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 Palearctic, 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 Palearctic 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 Palearctic 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 (*Sylisius*, *Isotus*, *Paramyotis*, *Myotis*, *Chrysopteron*, *Leucoge*, and *Rickettia*). More recently, Findley (1972) used numerical taxonomy on 48 cranial and external...
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 Barclay, 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 “ecormorphs” (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 sympathy (Saunders and Barclay, 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 H2O (Sambrook et al., 1989). Each individual bat was sequenced for two mitochondrial genes, except for *M. levis* and *M. velutipennis* 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 MgCl2, 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

<table>
<thead>
<tr>
<th>GenBank</th>
<th>ND-1</th>
<th>Voucher</th>
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</thead>
<tbody>
<tr>
<td>Locality</td>
<td>Cyt-b</td>
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</tbody>
</table>

#### Family Vespertilionidae

**Subfamily Miniopterinae**

- **Miniopterus schreibersii** Barcelona, Spain
  - GenBank: AF376830
  - ND-1: AY033969
  - Voucher: IZEA 3431

#### Vespertilioninae

- **Eptesicus fuscus** California, USA
  - GenBank: AF376835
  - ND-1: AY033968
  - Voucher: MVZ 148681
- **Eptesicus diminutus** Paraná, Brazil
  - GenBank: AF376833
  - ND-1: AY033976
  - Voucher: MVZ AD496
- **Eptesicus nilssonii** Bavaria, Germany
  - GenBank: AF376836
  - ND-1: AY033987
  - Voucher: ER 1300
- **Eptesicus serotinus** Chalkidiki, Greece
  - GenBank: AF376837
  - ND-1: AY033950
  - Voucher: ER 659
- **Lasiurus sp.** Parana, Brasil
  - GenBank: AF376838
  - ND-1: AY033975
  - Voucher: MVZ AD522
- **Nyctalus leisleri** Obwald, Switzerland
  - GenBank: AF376832
  - ND-1: AY033949
  - Voucher: IZEA 2639
- **Scotophilus heathi** Yunnan, China
  - GenBank: AF376831
  - ND-1: AY033974
  - Voucher: MVZ 176513
- **Vespertilio murinus** Valais, Switzerland
  - GenBank: AF376834
  - ND-1: AY033964
  - Voucher: IZEA 3599

#### Subgenus Myotis

- **Myotis bechsteinii** Jura, Switzerland
  - GenBank: AF376843
  - ND-1: AY033978
  - Voucher: IZEA 3390
- **Myotis blythii blythii** Os, Kirghizstan
  - GenBank: AF376840
  - ND-1: AY033966
  - Voucher: IZEA 4726
- **Myotis b. oxygnathus** Peloponnes, Greece
  - GenBank: AF376841
  - ND-1: AY033988
  - Voucher: (No voucher)
- **Myotis b. punicus** Meknes, Morocco
  - GenBank: AF376842
  - ND-1: AY033959
  - Voucher: IZEA 3791
- **Myotis emarginatus** Thessaloniki, Greece
  - GenBank: AF376849
  - ND-1: AY027859
  - Voucher: ER 99
- **Myotis myotis** Bavaria, Germany
  - GenBank: AF376860
  - ND-1: AY033986
  - Voucher: ER 1312
- **Myotis nattereri** Peloponnes, Greece
  - GenBank: AF376863
  - ND-1: AY033984
  - Voucher: ER 1633
- **Myotis schaubii** Chapla, Iran
  - GenBank: AF376868
  - ND-1: AY033955
  - Voucher: PB 1278
- **Myotis thyssanodes** Texas, USA
  - GenBank: AF376869
  - ND-1: AY033957
  - Voucher: TK 78796
- **Myotis welwitschii A** Rwenzori Mts, Uganda
  - GenBank: AF376873
  - ND-1: —
  - Voucher: FMNH 144313
- **Myotis welwitschii B** Transvaal, South Africa
  - GenBank: AF376874
  - ND-1: AY033953
  - Voucher: TM 39421

#### Subgenus Selysius

- **Myotis brandtii** Neuhaus, Germany
  - GenBank: AF376844
  - ND-1: AY027851
  - Voucher: ER 97
- **Myotis dominicensis** St. Joseph’s Parish, Dominica
  - GenBank: AF376848
  - ND-1: AY033965
  - Voucher: TK 15613
- **Myotis keaysi** Yucatan, Mexico
  - GenBank: AF376852
  - ND-1: AY033963
  - Voucher: TK 13532
- **Myotis muricola browni** Mindanao, Philippines
  - GenBank: AF376859
  - ND-1: AY033958
  - Voucher: FMNH 147067
- **Myotis mystacinus** Württemberg, Germany
  - GenBank: AF376861
  - ND-1: AY027848
  - Voucher: ER 122
- **Myotis nigricans** Paraiba, Brazil
  - GenBank: AF376864
  - ND-1: AY033983
  - Voucher: MVZ AD50

#### Subgenus Leuconoe

- **Myotis albecens** Tarja, Bolivia
  - GenBank: AF376839
  - ND-1: AY033952
  - Voucher: FMNH 162543
- **Myotis capaccinii** Peloponnes, Greece
  - GenBank: AF376845
  - ND-1: AY033989
  - Voucher: (No voucher)
- **Myotis dasycneme** Leiden, Holland
  - GenBank: AF376846
  - ND-1: AY033977
  - Voucher: IZEA 5049
- **Myotis daubentonii** Bavaria, Germany
  - GenBank: AF376847
  - ND-1: AY033985
  - Voucher: ER 144
- **Myotis d. nathaliacae** Ciudad Real, Spain
  - GenBank: AF376862
  - ND-1: AY033954
  - Voucher: (No voucher)
- **Myotis hasseltii** Selangor, Malaysia
  - GenBank: AF376850
  - ND-1: AY033973
  - Voucher: SMFH345
- **Myotis levis** Sao Paolo, Brazil
  - GenBank: AF376853
  - ND-1: AY033967
  - Voucher: FMNH 141600
- **Myotis lucifugus** Alaska, USA
  - GenBank: AF376854
  - ND-1: AY033967
  - Voucher: UAM 22927
- **Myotis macrotragus A** Mindanao, Philippines
  - GenBank: AF376855
  - ND-1: AY033962
  - Voucher: FMNH
- **Myotis macrotragus B** Mindanao, Philippines
  - GenBank: AF376856
  - ND-1: AY033951
  - Voucher: EAB1223
- **Myotis montivagus A** Selangor, Malaysia
  - GenBank: AF376857
  - ND-1: AY033972
  - Voucher: SMFH 69340
- **Myotis montivagus B** Selangor, Malaysia
  - GenBank: AF376858
  - ND-1: AY033971
  - Voucher: SMFH 69341
- **Myotis oxytus** Lima, Peru
  - GenBank: AF376865
  - ND-1: AY033956
  - Voucher: FMNH 129208
- **Myotis riparius** Pernambuco, Brazil
  - GenBank: AF376866
  - ND-1: AY033982
  - Voucher: MVZ AD119
- **Myotis ruber** Salesopolis, Brazil
  - GenBank: AF376867
  - ND-1: AY033981
  - Voucher: MVZ AD472
- **Myotis vellifer** Sonora, Mexico
  - GenBank: AF376870
  - ND-1: AY033980
  - Voucher: MVZ 146766
- **Myotis volans A** Texas, USA
  - GenBank: AF376871
  - ND-1: AY033960
  - Voucher: TK 78980
- **Myotis volans B** Texas, USA
  - GenBank: AF376872
  - ND-1: AY033961
  - Voucher: TK 78925
- **Myotis yumanensis** California, USA
  - GenBank: AF376875
  - ND-1: AY033979
  - Voucher: MVZ 15585

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\(^a\) 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.

\(^\text{A}\) A. Dietrich 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’-CCCGATGTTGGATCAGG-3’) and ER66 (H4419, 5’-GTATGGCCCGTACGTT-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, Lasiusurus, Scotophilus, Nyctalus, Vesperilio, 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 + G) 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 + G). 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.
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 *cyt b* 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 *cyt b* and *ndl*, 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 sympathy 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 *cyt b* and *ndl* data sets separately. The *cyt b* 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 *cyt b* 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. capaccini*, or of several basal clades received low bootstrap support in all reconstruc-
### Table 2

<table>
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<th>Robust clade</th>
<th>MP cytb</th>
<th>MP-5:1 cytb</th>
<th>NJ-HKY85+ cytb</th>
<th>ML-HKY85+Γ cytb</th>
<th>MP ndl</th>
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<tr>
<td><em>F: E + C</em></td>
<td>91</td>
<td>91</td>
<td>96</td>
<td>96</td>
<td>93</td>
<td>99</td>
<td>99</td>
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<td>85</td>
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</tr>
<tr>
<td><em>G. riparius + ruber</em></td>
<td>88</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>76</td>
<td>87</td>
<td>96</td>
<td>95</td>
<td>92</td>
<td>92</td>
<td>92</td>
<td>99</td>
</tr>
<tr>
<td><em>H: G + caesium</em></td>
<td>59</td>
<td>96</td>
<td>98</td>
<td>98</td>
<td>74</td>
<td>87</td>
<td>85</td>
<td>85</td>
<td>99</td>
<td>99</td>
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<td>99</td>
</tr>
<tr>
<td><em>I: H + F</em></td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>50</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td><em>J: lucifugus + thyanoideas</em></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
<tr>
<td><em>K: J + volans</em></td>
<td>95</td>
<td>92</td>
<td>92</td>
<td>92</td>
<td>95</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>100</td>
<td>98</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td><em>L: I + K + brandtii = Clade I</em></td>
<td>60</td>
<td>98</td>
<td>96</td>
<td>96</td>
<td>83</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>99</td>
<td>85</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td><em>M: oxygnathus + myotis</em></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>N: M + blythii</em></td>
<td>100</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>O: N + punicus + nattereri + schaubi = Clade II</em></td>
<td>51</td>
<td>84</td>
<td>54</td>
<td>54</td>
<td>65</td>
<td>93</td>
<td>91</td>
<td>91</td>
<td>81</td>
<td>81</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td><em>P: daubentonii + nathalinae</em></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Q: P + bechsteinii = Clade III</em></td>
<td>98</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>&lt; 99</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>R: macrotarsus + hasseltii + horsfieldii = Clade IV</em></td>
<td>78</td>
<td>92</td>
<td>88</td>
<td>88</td>
<td>85</td>
<td>97</td>
<td>87</td>
<td>87</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td><em>S: welwitschi + emarginatus = Clade V</em></td>
<td>58</td>
<td>87</td>
<td>98</td>
<td>98</td>
<td>&lt; 95</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>85</td>
<td>85</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td><em>T: all Myotis</em></td>
<td>77</td>
<td>96</td>
<td>84</td>
<td>84</td>
<td>59</td>
<td>91</td>
<td>79</td>
<td>79</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>96</td>
</tr>
</tbody>
</table>

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 ndl of *M. levis* was missing, bootstrap level for clade A is not available for that gene.

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. welwitschi* 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).
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

<table>
<thead>
<tr>
<th>Constrained group</th>
<th>Templeton test</th>
<th>Likelyhood ratio test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score</td>
<td>Extra steps</td>
</tr>
<tr>
<td>Unconstrained (best) trees</td>
<td>6408</td>
<td>—</td>
</tr>
<tr>
<td>All subgenera monophyletic</td>
<td>6784</td>
<td>376</td>
</tr>
<tr>
<td>Sclysius monophyletic</td>
<td>6555</td>
<td>147</td>
</tr>
<tr>
<td>Myotis monophyletic</td>
<td>6547</td>
<td>139</td>
</tr>
<tr>
<td>Leuconoe monophyletic</td>
<td>6720</td>
<td>312</td>
</tr>
<tr>
<td>American monophyletic</td>
<td>6414</td>
<td>6</td>
</tr>
<tr>
<td>Indomalayan monophyletic</td>
<td>6413</td>
<td>5</td>
</tr>
<tr>
<td>Palaeartic monophyletic</td>
<td>6465</td>
<td>57</td>
</tr>
<tr>
<td>Palaeartic (without brandti)</td>
<td>6431</td>
<td>23</td>
</tr>
</tbody>
</table>

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 branches 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 envisionned 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. brandti) 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 Palaeartic 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. brandti, 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 Palaeartic 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 Barclay, 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 Leucoone (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 Palaeartic equivalent of M. lucitngus, 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 Palaeartic 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, the 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. brandti*, 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 Palearctic 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 missoni*; 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 Ruscian-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. paradaubentoni* is considered a direct ancestor of *M. daubentoni*. 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 Palearctic 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 rates 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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