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MOLECULAR SYSTEMATICS OF THE FISHING BAT *MYOTIS* (*PIZONYX*) *VIVESI*

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Phylogenetic reconstructions based on molecular data have shown recurrent morphological convergence during evolution of the species-rich genus *Myotis*. Species or groups of species with similar feeding strategies have evolved independently several times to produce remarkable similarities in external morphology. In this context, we investigated the contentious phylogenetic position of 1 of the 2 piscivorous bat species, *Myotis vivesi*, which was not included in previous molecular studies. This bat, endemic to the coasts and islands of the Gulf of California, Mexico, was long classified in its own genus, *Pizonyx*, because of its distinctive morphology. To reconstruct its phylogenetic origins relative to other *Myotis*, we sequenced the mitochondrial cytochrome-*b* gene of 2 *M. vivesi* and related vespertilionids. These outgroups included *Pipistrellus subflavus*, a member of the subgenus *Perimyotis*, sometimes classified within the genus *Myotis*. Unexpectedly, all reconstructions placed *M. vivesi* within a strongly supported clade including all other typical neotropical and Nearctic *Myotis*. This molecular phylogeny supports an endemic radiation of New World *Myotis*. Other *Myotis* species with similar adaptations to gaffing prey from the water surface present no close phylogenetic relationships with *M. vivesi*, indicating that such adaptations are convergences. On the other hand, *P. subflavus* is genetically as distant from the genus *Myotis* as from other *Pipistrellus* species, suggesting that generic rank to *Perimyotis* is warranted.

Key words: adaptive radiation, Chiroptera, cytochrome *b,* mitochondrial DNA, *Myotis*, *Perimyotis*, phylogeny, piscivory, *Pizonyx*

Invasion of a novel habitat triggers adaptive divergence and speciation (Orr and Smith 1998) because new key adaptations may appear most rapidly when vacant niches are available (Kawata 2001). Bats, the only mammals capable of powered flight, have undergone tremendous diversification since the Eocene. They have colonized numerous habitats and are distributed globally except in the polar regions (Koopman 1994). The evolutionary success of bats is exemplified by their trophic radiation, which includes nectarivory, frugivory, carnivory, sanguivory, and, for most species, insectivory (Koopman 1994). These different feeding modes have been accompanied by evolution of remarkable adaptations such as elongated, protrusible, brushy tongues in nectarivorous macroglossine fruit bats (Andersen 1912). Molecular phylogenetic reconstructions (Hollar and Springer 1997; Juste et al. 1999) have shown that the latter (and other) anatomical specialization for nectarivory evolved independently at least twice in 2 unrelated macroglossine fruitbats. Thus, specialized characters linked to a particular foraging ecology may appear repeatedly and independently during the evolution of bats.

Recent studies indicate that recurrent morphological convergences have occurred during evolution of the species-rich genus *Myotis* (Ruedi and Mayer 2001). In this case, the independent evolution of several groups of species with similar modes of food procurement has led to remarkable similarities in external morphology. Because these similarities were the

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basis of the taxonomic subdivision of that genus (Findley 1972; Jones et al. 2002; Koopman 1994; Tate 1941), the classic subgenera correspond to assemblages of similar ecomorphs, rather than to natural groupings of phylogenetically related species (Ruedi and Mayer 2001). Another salient result from the molecular analysis of Ruedi and Mayer (2001) that did not emerge from previous morphological studies was the implication of geographic distributions for phylogenetic relationships. All Nearctic and Neotropical *Myotis* species analyzed thus far group into an exclusive, monophyletic clade, suggesting that species radiation took place after colonization of the New World (Ruedi and Mayer 2001).

Previous analyses initially included only one-third of all species from the Americas and did not include the distinctive fishing bat, *Myotis vivesi*. Endemic to the coasts and islands of the Gulf of California in Mexico, this species is 1 of the only 2 truly piscivorous bats in the world, although it also takes invertebrates from the water surface (Blood and Clark 1998; Schnitzler et al. 1994). Originally described as a member of the genus *Myotis* by Menegaux (1901), *M. vivesi* was placed by Miller (1906) in its own genus *Pizonyx*. Miller regarded the suite of morphological characters unique to *M. vivesi* sufficient to distinguish it from all other *Myotis*, including greater relative length of foot and claw, strong lateral compression of claws, presence of glands (hemopoietic nodules—Quay and Reeder 1954) on wing and tail membranes, wing membrane abruptly narrowed at knee, hind limbs essentially free of patagium, and a tendency toward increased height and slenderness of cusps of teeth. The restricted distribution and special mode of life of *M. vivesi* also were distinct from all other known *Myotis* (Miller and Allen 1928), which together justified its attribution to the monotypic genus *Pizonyx* (Corbet 1978; Corbet and Hill 1991; Menu 1987; Tate 1942). However, the karyotype of *M. vivesi* is identical to that of other North American *Myotis* (Baker and Patton 1967; Zima and Horacek 1985), which supports its original classification among *Myotis* (Menegaux 1901).

The other piscivorous bat is the Neotropical *Noctilio leporinus* (Hood and Jones 1984; Schnitzler et al. 1994). This species and *M. vivesi* share a suite of characters linked to their unique feeding strategy; compared with their congeners, they are larger in body size, have longer hind legs, and have larger feet with enlarged and laterally compressed claws and toes (Blood and Clark 1998; Lewis-Oritt et al. 2001).

Noctilio leporinus was compared in a molecular analysis to its insectivorous, sister species, *N. albiventris*, which revealed that its fish-eating adaptations evolved relatively rapidly (0.28–0.7 million years ago) from an insectivorous ancestor (Lewis-Oritt et al. 2001). No comparable study of the evolution of piscivory has been done for *Myotis*. Yet, apart from *M. vivesi*, several other species that have elongated hind legs with strong feet also include, at least occasionally, fish in their diet: *M. macrotarsus, M. stalkeri* (Flannery 1995), *M. ricketti* (= *M. pilosus* according to Koopman 1993), *M. macropus* (Dwyer 1970; Law and Urquhart 2000), and *M. daubentonii* (Brosset and Delamare Deboutteville 1966). Although none of them makes fish a primary food source, they may represent intermediate stages in the evolution of piscivory in *Myotis* (Kalko et al. 1998). In the absence of a phylogenetic hypothesis independent of external morphology, this possibility cannot be tested.

We envisioned 4 distinct hypotheses to explain the origin and evolution of *M. vivesi*. The 1st is the phenetic hypothesis, which postulates that all *Myotis* displaying morphological adaptations for gaffing prey on the water surface (i.e., the trawling *Myotis*—Siemers et al. 2001) form a monophyletic group. The 2nd is the ecological hypothesis, which postulates that the fishing bat shares a common history with other potentially piscivorous *Myotis*; these bats would have evolved uniquely the ability to catch fish. The 3rd is the biogeographic hypothesis, which predicts that because *M. vivesi* is Nearctic in distribution, it should share a common ancestor with other New World *Myotis*, regardless of their morphological or ecological distinctions. Finally, the 4th hypothesis is that *M. vivesi* might simply not be closely related to any *Myotis*, which would support Miller's hypothesis to raise *Pizonyx* to generic rank (Miller 1906). We used mitochondrial DNA (mtDNA) sequences of an extensive sample of *Myotis* from around the world to reconstruct the phylogenetic history of *M. vivesi* and test these hypotheses.

Materials and Methods

Taxonomic sampling.—During a survey of genetic variability and assessment of population size of the largest known colony of *M. vivesi* in the Gulf of California, Mexico (Flores-Martínez et al., 2001), we took biopsies of wing membrane from 1 male and 1 female (see Appendix I). These animals were compared with 37 other taxa of *Myotis*: all species reported in Ruedi and Mayer (2001) plus 3 additional species from Africa, and the big-footed bat *M. ricketti* from Southeast Asia (see Appendix I). To root the tree of *Myotis*, 4 *Eptesicus* species and *Vespertilio murinus* were taken from Ruedi and Mayer (2001), and the complete cytochrome *b (Cytb)* genes of 10 pipistrellid specimens were newly sequenced: *P. pipistrellus*, *P. pygmaeus* (sensu Jones and Barratt 1999), *P. kuhlii*, *P. nathusii*, *P.* cf. *javanicus*, *P. abramus*, *P. subflavus*, and *P. savii* (see Appendix I). The latter 2 species are sometimes classified in distinct genera as *Perimyotis subflavus* (Menu 1984) and *Hypsugo savii* (Horacek and Hanak 1985–1986; Ruedi and Arlettaz 1991), respectively.

In addition, we took from GenBank complete or partial *Cytb* sequences of *M. adversus* (AY007528 and AY007529), *M. horsfieldii* (AY007530), and *M. macropus* (AY007526) reported by Cooper et al. (2001), and of *M. leibii* (L19726—Sudman et al. 1994), *Chalinolobus tuberculatus* (AF321051—Lin and Penny 2001), and *Pipistrellus abramus* (AB061528—Nikaido et al. 2001).

Genetic analyses.—Total genomic DNA was isolated in guanidinium (Chomczynski and Sacchi 1987), precipitated overnight in isopropanol at −20°C, centrifuged, and dissolved in 0.5 M NaOH. For amplification, 10 µl of the NaOH solution was diluted in 200 µl of Tris (0.1 M, pH 8—Wang et al. 1993). Two polymerase chain reactions (PCRs) were performed to amplify the complete *Cytb*, a gene that has proven to be well suited for intrageneric comparisons of bats (Van Den Bussche and Baker 1993). The 2 overlapping PCR fragments were amplified with primer pairs L14724–MVZ16 and L15162–H15915 (Irwin et al. 1991; Smith and Patton 1991). A new primer (BSves268H 5′-ATT TCT GGY TTA CAA KAC CRG TGT AA-3′) was designed to replace H15915 for 3 species (*M. tricolor*, *M. goudoti*, and *P. savii*). All PCR cocktails (50-µl reaction volume) included 2–10 µl of DNA extract, 0.2 µM of each primer, 2.5–4 mM

of $MgCl_2$, 0.2 mM of each of 4 deoxynucleoside triphosphates, 1 unit of Taq DNA polymerase (QIAgen, Inc., Basel, Switzerland) with appropriate buffer, and double-distilled H_2O. Thermal profiles of amplifications included 3 min of initial denaturation at 94°C, followed by 36–39 cycles of 94°C (45 s), 45–53°C (45 s), and 72°C (1 min), with a final extension at 72°C (5 min). PCR products were purified and sequenced directly in both directions by using primers L14724 and H15915 (or BSves268H).

Phylogenetic reconstructions.—The complete *Cytb* sequences were edited and aligned manually with BioEdit software (Hall 1999). Maximum parsimony, maximum likelihood, and minimum evolution methods were performed to determine the phylogenetic relationships among the 52 taxa by using PAUP* (version 4.0b10a for PC—Swofford 2001).

Maximum parsimony analyses were performed with characters weighted according to the rescaled consistency index (Farris 1989). The most parsimonious solution was estimated through a heuristic search with 50 random additions of taxa and tree-bisection–reconnection branch swapping for each iteration (Swofford et al. 1996).

The best model of DNA evolution and the parameters used to calculate likelihoods and genetic distances in maximum likelihood and minimum evolution analyses were estimated on a parsimony tree of ingroups (i.e., including only the 42 *Myotis* sequences). We used the likelihood ratio test (Huelsenbeck and Crandall 1997) to choose the most appropriate model of evolution. This model is based on general time-reversible substitutions (Rodriguez et al. 1990), and includes 6 different rates of nucleotide-substitution classes (A–C = 0.66, A–G = 21.3, A–T = 0.67, C–G = 0.7, C–T = 19.7, G–T = 1), uneven nucleotide frequencies (A = 0.348, C = 0.2814, G = 0.0757, T = 0.2949), a proportion of invariant sites ($I = 0.5137$), and a gamma-distributed rate of substitutions ($\alpha = 1.196$—Hasegawa et al.).

The best maximum likelihood tree was estimated from an initial neighbor-joining tree (with maximum likelihood distances), followed by tree-bisection–reconnection branch swapping. Due to computing-time limitations, this swapping algorithm was stopped if the likelihood score of the tree did not improve within 36 h. Likewise, the minimum evolution tree was approximated with a heuristic search based on maximum likelihood distances, by using the same model of DNA evolution. The initial tree was obtained by stepwise addition (random input order) of the taxa, followed by a complete tree-bisection–reconnection branch swapping. This process was repeated 50 times.

Levels of reliability of nodes were assessed with nonparametric bootstraps (Felsenstein 1985): under maximum parsimony and minimum evolution criterion, 1,000 bootstraps were generated, each with 15 stepwise random additions and complete tree-bisection–reconnection branch swapping. Under the maximum likelihood framework, only 200 bootstrap replicates were generated with tree-bisection–reconnection branch swapping limited to 900 s. As suggested by Hillis and Bull (1993), nodes with more than 85% bootstrap support were considered as strongly supported.

We also used the likelihood ratio test to test whether a priori alternative topologies were significantly worse than the optimal solution (KH test—Kishino and Hasegawa 1989; SH test—Shimodaira and Hasegawa 1999), by using routines implemented in PAUP* (Swofford 2001).

Results

The 16 complete (1,140 base pairs) *Cytb* sequences obtained had no insertion or stop codons; therefore, it was assumed that all sequences were of mtDNA origin. These sequences have been deposited in GenBank under accession numbers AJ504406–AJ504409 and AJ504441–AJ504452. Base composition of *Cytb* sequences of *M. vivesi* (A = 0.295, C = 0.258, G = 0.135, T = 0.312) is similar to that of other *Myotis* species (A = 0.300, C = 0.257, G = 0.131, T = 0.313) and other mammals (Irwin et al. 1991; Johns and Avise 1998). The 2 haplotypes of *M. vivesi* differ by 3 transitions at the 1st codon position and 2 transitions and 1 transversion at the 3rd codon position.

Mean percentage sequence divergence when using the Kimura 2-parameter model indicates that genetic distances are smaller between *M. vivesi* and the other New World *Myotis* (mean D = 17.2 ± 1.5) than between *M. vivesi* and Old World *Myotis* (mean D = 20.7 ± 1.3). Distances are greatest when *M. vivesi* is compared with outgroups (mean D = 25.1 ± 1.2).

Phylogenetic analyses.—Fig. 1 presents a bootstrapped weighted maximum parsimony tree representing phylogenetic relationships based on all complete *Cytb* examined. Other methods of phylogenetic reconstructions (maximum likelihood and minimum evolution, not shown) gave tree topologies similar to the weighted maximum parsimony tree, although the bootstrap support of some nodes depended on which optimization criterion was used (Fig. 1). The monophyly of *Myotis* is strongly supported by all reconstructions, but the basal relationships of most clades were largely unresolved.

The phylogenetic position of *M. vivesi* within *Myotis* is unambiguous: it falls within the New World clade in all reconstructions with a high confidence level (Fig. 1). Other solid groups supported by high bootstrap values in all phylogenetic reconstructions include an African clade (containing *M. welwitschii*, *M. bocagei*, *M. tricolor*, *M. goudoti*, and *M. emarginatus*) and different Palearctic and Asian clades as indicated by Ruedi and Mayer (2001). By contrast, all phylogenetic reconstructions agree in placing *P. subflavus* outside the genus *Myotis* (Fig. 1), although *P. subflavus* also is distinct from the other pipistrelles.

In a 2nd set of phylogenetic analyses focusing on *Myotis*, the partial sequence of *M. leibii* groups within the New World clade with high bootstrap support (Fig. 2), and other partial sequences representing 3 Australasian species (Cooper et al. 2001) all group within a clade containing Palearctic and Oriental species.

The trawling species of *Myotis* do not share a common phylogenetic history with *M. vivesi* (Figs. 1 and 2). Indeed, likelihood scores of topologies that force a monophyly of trawling bats are all significantly worse (KH and SH $P < 0.001$) than the best topology of Fig. 2. Likewise, the species marked by stars in Figs. 1 and 2 are bats that are at least occasionally piscivorous and do not appear to be closely related to one another. If we force them into a monophyletic group, with or without *M. vivesi*, the scores of such constrained trees are also significantly worse (KH and SH $P < 0.001$) than the best tree.

Discussion

Our results strongly support the retention of *M. vivesi* within *Myotis* and do not support Miller's (1906) suggestion of

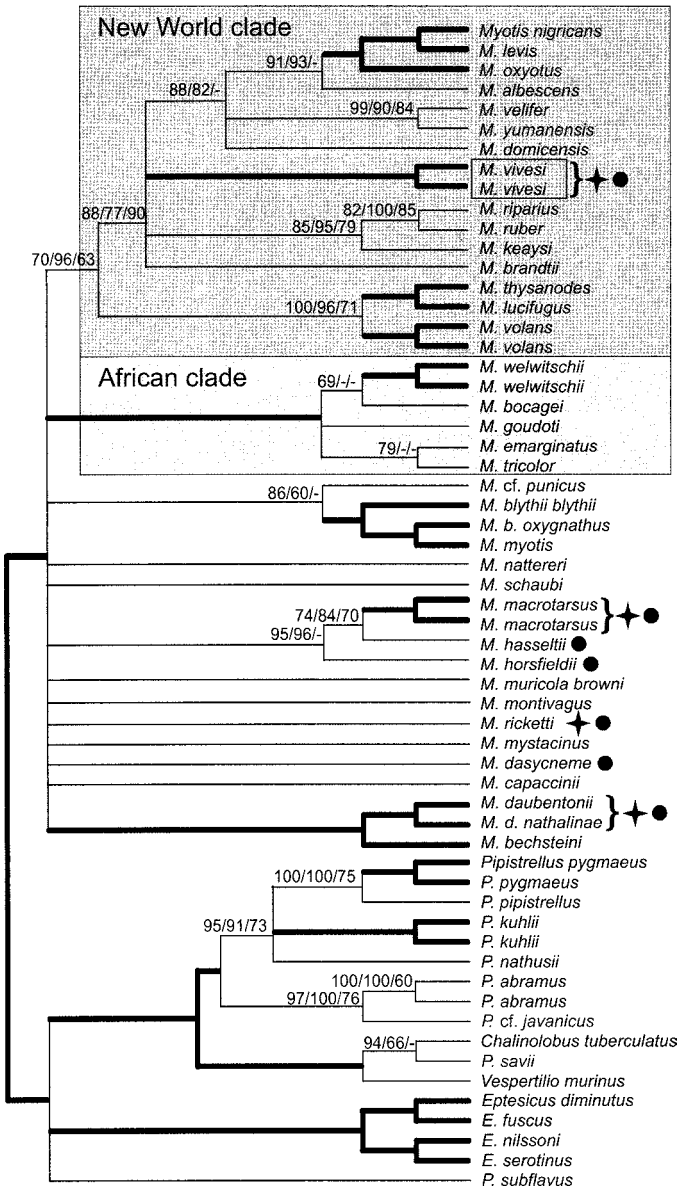

FIG. 1.—Topology of the 60% majority-rule consensus tree of 52 complete cytochrome-*b* (*Cytb*) gene sequences. This weighted, maximum parsimony tree was reconstructed by using the rescaled consistency index of each character. Nodes supported by >85% bootstrap values in all phylogenetic reconstructions are indicated as bold lines. Bootstrap values of other nodes are detailed for maximum parsimony, maximum likelihood, and minimum evolution analyses, respectively. Stars indicate species that eat fish at least occasionally; filled circles indicate the trawling *Myotis* (i.e., those displaying morphological adaptations for gaffing prey over the water surface—Siemers et al. 2001). Geographic clades are highlighted in gray.

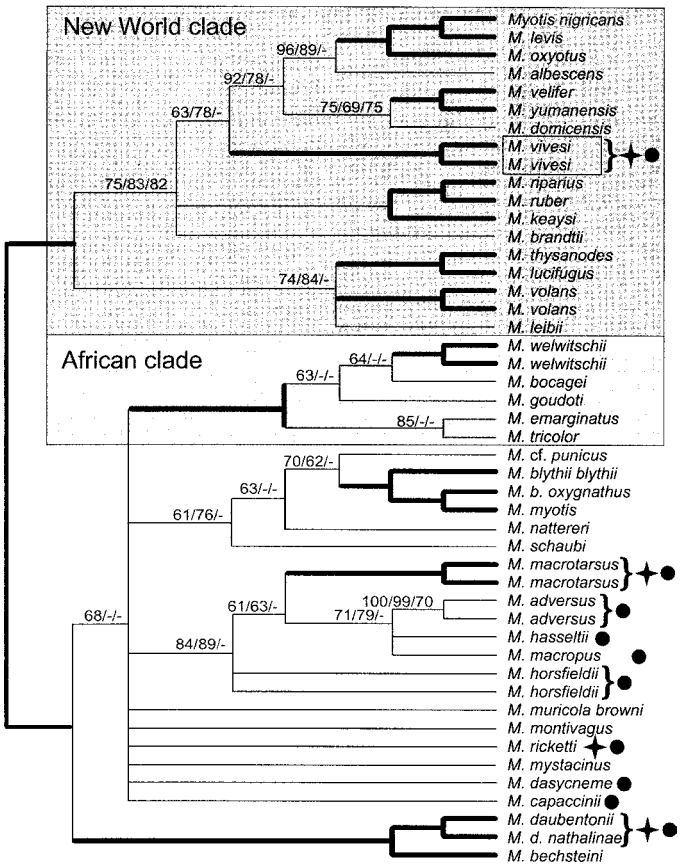

FIG. 2.—Same legend as in Fig. 1, but analyses restricted to *Myotis* species only, with the addition of 5 partial cytochrome-*b* (*Cytb*) gene sequences (for *M. leibii*, 2 *M. adversus*, *M. macropus*, and *M. horsfieldii*).

generic status as *Pizonyx*. Moreover, *M. vivesi* is not closely related to any other big-footed *Myotis* that we sequenced, indicating that trawling *Myotis* species with specializations for gaffing prey evolved multiple times. As already suspected by Findley (1972), the surprising phenetic resemblance of *M. vivesi* to *M. macrotarsus* or *M. ricketti* bears no phylogenetic significance but is the result of convergence. Furthermore, *M. vivesi* does not share a common history with other species that are occasionally piscivorous (Fig. 2), which in turn contradicts the ecological hypothesis that bats of the genus *Myotis* are unique in evolving the ability to catch fish.

A New World clade (Ruedi and Mayer 2001) appears in all analyses with moderate to high bootstrap support. Thus, the biogeographic hypothesis, that all New World species are monophyletic, is strongly supported by our molecular reconstructions, even with the inclusion of the fishing bat *M. vivesi*. Despite its behavioral and morphological peculiarities, *M. vivesi* appears to have evolved from a common ancestor with other typical Nearctic and Neotropical species of *Myotis*. At present it is not possible to identify sister-group relationships of *M. vivesi* within this New World clade because taxonomic coverage included in the molecular tree is limited (~40% of New World species).

The hypothesis that biogeography may be a good predictor of phylogenetic relationships finds even more general support in the tree of *Myotis*. *M. bocagei*, *M. goudoti*, *M. tricolor*, and *M. welwitschii* are endemic to the Ethiopian region. They are morphologically divergent from one another and are currently classified in 3 subgenera (*Myotis*, *Chrysopteron*, and *Leuconoe*—Corbet and Hill 1991), yet all molecular reconstructions group these African species with *M. emarginatus* (distributed

in North Africa and the western Palearctic) to form a strongly supported, monophyletic clade. Thus, the current classification of *Myotis* species based on morphological characters does not represent natural groupings, but rather reflects multiple morphological convergences (Ruedi and Mayer 2001).

Despite the addition of many new ingroup and outgroup species in our study, resolution at the basis of the *Myotis* radiation remains limited. But beyond the *Myotis* radiation, our molecular reconstructions clarify 2 other taxonomic issues. The present molecular evidence clearly rejects the hypothesis put forward by Menu (1984, 1987) that dental characteristics suggest a close relationship between *P. subflavus* and some *Myotis* species within the subgenus *Leuconoe*. Indeed, topologies enforcing the monophyly of *P. subflavus* with any *Myotis* species or with other *Pipistrellus* are clearly rejected as possible alternatives to the optimal tree of Fig. 1. *P. subflavus* appears genetically as distinct from *Myotis* (mean D = 22.9 ± 1.2) as from other *Pipistrellus* (mean D = 23.1 ± 0.8). Together, these results add to growing evidence that *P. subflavus* should be separated from other pipistrelles into a monotypic subgenus (Koopman 1994) or even as a separate genus, *Perimyotis* (Menu 1984, 1987).

Similar conclusions concern the taxonomic position of *P. savii*: our mtDNA tree places this taxon in a well-supported clade including *Vespertilio murinus* and *Chalinolobus tuberculatus* (Fig. 1). This is consistent with other studies that considered another mitochondrial gene (*ND1*—Mayer and Helversen 2001), and with morphological (Hill and Harrison 1987; Horacek and Hanak 1985–1986), karyological (Volleth and Heller 1994), and allozymic data (Ruedi and Arlettaz 1991), which all suggest that the *savii* group be raised to generic rank, *Hypsugo*.

To conclude, the phylogenetic evidence presented here adds to a growing body of literature that reveals the importance of using genetic techniques to reconstruct phylogenetic relationships for morphologically conserved taxa. Species of the genus *Myotis* are not an exception. Phenetically similar species or species with ecological similarities such as trawling *Myotis* do not share a close common ancestor. It seems that adaptation to a particular foraging strategy leads to a deterministic morphological solution, producing recurrent cases of convergent evolution. Such convergences happened independently, in multiple geographic regions, which renders studies based solely on phenetic characters particularly prone to make taxonomic grouping devoid of phylogenetic information.

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APPENDIX I

Most specimens in this study are deposited as vouchers in the following institutions: Senckenberg Museum of Frankfurt (SMF), Museum of Texas Tech University (TK), Transvaal Museum, South Africa (TM), Field Museum of Natural History in Chicago (FMNH), Natural History Museum of Geneva (MHNG), and Petr Benda's private collection (PB). Where listed, latitude and longitude are given in degrees and decimal minutes.

The following specimens were examined: *Myotis vivesi*.—MEXICO: Baja California, Isla Partida, 28°52′N, 113°02′W (1 male and 1 female; no voucher). *M. tricolor*.—SOUTH AFRICA: Transvaal, Graskop, Blyderiversproot Nature reserve, 24°55′S, 30°49′E (male; TM 40300). *M. goudoti*.—MADAGASCAR: Ambalavao, Fianarantsoa Province, Andrinditra reserve, 22°13′S, 47°0′E (male; FMNH 151709). *M. bocagei*.—GHANA: Agumasta wildlife sanctuary, 7°07′N, 0°36′E (female; SMF 89673). *M. ricketti*.—LAOS: Khammouane, 17°23′N, 104°47′E (male; TK AG980129.7). *Pipistrellus subflavus*.—UNITED STATES: Texas, White Oak Creek Wildlife Management Area (male; TK 90671). *P. pipistrellus*.—GREECE: Macedonia, Pili, Prespa, 39°15′N, 21°75′E (male; MHNG 1807.52). *P. pygmaeus*.—GREECE: Macedonia, Rendina, 40°65′N, 23°61′E (male; MHNG 1807.059). CYPRUS: Mt. Troodos, 34°91′N, 32°75′E (male; MHNG 1807.90). *P. kuhlii*.—GREECE: Macedonia, Kilkis, 41°00′N, 22°86′E (male; MHNG 1807.54). IRAN: Polan, Pir Sohrab, 25°45′N, 60°50′E (male; PB 1686). *P. nathusii*.—SWITZERLAND: Vaud, Lausanne, 46°53′N, 6°6′E (male; MHNG 1806.10). *P.* cf. *javanicus*.—TAIWAN: Miou-li County, 24°5′N, 120°8′E (male; no voucher). *P. abramus*.—TAIWAN: Shin-mei village, 24°5′N, 120°8′E (male; no voucher). *P. savii*.—SWITZERLAND: Valais, Fully, 46°13′N, 7°1′E (male; MHNG 1805.007).