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Molecular Systematics of Bats of the Genus *Myotis* (Vespertilionidae) Suggests Deterministic Ecomorphological Convergences

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Based on extensive phenetic analyses, bats of the genus *Myotis* have been classically subdivided into four major subgenera each of which comprise many species with similar morphological and ecological adaptations. Each subgenus thus corresponds to a distinct "ecomorph" encompassing bat species exploiting their environment in a similar fashion. As three of these subgenera are cosmopolitan, regional species assemblages of *Myotis* usually include sympatric representatives of each ecomorph. If species within these ecomorphs are monophyletic, such assemblages would suggest extensive secondary dispersal across geographic areas. Conversely, these ecomorphological adaptations may have evolved independently through deterministic processes, such as adaptive radiation. In this case, phylogenetic reconstructions are not expected to sort species of the same ecomorph into monophyletic clades. To test these predictions, we reconstructed the phylogenetic history of 13 American, 11 Palaearctic, and 6 other *Myotis* species, using sequence data obtained from nearly 2 kb of mitochondrial genes (cytochrome *b* and *nd1*). Separate or combined analyses of these sequences clearly demonstrate the existence of several pairs of morphologically very similar species (i.e., sibling species) which are phylogenetically not closely related. None of the three tested subgenera constitute monophyletic units. For instance, Nearctic and Neotropical species currently classified into the three subgenera were clustered in a single, well-supported monophyletic clade. These species thus evolved independently of their ecological equivalents from the Palaearctic region. Independent adaptive radiations among species of the genus *Myotis* therefore produced strikingly similar evolutionary solutions in different parts of the world. Furthermore, all phylogenetic reconstructions based on mtDNA strongly supported the existence of an unsuspected monophyletic clade which included all assayed New World species plus *M. brandtii* (from the Palaearctic Region). This "American" clade thus radiated into a morphologically diverse species assemblage which evolved after the first *Myotis* species colonized the Americas. Molecular reconstructions support paleontological evidence that species of the genus *Myotis* had a burst of diversification during the late Miocene–early Pliocene epoch.

Key Words: Myotis; Chiroptera; cytochrome *b; nd1*; adaptive radiations; phylogeny; fossils.

INTRODUCTION

The evolution of species diversity is achieved through a complex balance between processes of extinction and speciation. The result is that at any time, some groups may be highly diversified while sister clades are depauperate. The causes that promote or inhibit these processes in particular clades and not in others are still highly debated, but may include intrinsic key innovations, extrinsic processes linked to climatic fluctuations, differential rates of gene evolution, etc. One difficulty in the identification of the common processes underlying the evolution of species diversification is the need for accurate knowledge of the phylogenetic history of these groups (Sanderson and Donoghue, 1996). The advent of molecular techniques in systematic biology has provided unprecedented possibilities for obtaining DNA information suitable for phylogenetic reconstructions. As yet, extensive data sets are available for only a minor fraction of organisms.

With about 90 species spread all over the world, the genus *Myotis* represents one of the most diverse and successful radiations among mammals. Because species have a rather undifferentiated morphology and often share many plesiomorphic characters (Menu, 1987), the taxonomic subdivision of that genus has been difficult. Based on an extensive phenetic study of Eurasian and American species, Tate (1941) distinguished seven groups which he described as subgenera (*Selysius, Isotus, Paramyotis, Myotis, Chrysopteron, Leuconoe,* and *Rickettia*). More recently, Findley (1972) used numerical taxonomy on 48 cranial and external

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characters to classify most described species and retained only *Myotis, Selysius,* and *Leuconoe* as distinct subgenera. He also noticed that these three subgenera grouped species possessing a suite of related morphological traits which, supposedly, corresponded to three major modes of flight and food procurement. According to Findley (1972), the subgenus *Leuconoe* (type species *M. daubentonii*) is characterized by bats with relatively large feet, hairy legs, and a small plagiopatagium that typically forage on the water surface. Species of the subgenus *Selysius* (type species *M. mystacinus*) are usually smaller footed, with an enlarged uropatagium, and forage on aerial insect plankton. Species of the subgenus *Myotis* (type species *M. myotis*) are relatively larger animals with long ears, broad wings, and a more derived dentition (Menu, 1987) that are typically gleaners, catching their food on solid surfaces or even on the ground (Arlettaz, 1996). Although at a fine scale, similar species within each subgenus may exploit their environment in different ways (Arlettaz, 1999; Saunders and Barcley, 1992), field studies on behavioral and ecological characteristics of many species within each subgenus (see reviews in, e.g., Harvey *et al.,* (1999) and Schober and Grimmberger (1987)) support this division into three major "ecomorphs" (Losos *et al.,* 1998). In addition to these three classical subgenera, two rare South African species (which were not examined by Tate nor by Findley) are currently regarded as belonging to a fourth subgenus, *Cistugo* (type species *M. seabrai*). They are characterized by distinctive wing glands and a unique karyotype among bats of the genus *Myotis,* which may warrant full generic rank as *Cistugo* (Rautenbach *et al.,* 1993).

Although this subdivision into four major subgenera is currently widely accepted (Koopman, 1994), it is entirely based on a limited suite of phenetic characters, and thus it is unclear whether it corresponds to a true phylogenetic grouping. For instance, Menu (1987) or Godawa Stormark (1998) examined solely dental characters and did not find sharp boundaries between the subgenera *Selysius* and *Leuconoe* as some species showed intermediate characteristics. Hence, a comprehensive cladistic analysis of all morphological characters is still needed to test the robustness of the current classification of *Myotis* bats. An alternative approach, which we have adopted, is to use molecular techniques to test the validity of the current subdivision into four subgenera.

Except for the subgenus *Cistugo,* which is endemic to South Africa, the remaining three subgenera are widely distributed throughout both the Old and the New World (Koopman, 1993). Over most of their world distribution, several species of each subgenus are usually found in sympatry (Saunders and Barcley, 1992). If these subgenera are valid monophyletic units, this implies that ecomorphs evolved prior to species radiation and the current worldwide distribution of the different subgenera resulted from extensive, secondary dispersal across continents. Conversely, these different ecomorphs may have evolved independently through some deterministic processes (Losos *et al.,* 1998), and similar species currently classified in the same subgenus may not be monophyletic. Based on these two alternative (but nonexclusive) hypotheses, the occurrence of similar ecomorphs found on different continents could have resulted either from extensive migrations or from recurrent, convergent adaptations. We tested these two predictions with molecular phylogenetic reconstructions of species sampled on both sides of the Atlantic Ocean and showed that the hypothesis of multiple independent evolution of ecomorphs has strong support.

MATERIAL AND METHODS

Taxonomic Sampling

Thirty-three taxa of *Myotis* representing all three widespread subgenera have been sampled, as listed in Table 1. These taxa correspond to all currently described species of European *Myotis,* 13 of 35 North and South American species, 5 of 29 Oriental species, and 1 of 8 African species. Unfortunately, as no tissue of the 2 rare, endemic species of the subgenus *Cistugo* was available, we could not consider this subgenus further in the genetic analyses. To serve as outgroups, 9 additional vespertilionid bat species were analyzed. Most reference specimens are deposited as vouchers in different institutions as shown in Table 1. With few exceptions, each taxon was represented by two or more specimens, but multiple sequences are reported only if they diverged by at least 1%.

Genetic Analysis

Total genomic DNA was extracted from frozen or ethanol-preserved tissues by digestion with proteinase K for 3–4 hours at 55°C, purified by extraction twice with phenol/chloroform and once with chloroform, desalted and concentrated with ethanol precipitation, and then resuspended in 100 μl H_2O (Sambrook *et al.,* 1989). Each individual bat was sequenced for two mitochondrial genes, except for *M. levis* and *M. welwitschii* A for which only one gene could be sequenced (Table 1). The complete cytochrome *b* gene (*cytb*) was obtained from two overlapping PCR products with primer pairs L14724–MVZ 16 (Kocher *et al.,* 1989; Smith and Patton, 1993) and L15162–H15915 (Irwin *et al.,* 1991). The PCR cocktail (50-μl reaction volumes) included 5 μl of DNA extract with 0.2 μM each primer, 2.5 mM $MgCl_2$, 0.8 mM dNTP, and 1 unit of *Taq* polymerase with appropriate buffer (QIAgen, Inc.). Amplifications included 3′ initial denaturation at 94°C, followed by 37 cycles at 94°C (45 s), 45–50°C (45 s), and

TABLE 1

Current Taxonomy[a] of the Animals Sequenced in This Paper, with Collecting Localities, GenBank Accession Nos., Reference, and Location[b] of Voucher Specimens

	Locality	GenBank		Voucher
		Cyt-*b*	ND-1	
Family Vespertilionidae				
Subfamily Miniopterinae				
Miniopterus schreibersii	Barcelona, Spain	AF376830	AY033969	IZEA 3431
Subfamily Vespertilioninae				
Eptesicus fuscus	California, USA	AF376835	AY033968	MVZ 148681
Eptesicus diminutus	Paranà, Brasil	AF376833	AY033976	MVZ AD496
Eptesicus nilssonii	Bavaria, Germany	AF376836	AY033987	ER 1300
Eptesicus serotinus	Chalkidiki, Greece	AF376837	AY033950	ER 659
Lasiurus sp.[c]	Parana, Brasil	AF376838	AY033975	MVZ AD522
Nyctalus leislerii	Obwald, Switzerland	AF376832	AY033949	IZEA 2639
Scotophilus heathi	Yunnan, China	AF376831	AY033974	MVZ 176513
Vespertilio murinus	Valais, Switzerland	AF376834	AY033964	IZEA 3599
Subgenus *Myotis*				
Myotis bechsteinii	Jura, Switzerland	AF376843	AY033978	IZEA 3390
Myotis blythii blythii	Os, Kirghizstan	AF376840	AY033966	IZEA 4726
Myotis b. oxygnathus	Peloponnese, Greece	AF376841	AY033988	(No voucher)
Myotis b. punicus	Meknes, Morocco	AF376842	AY033959	IZEA 3791
Myotis emarginatus	Thessaloniki, Greece	AF376849	AY027859	ER 99
Myotis myotis	Bavaria, Germany	AF376860	AY033986	ER 1312
Myotis nattereri	Peloponnese, Greece	AF376863	AY033984	ER 1633
Myotis schaubi	Choplu, Iran	AF376868	AY033955	PB 1278
Myotis thysanodes	Texas, USA	AF376869	AY033957	TK 78796
Myotis welwitschii A	Rwenzori Mts, Uganda	AF376873	—	FMNH 144313
Myotis welwitschii B	Transvaal, South Africa	AF376874	AY033953	TM 39421
Subgenus *Selysius*				
Myotis brandtii	Neuhaus, Germany	AF376844	AY027851	ER 97
Myotis dominicensis	St. Joseph's Parish, Dominica	AF376848	AY033965	TK 15613
Myotis keaysi	Yucatan, Mexico	AF376852	AY033963	TK 13532
Myotis muricola browni	Mindanao, Philippines	AF376859	AY033958	FMNH 147067
Myotis mystacinus	Württemberg, Germany	AF376861	AY027848	ER 122
Myotis nigricans	Paraiba, Brazil	AF376864	AY033983	MVZ AD50
Subgenus *Leuconoe*				
Myotis albescens	Tarija, Bolivia	AF376839	AY033952	FMNH 162543
Myotis capaccinii	Peloponnese, Greece	AF376845	AY033989	(No voucher)
Myotis dasycneme	Leiden, Holland	AF376846	AY033977	IZEA 5049
Myotis daubentonii	Bavaria, Germany	AF376847	AY033985	ER 144
Myotis d. nathalinae	Ciudad Real, Spain	AF376862	AY033954	(No voucher)
Myotis hasseltii	Selangor, Malaysia	AF376850	AY033973	SMF69345
Myotis levis	Sao Paolo, Brazil	AF376853	—	FMNH 141600
Myotis lucifugus	Alaska, USA	AF376854	AY033967	UAM 22927
Myotis macrotrasus A	Mindanao, Philippines	AF376855	AY033962	FMNH EAR1222
Myotis macrotrasus B	Mindanao, Philippines	AF376856	AY033951	FMNH EAR1223
Myotis montivagus A	Selangor, Malaysia	AF376857	AY033972	SMF69340
Myotis montivagus B	Selangor, Malaysia	AF376858	AY033971	SMF69341
Myotis oxyotus	Lima, Peru	AF376865	AY033956	FMNH 129208
Myotis riparius	Pernambuco, Brazil	AF376866	AY033982	MVZ AD119
Myotis ruber	Salesopolis, Brazil	AF376867	AY033981	MVZ AD472
Myotis velifer	Sonora, Mexico	AF376870	AY033980	MVZ 146766
Myotis volans A	Texas, USA	AF376871	AY033960	TK 78980
Myotis volans B	Texas, USA	AF376872	AY033961	TK 78925
Myotis yumanensis	California, USA	AF376875	AY033979	MVZ 15585

[a] Koopman (1994).

[b] ER, Institute of Zoology, University of Erlangen, Germany; FMNH, Field Museum of Natural History, USA; IZEA, Institute of Ecology, University of Lausanne, Switzerland; MVZ, Museum of Vertebrate Zoology, UC Berkeley, USA; PB, Petr Benda, Prague National Museum, Czech Republic; SMF, Senckenberg Museum of Frankfurt, Germany; TK, Museum of Texas Tech University; TM, Transvaal Museum, South Africa; UAM, University of Alaska Museum, USA.

[c] A. Dietchfield and C. Handley, pers. com.

72°C (1 min), with a final extension at 72°C (5 min). PCR products were sequenced directly with L14724 and H15915 primers and followed automated sequencing protocols (Applied Biosystems Model 377). The sequence overlap between these two fragments of the *cytb* was about 300 bp and allowed a control of homology among independent PCR products of the same extract. Similarly, the nicotinamide adenine dinucleotide dehydrogenase subunit I gene (*nd1*) was amplified with primers ER65 (L2985, 5′-CCTCGATGTTGGATCAGG-3′) and ER66 (H4419, 5′-GTATGGGCCCGATAGCTT-3′) and sequenced with internal primers as described in Petit (1998). To insure accuracy of the sequences, most fragments were amplified and sequenced at least two times. As the two target genes code for proteins, sequences were easily aligned by eye. For four American species (*E. diminutus, M. nigricans, M. ruber,* and *M. riparius*), we tested whether the *cytb* sequences amplified from total genomic extracts were identical to those amplified from mtDNA-enriched extracts (Beckman *et al.,* 1992). As no difference was detected between PCR products of these two extracts, and because no insertions, deletions, or stop codons were present within the coding region, we assumed that all sequences were the original mitochondrial genes and not nuclear pseudogenes.

Phylogenetic Reconstruction

To minimize long branches due to a single distant outgroup, *Miniopterus, Lasiurus, Scotophilus, Nyctalus, Vespertilio,* and *Eptesicus* provided a composite outgroup. To explore the sensitivity of our results, the methods of maximum-likelihood (ML), maximum-parsimony (MP), and neighbor-joining (NJ) were used to reconstruct the phylogenetic evolution of these bats. The model of DNA evolution (HKY 85 + Γ) used to calculate likelihoods and genetic distances takes into account the uneven nucleotide composition of the gene pool (empirical composition), the bias in transition mutations relative to transversions (TS/TV ratio), and rate heterogeneity (gamma shape parameter) of DNA evolution within sequences (Hasegawa *et al.,* 1985; Yang, 1993). These parameters were estimated with PAUP4.0 (version 4.0b4 for power Macintosh) (Swofford, 1996) from the data with the MP tree as an initial topology (Swofford *et al.,* 1996). Estimated values for the *cytb* data set were 8.12 for TS/TV ratio and 0.24 for gamma; for the *nd1* gene these values were 9.00 and 0.25, respectively. As estimates from both genes are similar, we used the same model of DNA evolution (with TS/TV ratio set at 8.56 and gamma shape set at 0.24) for all subsequent analyses. The best ML tree was estimated from a NJ tree as a starting topology, followed by the nearest-neighbor interchange swapping algorithm. Maximum-parsimony analyses (heuristic search with 25 random input orders of taxa and TBR branch swapping) were run both with equally weighted characters and with transitions weighted 2, 5, and 10 times more than transitions. Neighbor-joining trees were generated from the distance matrix of corrected pairwise sequence divergence (HKY 85 + Γ). Levels of repeatability of the branching patterns were assessed with 1000 bootstrap replicates. As suggested by Hillis and Bull (1993), nodes with more than 70% bootstrap support were considered well supported. Because each ML analysis of the 46 sequenced taxa needed considerable computing time, no swapping algorithm was used to bootstrap the ML tree.

To test whether the estimated trees were consistent with the hypothesis of a monophyletic origin of the subgenera in *Myotis,* we used a likelihood ratio test (Kishino and Hasegawa, 1989) and the Templeton test (Templeton, 1983), available in PAUP4.0. The most stringent way to falsify this hypothesis is to force all three subgenera to be respectively monophyletic and compare the score of this constrained tree with that of the optimal, unconstrained reconstruction. Significant deviation from the constrained tree may, however, be due to the placement of a particular taxon, and rejection of the whole hypothesis in this case would be too conservative. We therefore also tested the monophyly of each subgenus separately. Similarly, the alternative hypothesis of a monophyletic evolution according to the biogeographic origin of the species was tested in the same way.

RESULTS

Characteristics of the cytb and nd1 Data Sets

The 46 complete *cytb* sequences of the bats sequenced here are deposited in GenBank (accession numbers in Table 1). All sequences consisted of 1140 bp with ATG as a start codon and AGA as a stop codon. As observed in other mammalian mitochondrial sequences (Irwin *et al.,* 1991; Johns and Avise, 1998), the overall nucleotide composition of the *cytb* is biased toward a deficit of guanine residues (A = 0.297, C = 0.257, G = 0.133, and T = 0.313). As usual, third positions are extreme in this respect with only 3.8% guanine. Sequences of 800 bp of the *nd1* gene were obtained from the same individuals (Table 1), except for *M. levis* and *M. welwitschii* A, which could not be sequenced for that gene. All sequences for this gene had ATG as a start codon. Comparable overall patterns of nucleotide composition was observed in *nd1* (A = 0.319, C = 0.248, G = 0.125, and T = 0.308), with only 4.8% guanines at the third codon position.

Although *cytb* and *nd1* genes are linked on the same mitochondrial molecule, several authors have shown that they may evolve at different rates (e.g., Zardoya and Meyer, 1996). In a bivariate plot of pairwise divergence (Fig. 1), each gene appeared to give similar uncorrected sequence divergence estimates among bats.

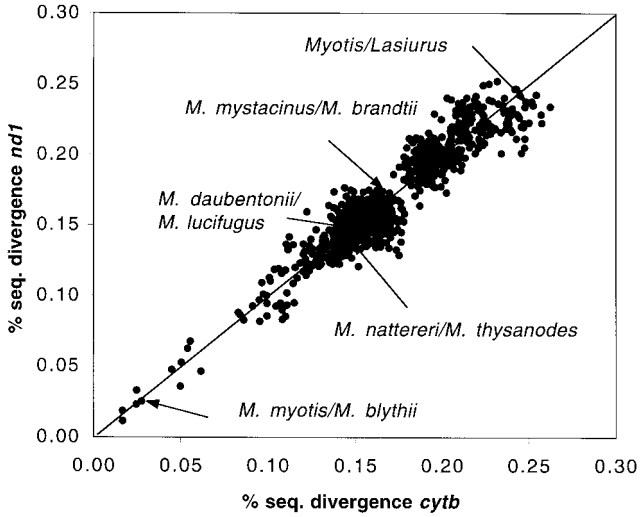

FIG. 1. Uncorrected pairwise sequence divergence of bats measured by variation of the *cytb* (x axis) or the *nd1* gene (y axis). The correlation coefficient of the two measures is high ($r^2 = 0.998$) and highly significant (Mantel's test, $P < 0.005$). The lowest dot (at 1.7%) represents divergence estimates of the pair *Eptesicus serotinus* and *E. nilssonii*. The next lowest divergence is that between *Myotis myotis* and *M. b. oxygnathus*, at about 2.5%. Two clusters above 18% sequence divergence represent all intergeneric comparisons (e.g., *Myotis*/outgroups).

A paired signed rank test revealed no significant departure ($P = 0.820$) from the null hypothesis of equal median sequence divergence between the two data sets. The phylogenetic content of both genes, as judged from the relative number of parsimony-informative sites, was also similar (see below).

Sequence Divergence within and among Species

As usual for conspecific bats sampled in the same geographic area (Barratt *et al.,* 1997; Ditchfield, 2000; Petit, 1998; Wright *et al.,* 1999), raw sequence divergence among individuals of the same species showed minimal variation (typically less than 1%). For instance, *E. serotinus* from Germany differed by only four TS from the *cytb* sequence (635 bp) of a British specimen deposited in GenBank (Barratt *et al.,* 1997). For the same gene, two *M. welwitschii* sampled in Eastern and Southern Africa were more divergent (4.5% sequence divergence), as were the two examined *M. macrotarsus* (about 1.7% at both genes) and *M. volans* (2.3%). More surprising was the 5.0% difference evidenced at both genes between two Malay *M. montivagus* sampled in the same area (Heller and Volleth, 1989). This may reflect the existence of additional cryptic taxonomic variability or indicate a region of admixture of two well-differentiated subpopulations. At the subspecific level, haplotypes of *M. b. blythii* from Kirghiztan and of *M. b. oxygnathus* from Greece were clearly distinct, with 5.5 and 6.7% sequence divergence at *cytb* and *nd1,* respectively. Both differed substantially from the North African subspecies *M. b. punicus* (about 11%), which probably warrants full species rank (Castella *et al.,* 2000).

Interspecific divergence within the genus *Myotis* was usually higher than 10% (mean 15% for both genes). The least divergent taxa were the two sibling species *M. myotis* and *M. blythii* from Europe, with about 2.5% sequence divergence (Fig. 1). Sequences of other sibling species of *Myotis* were more distinct. For instance, *M. mystacinus* and *M. brandtii* were recognized as representing two distinct species only during the 1970s (Gauckler and Kraus, 1970; Hanak, 1970) and are still difficult to identify by external characters (Schober and Grimmberger, 1987). As seen from the sequence divergence of their haplotypes (about 16%), they appear to be genetically very distinct from each other.

Unexpected patterns of divergence of DNA sequences were found between two very distinct species of European *Eptesicus, E. serotinus* and *E. nilssonii*. Although these two species are phenetically easily recognized and live in sympatry with no evidence of hybridization, they have similar haplotypes (<2% sequence divergence). This low level of genetic differentiation was consistent both in mitochondrial genes (the lowest point in Fig. 1) and among many individuals of each species sequenced independently in two different laboratories (results not shown); this multiple evidence excludes the possibility of a PCR artifact or of a cross-contamination among samples. Further work is necessary to determine whether this unexpected similarity of haplotypes is peculiar to the mitochondrial DNA of these bats or whether it is representative of the whole genome.

Phylogenetic Analyses

As an initial step, we analyzed the *cytb* and *nd1* data sets separately. The *cytb* data set consisted of 1140 characters, of which 500 were parsimony informative. These sites were 113 first, 33 second, and 354 third codon positions. The unweighted parsimony analysis of the *cytb* data set produced a single most parsimonious tree of 3791 steps. Although other methods of reconstructions (weighted parsimony, maximum-likelihood, or neighbor-joining trees) resulted in slightly different tree topologies, robust nodes in the bootstrapped MP tree were also strongly supported in other reconstructions (Table 2). Methods correcting for multiple hits (i.e., weighted parsimony or neighbor-joining trees) resulted in greater levels of support for several deep clades. A typical example is the monophyly of all American *Myotis* which is supported by 77% bootstrap in the unweighted MP tree and by 96% in the 5:1 weighted MP tree (Table 2). In contrast, the phylogenetic positions of some individual taxa, such as *M. montivagus, M. mystacinus,* or *M. capaccinii,* or of several basal clades received low bootstrap support in all reconstruc-

TABLE 2

Comparative Bootstrap Support (Percentage of 1000 Replicates) for all *Myotis* Clades that Received at Least 70% Support in the Combined Analyses (see Fig. 2)

	MP			MP-5:1			NJ-HKY85+			ML-HKY85+Γ		
Robust clade	*cytb*	*nd1*	C+N	*cytb*	*nd1*	C+N	*cytb*	*nd1*	C+N	*cytb*	*nd1*	C+N
A: levis + *nigricans*	98	n.a.	97	96	n.a.	98	98	n.a.	80	98	n.a.	78
B: A + *oxyotus*	100	100	100	100	100	100	100	100	100	100	100	100
C: B + *albescens*	79	<	70	92	<	75	95	<	85	96	<	87
D: velifer + *yumanensis*	88	98	98	97	97	99	98	100	100	96	100	100
E: D + *dominicensis*	72	<	70	56	73	87	98	51	92	98	<	94
F: E + *C*	91	93	99	91	99	100	96	99	100	99	99	100
G: riparius + *ruber*	88	76	94	96	87	98	96	95	100	96	92	99
H: G + *keaysi*	59	74	85	96	78	98	98	85	100	89	85	99
I: H + *F*	<	<	<	72	62	92	85	54	85	50	<	70
J: lucifugus + *thysanodes*	100	100	100	100	100	100	100	100	100	100	100	100
K: J + *volans*	95	95	100	92	98	100	92	98	100	97	98	100
L: I + *K* + *brandtii* = Clade I	60	83	98	98	97	100	86	97	99	85	91	99
M: oxygnathus + *myotis*	100	100	100	100	99	100	100	99	100	100	99	100
N: M + *blythii*	100	98	100	100	100	100	100	99	100	100	100	100
O: N + *punicus* + *nattereri* + *schaubi* = Clade II	51	<	65	84	<	93	54	<	91	<	<	81
P: daubentonii + *nathalinae*	100	100	100	100	100	100	100	98	100	100	100	100
Q: P + *bechsteinii* = Clade III	98	<	99	100	56	100	100	<	100	100	<	100
R: macrotarsus + *hasseltii* + *horsfieldii* = Clade IV	78	85	94	92	92	98	88	88	97	87	83	96
S: welwitschii + *emarginatus* = Clade V	58	<	<	87	<	95	98	<	96	88	<	85
T: all Myotis	77	59	98	96	91	100	84	59	97	79	<	96

Note. Criteria used to perform phylogenetic reconstructions are unweighted (MP) and weighted (MP 5:1) maximum-parsimony, neighbor-joining (NJ), and maximum-likelihood (ML), both with HKY85 + Γ model of DNA evolution; <, less than 50% bootstrap support. As *nd1* of *M. levis* was missing, bootstrap level for clade A is not available for that gene.

tions, and thus their phylogenetic positions are uncertain.

The *nd1* data set consisted of 800 characters for 44 taxa (sequences of *M. welwitschii* A and *M. levis* were missing). Of the 359 parsimony-informative characters, 89 were first, 25 second, and 245 third position substitutions. A similar phylogenetic picture emerged from results of the *nd1* data set compared to those of the *cytb,* except that the overall level of bootstrap support was generally lower (Table 2). The congruence of phylogenetic information among genes was confirmed by the homogeneity test of Farris *et al.* (1995), which indicates no significant conflict between the two data sets ($P = 0.65$). We therefore combined *cytb* and *nd1* sequences for further phylogenetic inferences. To use all available information obtained for *M. levis* and for *M. welwitschii* A (i.e., 1140 bp of *cytb* but no *nd1* sequence; Table 1), we replaced their missing values by Ns. Figure 2 illustrates the maximum-likelihood tree (score $-\ln = 26349.18$) resulting from a heuristic search, with the HKY85 + Γ model of DNA evolution with a gamma shape parameter of 0.24 and a TS/TV ratio of 8.56. This tree is almost identical to the single most parsimonious tree found in a heuristic search with transversions weighted five times more than transitions (only three topological discrepancies; see Fig. 2). As in the separate analyses, the total evidence ML tree of Fig. 2 reveals many short internal branches at the basis of the *Myotis* radiation. These nodes received low bootstrap support in any analysis. Thus, despite a high number of characters used in this combined analysis (1940 characters), only few additional nodes were resolved with a good bootstrap support compared to those obtained for instance in the *cytb*-only data (Table 2).

The monophyly of the 33 *Myotis* taxa relative to the outgroups was firmly confirmed by the molecular data. Five major clades were strongly supported by all analyses (Table 2). One clade (clade II) includes the two largest species of the genus (*M. myotis* and *M. blythii*) and the pair *M. schaubi* and *M. nattereri*. *M. bechsteinii* and the two subspecies of *M. daubentonii* constitute another monophyletic unit (clade III) with very high bootstrap support in all analyses. Similarly, *M. macrotarsus, M. hasseltii,* and *M. horsfieldii* from southeast Asia appear monophyletic in all analyses (clade IV), as were *M. welwitschii* and *M. emarginatus* (clade V). The last major clade groups all 13 American species plus *M. brandtii* into a monophyletic unit (clade I). For convenience, we will refer to this group as the "American clade." Most nodes within this clade received strong bootstrap support, indicating that the resolution within this group is very good (Fig. 2).

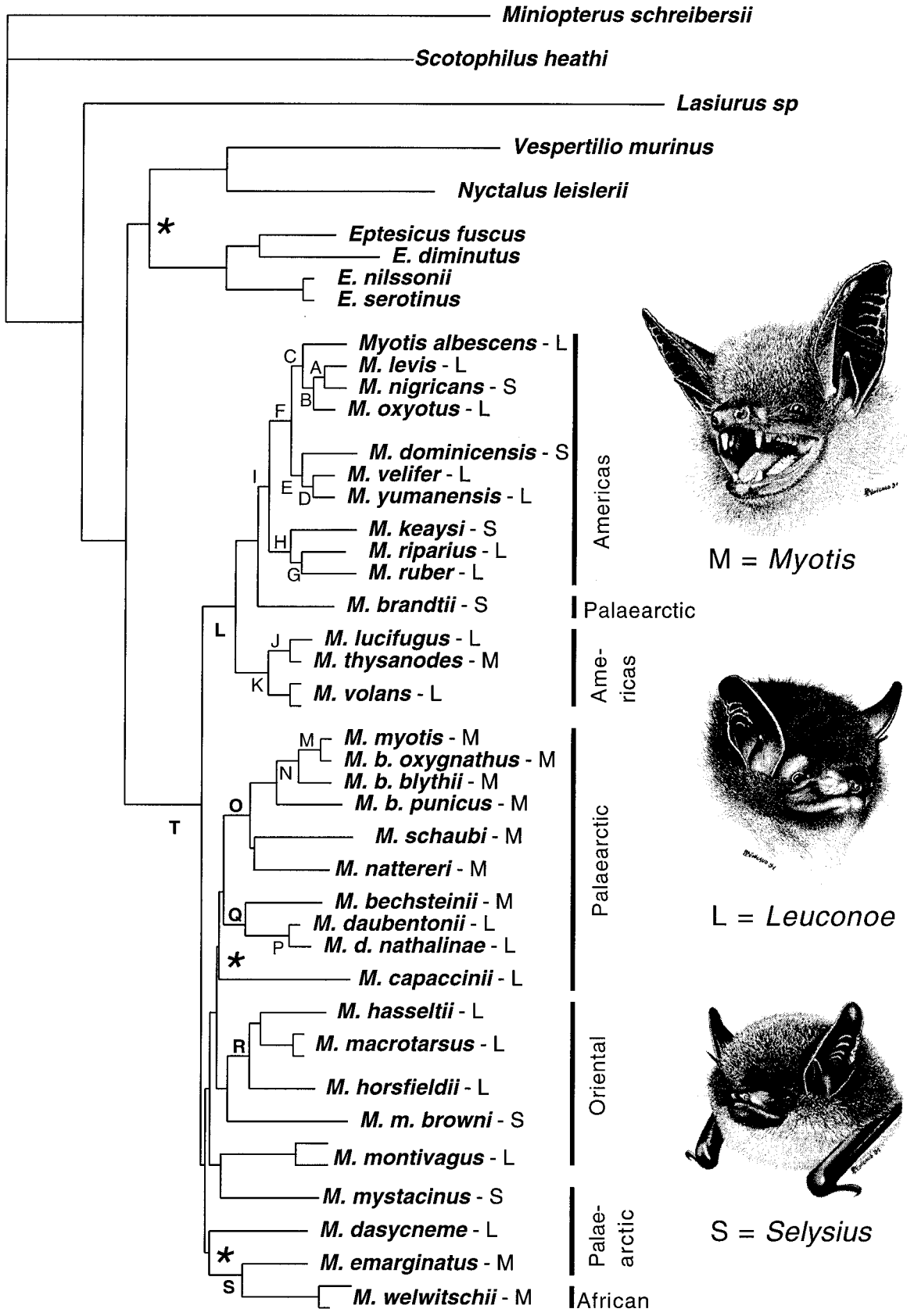
Miniopterus schreibersii
Scotophilus heathi
Lasiurus sp
Vespertilio murinus
Nyctalus leislerii
Eptesicus fuscus
E. diminutus
E. nilssonii
E. serotinus
Myotis albescens - L
M. levis - L
M. nigricans - S
M. oxyotus - L
M. dominicensis - S
M. velifer - L
M. yumanensis - L
M. keaysi - S
M. riparius - L
M. ruber - L
M. brandtii - S
M. lucifugus - L
M. thysanodes - M
M. volans - L
M. myotis - M
M. b. oxygnathus - M
M. b. blythii - M
M. b. punicus - M
M. schaubi - M
M. nattereri - M
M. bechsteinii - M
M. daubentonii - L
M. d. nathalinae - L
M. capaccinii - L
M. hasseltii - L
M. macrotarsus - L
M. horsfieldii - L
M. m. browni - S
M. montivagus - L
M. mystacinus - S
M. dasycneme - L
M. emarginatus - M
M. welwitschii - M
Americas
Palaearctic
Ame-
ricas
Palaearctic
Oriental
Palae-
arctic
African
M = Myotis
L = Leuconoe
S = Selysius

TABLE 3

Tests of Alternative Hypotheses of Monophyly Compared to the Most Parsimonious Trees (Templeton Test) or the Maximum-Likelihood Tree (Likelihood Ratio Test), Using the Combined *cytb* + *nd1* Data Sets

	Templeton test			Likelihood ratio test		
Constrained group	Score	Extra steps	*P* value	−ln L	Δ ln L	*P* value
Unconstrained (best) trees	6408	—	—	26349.2	—	—
All subgenera monophyletic	6784	376	<0.001	27190.4	841.2	<0.001
Selysius monophyletic	6555	147	<0.001	26719.9	370.7	<0.001
Myotis monophyletic	6547	139	<0.001	26638.8	289.6	<0.001
Leuconoe monophyletic	6720	312	<0.001	27067.8	718.6	<0.001
American monophyletic	6414	6	0.786	26356.4	7.2	0.327
Indomalayan monophyletic	6413	5	0.773	26358.0	8.8	0.158
Palaearctic monophyletic	6465	57	0.004	26503.7	154.5	<0.001
Palaearctic (without *brandtii*)	6431	23	0.18	26403.5	48.3	<0.001

The phylogenetic position of these five major clades relative to each other could not be firmly established. Indeed, since all basal branches of the *Myotis* radiation are short (see Fig. 2), the exact position of the root depended on the kind of analysis applied to the character matrix. For instance, based on all characters, the American clade was the most basal species in the maximum-likelihood tree (Fig. 2), but clade V was more basal in the NJ or weighted MP trees. However, these distinct rooted trees did not differ significantly under the Kishino–Hasegawa test ($P > 0.31$), and, therefore, the position of the root is best treated as uncertain.

Tests of the Monophyly of Ecomorphs

A visual inspection of all phylogenetic reconstructions suggests that none of the three classical subgenera define monophyletic units. However, as considerable uncertainty remains as to the precise phylogenetic position of some taxa or clades, a formal test of this hypothesis is necessary. Using all available molecular information (combined data set), Templeton tests clearly rejected the monophyly of subgenera as an alternative topology to the most parsimonious, unconstrained trees (Table 3). This hypothesis was clearly rejected either in global tests or when the monophyly of any one subgenus was enforced. Under the framework of maximum-likelihood, the tree of Fig. 2 was significantly better than any tree reconstructed under alternative hypotheses of monophyletic evolution of subgenera (Table 3).

The other scenario of species radiation envisioned here is the monophyletic evolution of all taxa within each respective biogeographic region. In this case, all but one test could not reject the constrained tree as an alternative to the best topology. For instance, reconstructions constraining all 13 American species to be monophyletic (i.e., leaving out *M. brandtii*) did not differ significantly from the unconstrained tree, under either the parsimony or the maximum-likelihood criterion (Table 3). The same is true for the monophyletic evolution of the five Oriental species, but the limited taxonomic sampling in this biogeographic region precludes any definitive conclusion. Results concerning the Western Palaearctic region are more meaningful as they include all known species from this region. When all 14 taxa are forced into a monophyletic clade, the best tree under this hypothesis is significantly worse than the unconstrained tree (Table 3). This is essentially due to the position of *M. brandtii*, which clusters within the American clade in all unconstrained phylogenetic reconstructions (e.g., Fig. 2). If this Eurasian species is removed from the group of Palaearctic species, then the score of the constrained tree improves considerably.

DISCUSSION

The genus *Myotis* has had an incontestable evolutionary success as representatives of this genus may be found over all continents, from the boreal to sub-Antarctic zones, tropical rain forests, or semidesertic habitats. Ecologically, the approximately 90 species currently included in this genus are also diversified, with some taxa being piscivorous, others aerial planktonic feeders, and others terrestrial gleaners. This ecological

FIG. 2. Maximum-likelihood tree (score: $-\ -\ln = 26349.18$) obtained from the combined *cytb* + *nd1* data sets with the HKY85 + Γ model of evolution. Branch lengths are proportional to the quantity of mutational changes along them (i.e., the probability of change along the terminal branch leading to *Scotophilus heathi* is 0.40). The level of bootstrap support of the well-supported nodes (indicated by a letter) can be found in Table 2. An asterisk (*) identifies the three nodes which differed in other tree reconstruction methods analyzed with the same data set. The letter behind species names of *Myotis* refers to their current subgeneric classification as follows: M for *Myotis* (type species illustrated *M. myotis*), L for *Leuconoe* (type *M. daubentonii*), and S for *Selysius* (type *M. mystacinus*).

diversity allows most communities of insectivorous bats to include several coexisting species of *Myotis* which share space and resources without apparent competitive exclusion. The diversity of resource exploitation and use of space is partly reflected by morphological characteristics and echolocation call design (Arlettaz, 1999; Saunders and Barcley, 1992). Current subdivision of the genus into four subgenera therefore clusters species with similar, although not identical, ecomorphological adaptations (Findley, 1972; Koopman, 1994). A corollary of this subdivision is that morphological similarities among species in a subgenus can reflect ecological similarities, close phylogenetic relationships, or both. To understand how this diversity has evolved, it is important to tease apart these two components. Using phylogenetic reconstructions based on sequences of mitochondrial DNA of a sample of 33 *Myotis* taxa, we show that morphological similarities rarely reflect close phylogenetic relationships. All inferred phylogenetic trees illustrate several examples in which taxa currently classified into different subgenera cluster together as sister species. *M. daubentonii* for instance is a relatively small bat with short ears and it catches prey over water surfaces (Jones and Rayner, 1988); it typifies the subgenus *Leuconoe* (see Fig. 2). On all phylogenetic reconstructions, strong bootstrap support clusters *M. daubentonii* with *M. bechsteinii,* a species with characteristics distinctive of the subgenus *Myotis* (larger size, long ears, relatively small feet, typical behavior of a substrate gleaner, etc.). Furthermore, *M. daubentonii* is morphologically and ecologically the Palaearctic equivalent of *M. lucifugus,* living in North America (Fenton and Barclay, 1980). Molecular data place these two species into completely distinct clades (Fig. 2), suggesting that their remarkable similarities in morphology and behavior are the result of convergent evolution independent of their phylogenetic history. The same is true for the pair *M. nattereri* and *M. thysanodes,* which represent the Palaearctic and Nearctic members of "fringed bats," sometimes classified together in the distinct subgenus *Isotus* (Corbet and Hill, 1991; Tate, 1941). According to our molecular reconstructions, they both evolved in different clades, again suggesting that their morphological and behavioral resemblance are the result of convergent evolution. Convergence of morphology and behavior is also shown within the subgenus *Selysius* with the pair of sibling species *M. mystacinus* and *M. brandtii*. Despite their behavioral and morphological resemblance which make them hard to identify in the field (Gauckler and Kraus, 1970; Hanak, 1970), they do not appear as close relatives on the molecular trees; rather, *M. brandtii* consistently clusters with the Nearctic and Neotropical species within the American clade, and *M. mystacinus* is part of a more basal cluster of uncertain affinities (Fig. 2).

To evaluate more generally the lack of concordance between morphological evolution and phylogenetic relationships, we tested the significance of a series of a priori hypotheses concerning the monophyletic evolution of the three subgenera of *Myotis* represented in our samples, using the combined molecular data set (Table 3). If the phylogenetic relationships among species of *Myotis* are constrained to group all taxa into monophyletic subgenera, then the best tree compatible with this hypothesis is significantly worse than the unconstrained tree. As shown in Table 3, this result is further supported by less stringent tests, i.e., when only one subgenus at a time is constrained to be monophyletic. Thus, based on sequences of mtDNA genes, we reject with high statistical confidence the idea that a given subgenus has evolved uniquely prior to species diversification. Rather, each subgenus typifies major ecomorphs which evolved independently and multiple times during the radiation of *Myotis* species. In this respect, we concur with Menu (1987) and Godawa Stormark (1998) who examined dental characteristics of many Old World *Myotis* and concluded that the current classification based essentially on external morphology does not reflect phylogenetic affinities.

Overall, these molecular data based on mitochondrial genes demonstrate that the level of morphological similarity among these bats is a poor predictor of their genetic similarities, as already shown within the genus *Pipistrellus* (Barratt *et al.,* 1997). There is, however, a possibility that the history of the mtDNA genes in general does not reflect the real phylogenetic relationships among species (see, e.g., Avise, 1989). It would be a serious problem if, for instance, transfer of mtDNA across species boundaries through hybridization was frequent. As far as we know, such hybridization has never been substantiated in any species of bat (Arlettaz *et al.,* 1991; Herd and Fenton, 1983; Ruedi *et al.,* 1990). In addition, because of their haplotypic, nonrecombinant nature and strict maternal inheritance, mitochondrial lineages are expected to be fixed by the process of genetic drift four times faster than nuclear genes (Avise, 1989); thus, phylogenies inferred from mtDNA are more likely to represent species trees rather than a singular gene tree, because most lineages should have evolved toward reciprocal monophyly within each species (reviewed in Avise and Walker (1999)). This is supported by the fact that most interspecific comparisons are by far larger than intraspecific comparisons (see Results). We therefore assume that at least the majority of relationships suggested in our molecular trees do reflect phylogenetic relationships of the species and not only those of a particular gene.

The most striking pattern in the molecular *Myotis* phylogeny is that the biogeographic origin of the species appeared to be a much better predictor of their phylogenetic position than their morphology (Fig. 2). For instance, the 13 assayed New World species represent a broad spectrum of morphological diversity, yet

they appear together in a strongly supported monophyletic clade (the "American" clade; Table 2). Although only about a third of all current species of *Myotis* from the Americas were sampled, our molecular data suggest that they evolved from a single ancestor. Interestingly, the only non-American species appearing in this clade is *M. brandtii,* which has a wide distribution across northern Eurasia, including the Kamchatka Peninsula in Far Eastern Siberia (Borissenko and Kruskop, 1997; Koopman, 1994). It is possible that this boreal species evolved from a North American ancestor and colonized Eurasia secondarily through the Beringean Bridge. All other sequenced West Palaearctic species (13 taxa) were clearly more distantly related to the American clade (Fig. 2 and Table 2). Species from the other biogeographic regions are underrepresented in our sample, so no meaningful conclusion can be drawn before a more comprehensive sample of taxa is analyzed. We simply note that 3 of the 5 sequenced Oriental species formed a well-supported clade (clade IV, Table 2).

The genus *Myotis* has a long and relatively well-documented fossil history in Europe. Here, we consider the extent to which paleontological and molecular data can be combined to infer biogeographic patterns of evolution. Whereas the earliest fossil record attributed to the genus *Myotis* dates back to the early Oligocene (*Myotis misonnei*; Quinet, 1965), some 30 million years ago (Mya), the main pulse of diversification of the genus appears much later, during the late Miocene/early Pliocene of Europe (Ariagno, 1984). More precisely, this diversification is first apparent 5–6 Mya in several Ruscinian-age localities, such as Podlesice, Osztramos (Godawa, 1993) or Gundersheim (Heller, 1936) in eastern Europe. Evidence of *Myotis* fossils in Africa or Asia is fragmentary and more recent (late Pliocene/early Pleistocene), and no ancient fossil has been found so far in Australia (Savage and Russel, 1983). New World fossils of bats in general and of *Myotis* in particular were thought to be much younger, dating from the Irvingtonian land-mammal age, 1–1.5 Mya (early Pleistocene; Kurtén and Anderson, 1980). However, recent examination of the Tertiary fossil record of Nebraska (Czaplewski *et al.,* 1999) and Oklahoma (Dalquest *et al.,* 1996) suggest that *Myotis* bats may have been present in North America since the late Miocene. The current prevailing opinion among paleontologists is that the genus *Myotis* is an immigrant which invaded Europe, Africa, and the New World from another, yet unknown part of Asia (Findley, 1972; Menu, 1987).

Our molecular data corroborate some aspects of this paleontological scenario. First, the structure of the *Myotis* tree shown in Fig. 3 indicates that the species diversity has not accumulated randomly. There was a major pulse of speciation soon after the origin of the genus. Dating the divergence time of this pulse with molecular data is controversial (Hillis *et al.,* 1996), especially if calibration of divergence rates are issued from outgroup taxa. As the *Myotis* fossil record in Europe is quite extensive, we have, fortunately, two dates which may be used to calibrate the *Myotis* clock. First, Horacek and Hanak (1983–1984) revised a large collection of both recent and fossil material referred to *M. nattereri* and *M. schaubi* (and related taxa) and concluded that these two taxa shared probably a common ancestor in the upper Miocene, about 6 Mya. As both differ by 29.1% in their *cytb* + *nd1* sequences (ML distances), the clock would "tick" at about 4.8% per Myr. Similarly, according to Topál (1983), *M. paradaubentonii* is considered a direct ancestor of *M. daubentonii*. Thus, this lineage has been distinct from *M. bechsteinii* since at least the middle Pliocene, some 5 Mya. The ML corrected distance between these two species is 23.2%, which would correspond to 4.6% per Myr. Notice that the use of ML corrected distances make our calibrations not directly comparable to those of classical standards (e.g., Brown *et al.,* 1982; Hasegawa *et al.,* 1985), but are more appropriate to avoid the problem of saturation of mutations at the deeper nodes (Excoffier and Yang, 1999). Using this *Myotis*-calibrated rate of divergence, we estimated an approximate date of diversification from the linearized tree of Fig. 3, following the method of Takezaki *et al.,* (1995). This tree, obtained by enforcing a molecular clock, did not differ significantly from the unconstrained ML tree of Fig. 2 (Kishino–Hasegawa test; $P < 0.63$). Thus, under the assumption of a molecular clock, this tree indicates that the differentiation of most Palaearctic and Oriental species of *Myotis* took place during the Miocene, roughly between 5 and 9 Mya (mean 6.5 ± 1.6 Mya). In contrast the radiation of most assayed American species appears to be relatively younger, as it occurred mainly during the Pliocene (mean 4.6 ± 1.4 Mya). These time estimates are therefore compatible with the current understanding of the *Myotis* fossil record.

CONCLUSIONS

Despite quite an extensive DNA sampling (about 2000 bp sequenced in 46 vespertilionid bats), the evolution of species of the genus *Myotis* proved to be difficult to reconstruct, especially for the earlier radiations (Fig. 2). At least for the European taxa which represent the majority of species analyzed so far, we interpret this pattern as evidence of rapid species diversification, as suggested by the fossil record. This radiation not only is responsible for rampant low bootstrap support at many nodes in the molecular trees but renders attempts at rooting the *Myotis* radiation quite difficult. To circumvent these difficulties, recent reviews have suggested either increasing the taxonomic sampling or increasing the number of characters ana-

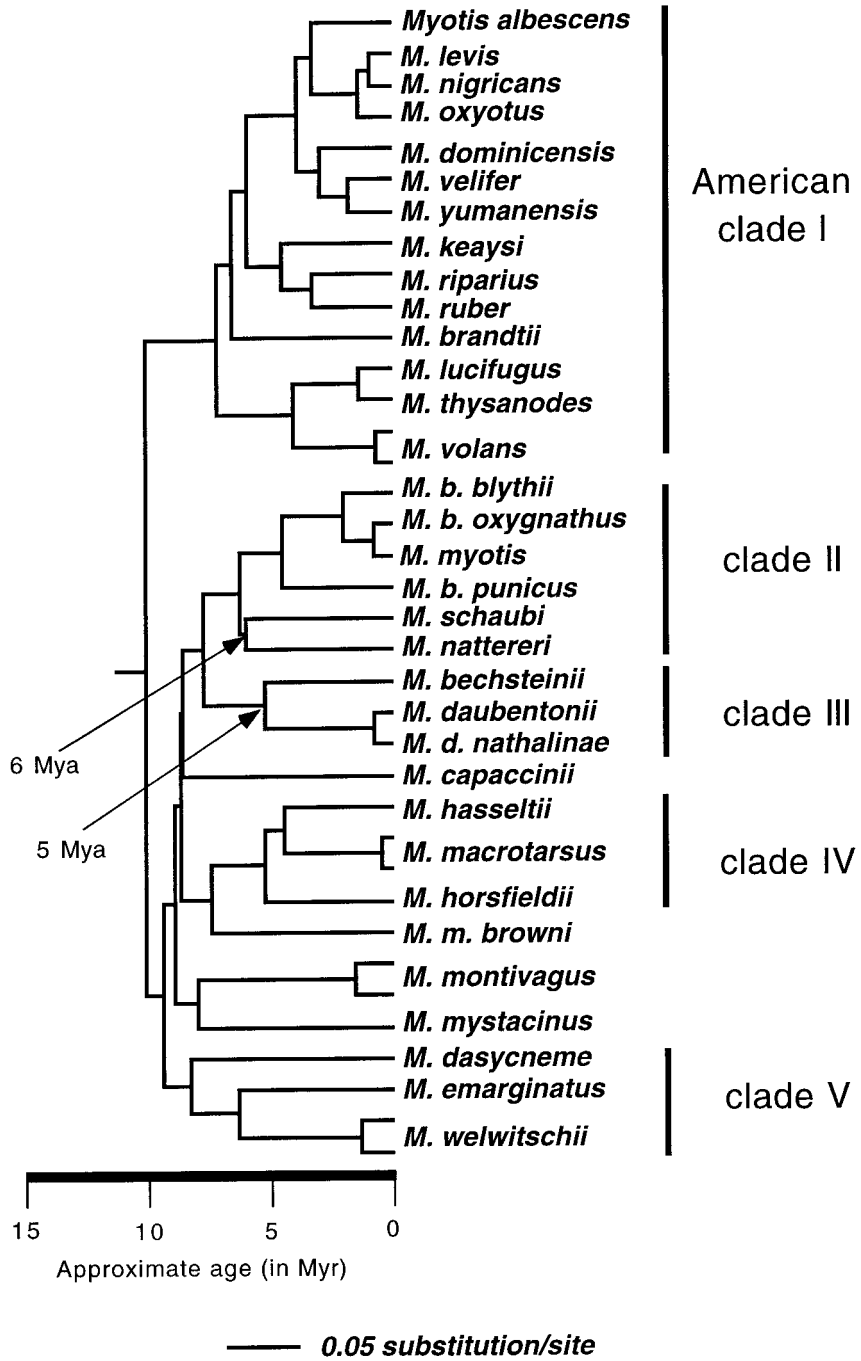

FIG. 3. Same maximum-likelihood tree as in Fig. 2, but was reconstructed under the constraint that all species evolved in a clock-like manner. Only the ingroup portion of the tree (all species of *Myotis*) is shown here. The arrows indicate the two nodes which were used to calibrate the divergence rate at 4.7% per Myr. The scale with divergence dates derives from this approximation.

lyzed. In our case, the number of nodes resolved with reasonable bootstrap support (Table 2) did not increase when the data set comprised the *cytb* gene alone (1140 bp) or when it was combined with the *nd1* gene (800 additional bp). The first option to break long branches with a more thorough taxonomic sampling seems therefore more promising and feasible since still two thirds of the species of *Myotis* wait to be analyzed. Despite these uncertainties, the phylogenetic signal contained in this molecular data set was sufficient to reject with high statistical confidence the hypothesis that the morphological diversification of the genus follows phylogenetic history. On the contrary, we showed that the same ecomorph appears to have evolved sev-

eral times independently. This kind of deterministic evolution has led to the situation in which a species found today in America appears morphologically almost identical to its European counterparts, yet both are completely unrelated on the phylogenetic tree. In fact, the independent evolution of *Myotis* species in the different biogeographic regions with subsequent convergent adaptive radiations is not an isolated case among vertebrates. Well-documented examples include cichlid fishes (Verheyen *et al.,* 1996), ranid frogs (Bossuyt and Milinkovitch, 2000), Caribbean anoles (Beuttell and Losos, 1999), river dolphins (Cassens *et al.,* 2000), and fruit bats (Alvarez *et al.,* 1999; Hollar and Springer, 1997). There is no doubt that other examples of morphological convergence will emerge as molecular assessments of phylogenetic relationships among morphologically similar organisms are extended to other taxa.

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