb*: $n = 1$), C, SHDF1, and W. White and K. Richardson (M: $n = 0$, *Cytb*: $n = 3$), A and D, M4, M5, and M17; Jozini Dam (27°25′S, 32°04′E; site 9), DSJ (M: $n = 28$, *Cytb*: $n = 0$); Pongola River Bridge (27°01′S, 32°16′E; site 9), Durban Natural Science Museum, South Africa (M: $n = 5$*, *Cytb*: $n = 0$); Sudwala (25°22′S, 30°42′E; site 10), DSJ (M: $n = 1$, *Cytb*: $n = 1$), B, SWF1; Peppercorn Cave (24°08′S, 29°12′E; site 11), DSJ (M: $n = 19$, *Cytb*: $n = 0$); Koegelbeen (28°39′S, 23°20′E; site 12), DSJ (M: $n = 40$, *Cytb*: $n = 1$), E, KBF1.

*Miniopterus fraterculus.*—SOUTH AFRICA: Die Hel (site 3), DSJ (M: $n = 1$, *Cytb*: $n = 0$); Knysna (site 5), DSJ (M: $n = 0$, *Cytb*: $n = 5$), A, B, and C, DSJ179, DSJ193, DSJ183*, DSJ80, DSJ182*; Maitland Mines (site 7), DSJ (M: $n = 3$, *Cytb*: $n = 1$), A, MMM17; Shongweni Dam (site 8), Durban Natural Science Museum, South Africa (M: $n = 1$, *Cytb*: $n = 1$), A, DM6213*, and W. White and K. Richardson (M: $n = 0$, *Cytb*: $n = 1$), A, M19; Sudwala (site 10), DSJ (M: $n = 8$, *Cytb*: $n = 2$), D and E, SWF11 and SWF17; Peppercorn Cave (site 11), DSJ (M: $n = 1$, *Cytb*: $n = 1$), F, PCM2.

*Miniopterus* (species unknown).—ZAMBIA: Leopard Hill Cave, Lusaka (15°36′S, 28°43′E), DSJ (M: $n = 0$, *Cytb*: $n = 2$), A and B, DSJZM1*, DSJZM4*.

*Miniopterus australis.*—VANATU: Australian Museum, Australia (M: $n = 0$, *Cytb*: $n = 1$), M28191.

*Miniopterus inflatus.*—MALAWI: Likabula Mission, Mulanje Mountain, Northern Flagship Institute, South Africa (M: $n = 0$, *Cytb*: $n = 1$), TM41802.

*Miniopterus macrocneme.*—PAPUA NEW GUINEA: Australian Museum, Australia (M: $n = 0$, *Cytb*: $n = 1$), M19552.

*Miniopterus manavi.*—MADAGASCAR: Ankarana, James Hutcheon (M: $n = 0$, *Cytb*: $n = 1$), A, JMH141; Ranomafana, James Hutcheon (M: $n = 0$, *Cytb*: $n = 1$), B, JMH029.

*Miniopterus schreibersii.*—SPAIN: Ruedi and Mayer (2001), (M: $n = 0$, *Cytb*: $n = 1$), AF376830; JAPAN: L. Tian (M: $n = 0$, *Cytb*: $n = 1$), GI37783851; CHINA: L Tian (M: $n = 0$, *Cytb*: $n = 1$), GI37783847.

*M. s. bassanii.*—AUSTRALIA: Naracoorte, Belinda Appleton (M: $n = 0$, *Cytb*: $n = 1$), NA-1.

*M. s. fuliginosus.*—JAPAN: Kashiwazaki, Niigata Pref., Sakai et al (2003), (M: $n = 0$, *Cytb*: $n = 1$), AB085735.

*M. s. orianae.*—AUSTRALIA: Kakadu, Belinda Appleton (M: $n = 0$, *Cytb*: $n = 1$), M24153.

*M. s. pallidus.*—ISRAEL: Alma Cave, N. Galilee, Benny Shalmon (M: $n = 0$, *Cytb*: $n = 1$), Alma1.

*Cistugo lesueuri.*—SOUTH AFRICA: Algeria Forestry Station (32°22′S, 19°03′E; site 2); DSJ (M: $n = 0$, *Cytb*: $n = 1$), 8.10.02MI1(A).

*Cynomops paranus.*—GUYANA: Potaro-Siparuni, Royal Ontario Museum, Canada (M: $n = 0$, *Cytb*: $n = 1$), ROM108466.