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## Phylogeny of African *Myotis* bats (Chiroptera, Vespertilionidae) inferred from cytochrome *b* sequences

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The genus *Myotis* is comprised of about 100 species that are unequally distributed between the Northern (81% of the species) and the Southern hemisphere (19% of the species). Only eight species of *Myotis* occur in the Ethiopian region, but this is the only biogeographic region with representatives of all four classical subgenera, suggesting a diverse assemblage of morphotypes. We used sequences of a mitochondrial DNA gene (cyt *b*) to investigate the evolution and the phylogenetic position of seven of the eight Ethiopian species, and compared them to a broad sampling of *Myotis* from the World and of other vespertilionids. Phylogenetic reconstruction was based on 91 complete sequences representing 79 species of bats. The two endemic southern African species of the subgenus *Cistugo* were not placed within the genus *Myotis*, but were basal to the vespertilionid radiation, as suggested by earlier work based on karyology. The remaining Ethiopian species formed a strong monophyletic clade within *Myotis*, further stressing the importance of biogeography as a good predictor of phylogenetic relationships. This Ethiopian clade includes one Western Palaearctic and one Oriental species, both of which probably secondarily colonized these areas from the Ethiopian region. Molecular dating based on Bayesian inferences suggest that these faunal exchanges occurred at the end of the Miocene, while the split of the Ethiopian clade from the other Old World *Myotis* dates back to the middle Miocene, quite early in the *Myotis* radiation. Thus, the relative paucity of species in sub-Saharan Africa cannot be attributed to a late entry into this continent. Instead, these molecular results suggest that other evolutionary processes are responsible for the poor species diversity of *Myotis* found in Africa today.

**Key words:** *Cistugo*, African *Myotis*, Vespertilionidae, cytochrome *b*, molecular dating

### INTRODUCTION

With about 100 currently recognized species (Koopman, 1994; Simmons, In press), bats of the genus *Myotis* represent one of the few mammalian groups with a natural distribution covering most of the world. The peak diversity of *Myotis* species is, however, reached in the northern continents. Eurasia supports the richest diversity

(55 species), followed by the Nearctic region (24 species), while only 14 species are found in the Neotropics, eight in the Ethiopian, and three in the Australian region (Koopman, 1994; Horáček *et al.*, 2000). This pattern is particularly surprising since *Myotis* bats have adapted to diverse habitats, from deserts to cloud forests, with a wide range of foraging habits (from insectivorous aerial hawkers to fish-eating). They

are also morphologically diverse and have been classically subdivided into four subgenera, corresponding to groups of species sharing similar morphology and feeding strategies (Findley, 1972; Koopman, 1994). Although there are only eight currently recognized species of *Myotis* in sub-Saharan Africa, it is the only biogeographic region that supports representatives of all these four traditionally recognized subgenera. These Ethiopian bats include four large species classified in the subgenus *Myotis* (*M. welwitschii*, *M. tricolor*, *M. morrisoni* and *M. goudoti*), one smaller species in the subgenus *Selysius* (*M. scotti*), one species in the subgenus *Leuconoe* (*M. bocagii* [original spelling]), and two species in the subgenus *Cistugo* (*M. seabrae* [original spelling] and *M. lesueuri*). In addition to these African species, the endemic Malagasy bat *M. goudoti* is characterized by a suite of plesiomorphic dental characters that are supposedly close to the ancestral situation for the whole genus *Myotis* (Godawa Stromark, 1998).

*Myotis seabrae* and *M. lesueuri* are distinguishable from other *Myotis* by the presence of unique glands on each wing of unknown function, by the reduced size of their anterior premolars, and by a large antero-internal cusp on the fourth lower premolar. In the original descriptions of *M. seabrae* and *M. lesueuri*, Thomas (1912) and Roberts (1919), respectively, included these two peculiar species in a new genus, *Cistugo*, but most subsequent authors considered *Cistugo* only as a subgenus of *Myotis*. Rautenbach *et al.* (1993) analysed the karyotypes of a number of Ethiopian vespertilionids and showed that the two endemic southern African species (*Myotis seabrae* and *M. lesueuri*) were unique in having  $2n = 50$  chromosomes. Except for *M. annectans* with  $2n = 46$ , all other known species of *Myotis* have  $2n = 44$  chromosomes (Zima and Horáček, 1985; Bickham *et al.*, 1986).

Rautenbach *et al.* (1993) thus proposed raising *Cistugo* to full generic rank, and suggested that their primitive karyotype indicated a basal position within the Vespertilionidae. This was confirmed by molecular studies based on mtDNA (Bickham *et al.*, 2004).

In an investigation of the phylogenetic relationships within the genus *Myotis*, Ruedi and Mayer (2001) sequenced 29 species of *Myotis* from around the world and discovered that the traditionally recognized subgenera did not cluster into monophyletic groups. Instead, various species currently classified in distinct subgenera but living in the same biogeographic region grouped together in strongly supported clades. More recent work by Hoofer and Van Den Bussche (2003) and Stadelmann *et al.* (2004) confirmed this surprising phylogenetic pattern, invalidating the classical, morphology-based systematic arrangement of this diverse group of bats. Unfortunately these molecular analyses were not informative about the Ethiopian species of *Myotis* as only *M. welwitschii*, *M. bocagii* and/or *M. goudoti* were sampled.

In this paper, we sequenced a mitochondrial DNA gene (cytochrome *b*) to investigate the phylogenetic relationships of the Afrotropical species of *Myotis*. We compare these taxa to a representative sampling of species from all other continents to test the biogeographic hypothesis that several *Myotis* radiations took place independently in each major biogeographic region (Ruedi and Mayer, 2001; Stadelmann *et al.*, 2004), and to investigate the controversial systematic position of the two southern African endemic species *M. seabrae* and *M. lesueuri*. We also use an internal fossil calibration of the molecular tree and the Bayesian relaxed clock approach developed by Thorne and Kishino (Thorne *et al.*, 1998; Kishino *et al.*, 2001; Thorne and Kishino,

2002) to estimate the time frame of these diversifications.

## MATERIALS AND METHODS

### *Taxon and Geographic Sampling*

Seven of the eight Afrotropical species are represented in this study (*Myotis bocagii*, *M. goudoti*, *M. welhwitschii*, *M. tricolor*, *M. scotti*, *M. lesueuri*, and *M. seabrae*). The only missing species is *M. morrisoni*, known from two specimens, one collected in western Ethiopia and the other in northeastern Nigeria (Hill and Morris, 1971; Hill *et al.*, 1988). The seven Afrotropical species sampled were compared to 42 other species of *Myotis* originating from various parts of the World. The latter include all species already sequenced in Ruedi and Mayer (2001) or in Stadelmann *et al.* (2004), and three Oriental species (*Myotis prinosus*, *M. chinensis*, and *M. ikonnikovi*) deposited in GenBank by Kawai *et al.* (2003). We also sequenced eight additional, non-Ethiopian species of *Myotis* (Table 1). This is the first taxon sampling of *Myotis* that includes all four traditional subgenera and all biogeographic regions.

To test the monophyly of the genus *Myotis*, a broad selection of vespertilionid outgroups were also included in the analyses. These outgroups represent 30 species belonging to 13 different genera. They comprise some taxa already sequenced by Ruedi and Mayer (2001) or Stadelmann *et al.* (2004), *Miniopterus fuliginosus*, *Murina leucogaster*, and *Vespertilio superans* from Japan sequenced by Sakai *et al.* (2003), and 11 species sequenced here for the first time (Table 1).

### *DNA Extraction*

All samples consisted of ethanol-preserved tissues collected with appropriate permits or obtained as loans from various institutions (Table 1). About 10–30 mg of tissue was soaked for 1–2 hours in sterile water. Total genomic DNA was isolated by digestion in guanidinium and proteinase K. After an overnight precipitation in isopropanol at -20°C, the lystate was centrifuged and re-dissolved in 0.5 M NaOH. Ten µl of the NaOH solution were diluted in 200 µl of 0.1 M Tris at pH 8 (Wang *et al.*, 1993) for amplification.

### *Amplification and Purification of Cytochrome B*

We used the mitochondrial cytochrome *b* gene (cyt *b*) as a marker so that we could compare our

sequences with the extensive existing database. Amplification by polymerase chain reactions (PCR) of the complete cyt *b* were performed with different primer pairs depending on the sample as described in Stadelmann *et al.* (2004). PCR cocktails (50 µl reaction volume) included 2–10 µl of DNA extract, 0.2 µM of each primer, 2.5–4 mM of MgCl<sub>2</sub>, 0.2 mM each of 4 dNTPs, 1 unit of Taq DNA polymerase (QIAGEN, Inc., Switzerland) with appropriate buffer and ddH<sub>2</sub>O. Thermal profiles of amplifications included 3 min initial denaturation at 94°C, followed by 39 cycles at 94°C (45 s), 45–53°C (45 s) and 72°C (1.5 min), with a final extension at 72°C (5 min). A few samples needed to be re-amplified with nested primers (MVZ16, L15162; Irwin *et al.*, 1991, and Smith and Patton, 1991; BSves268H see Stadelmann *et al.*, 2004). In these cases, only 19–25 cycles including an annealing temperature set between 54 and 56°C were used. All PCR products were purified using a QIAGEN PCR Purification Kit and sequenced directly (ABI Prism 377 automated DNA sequencer) in one direction using the same primers. Two or three overlapping PCR fragments were then assembled and checked for consistency to produce complete cyt *b* sequences. All sequences were assembled, edited and aligned manually with the software BioEdit.

### *Phylogenetic Analyses*

Phylogenetic reconstructions were performed with the Maximum Parsimony (MP) method implemented in PAUP\* (Swofford, 2002) and the Maximum Likelihood method (ML) implemented in PHYML. Bayesian inferences were carried out using MRBAYES 2.01 (Huelsenbeck and Ronquist, 2001). MP analyses were performed with characters weighted according to the rescaled consistency index (Farris, 1989) and using a stepmatrix weighting transversions 12 times over transitions. This ratio was estimated with PAUP\*. The most parsimonious tree was estimated through a heuristic search with 100 random additions of taxa, and complete tree-bisection-reconnection branch swapping for each iteration (Swofford, *et al.*, 1996). In the PHYML procedure, the starting tree was obtained with Bionj (Gascuel, 1997), which is an improved version of the neighbor-joining algorithm of Saitou and Nei (1987). The model of nucleotide substitution used was GTR+G+I, which allows rate variation among sites and includes a proportion of invariable sites (Lanave *et al.*, 1984; Rodriguez, 1990). The parameters were estimated and optimized during the search.

Reliability of nodes was assessed with non-parametric bootstraps (Felsenstein, 1985). Under MP, 500 bootstraps were generated, each with 30 stepwise

TABLE 1. Origin and GenBank accession numbers of newly sequenced specimens. Taxonomy follows Koopman (1994) except where mentioned. Vouchers are deposited in the following institutions: Senckenberg Museum Frankfurt/M (SMF), Transvaal Museum, South Africa (TM), Natural History Museum of Geneva (MHNG), Zoological Museum of Moscow State University (ZMMU), and the Royal Ontario Museum (ROM), or are yet uncatalogued specimen of M. Ruedi (MR), J. Fahr, University of Ulm (JF), or C. M. Francis, National Wildlife Research Centre, Ottawa (CMF). Samples without vouchers were released after capture

Taxon	Locality	GenBank	Voucher
<i>Myotis</i>			
Subfamily Vespertilioninae			
Subgenus <i>Myotis</i>			
<i>Myotis evotis</i>	Alberta, Canada	AJ841949	no voucher
<i>Myotis formosus</i>	Dong Amphan, south Laos	AJ841950	ROM 110544
<i>Myotis sicarius</i>	Annapurna, Nepal	AJ841951	ZMMU 164493
<i>Myotis tricolor</i> A	Transvaal, South Africa	AJ841952	TM 40300
<i>Myotis tricolor</i> B	De Hoop, South Africa	AJ841953	no voucher
<i>Myotis welwitschii</i> C	Pic de Fon, Guinea	AJ841954	JF 105
Subgenus <i>Selysius</i>			
<i>Myotis alcaethoe</i> <sup>1</sup>	Vaud, Switzerland	AJ841955	MHNG 1828.073
<i>Myotis annectans</i>	Ban Navang, central Laos	AJ841956	ROM 106376
<i>Myotis muricola</i>	Ban Keng Bit, Laos	AJ841957	SMF 86172
<i>Myotis scotti</i>	Territory of southern tribes, Coccia, Ethiopia	AJ841958	ZMMU 167226
Subgenus <i>Leuconoe</i>			
<i>Myotis macropus</i>	Queensland, Australia	AJ841959	no voucher
<i>Myotis macrotarsus</i> A	Madai Cave, Sabah, Malaysia	AJ841960	CMF 960522.46
Subgenus <i>Cistugo</i>			
<i>Myotis lesueuri</i>	Algeria Forest, South Africa	AJ841961	no voucher
<i>Myotis seabrae</i>	Goodhouse, South Africa	AJ841962	MR-M977
Outgroups			
Subfamily Vespertilioninae			
<i>Eptesicus hotentotus</i>	Algeria Forest, South Africa	AJ841963	MR-M984
<i>Laephotis wintoni</i>	Algeria Forest, South Africa	AJ841964	MR-M983
<i>Neoromicia capensis</i> A <sup>2</sup>	Springbok, Goegap Nat. Res., South Africa	AJ841965	MR-M963
<i>Neoromicia capensis</i> B	Algeria Forest, South Africa	AJ841966	MR-M981
<i>Nyctalus noctula</i>	Bern, Switzerland	AJ841967	MHNG
<i>Pipistrellus hesperidus</i> <sup>3</sup>	Knysna, South Africa	AJ841968	MR-M987
Subfamily Kerivoulinae			
<i>Kerivoula</i> cf. <i>papillosa</i> A	Dong Amphan, south Laos	AJ841969	ROM 110520
<i>Kerivoula</i> cf. <i>papillosa</i> B	Nam Et, north Laos	AJ841970	CMF 980326.61
Subfamily Murininae			
<i>Harpiocephalus mordax</i>	Nam Et, north Laos	AJ841971	CMF 980322.73
<i>Murina</i> cf. <i>cyclotis</i> A	Nam Et, north Laos	AJ841972	CMF 980322.66
<i>Murina</i> cf. <i>cyclotis</i> B	Nam HA, north-west Laos	AJ841973	CMF 980420.14
<i>Murina</i> cf. <i>cyclotis</i> C	Nam Et, north Laos	AJ841974	CMF 980329.19
Subfamily Miniopterinae			
<i>Miniopterus fraterculus</i>	Knysna, South Africa	AJ841975	MR-M988
<i>Miniopterus schreibersii natalensis</i> A	Knysna, South Africa	AJ841976	MR-M993
<i>Miniopterus schreibersii natalensis</i> B	Springbok, Goegap Nat. Res., South Africa	AJ841977	MR-M966

<sup>1</sup> — von Helversen *et al.* (2001)

<sup>2</sup> — Volleth *et al.* (2001)

<sup>3</sup> — Kock (2001)

random additions and complete tree-bisection-reconnection branch swapping. Similarly, under the ML framework, bootstraps were performed by generating 100 pseudoreplicates from the original data sets with Seqboot (Phylip package; Felsenstein, 1993). These matrices were then analyzed in PHYML as described above. As suggested by Hillis and Bull (1993), nodes with more than 70% bootstrap support were considered as strongly supported.

Bayesian posterior probabilities were calculated using a Metropolis-coupled, Markov chain, Monte Carlo (MCMCMC) sampling approach as implemented in MRBAYES 2.01 (Huelsenbeck and Ronquist, 2001) and using the same GTR+G+I model. Four simultaneous Markov chains were run for 1,000,000 generations with trees sampled every 10 generations. After about 50,000 generations the log-likelihoods of trees reached a plateau. These initial trees were thus discarded as 'burn-in'. Posterior probabilities were computed from the consensus of the remaining 95,000 trees.

### Molecular Dating

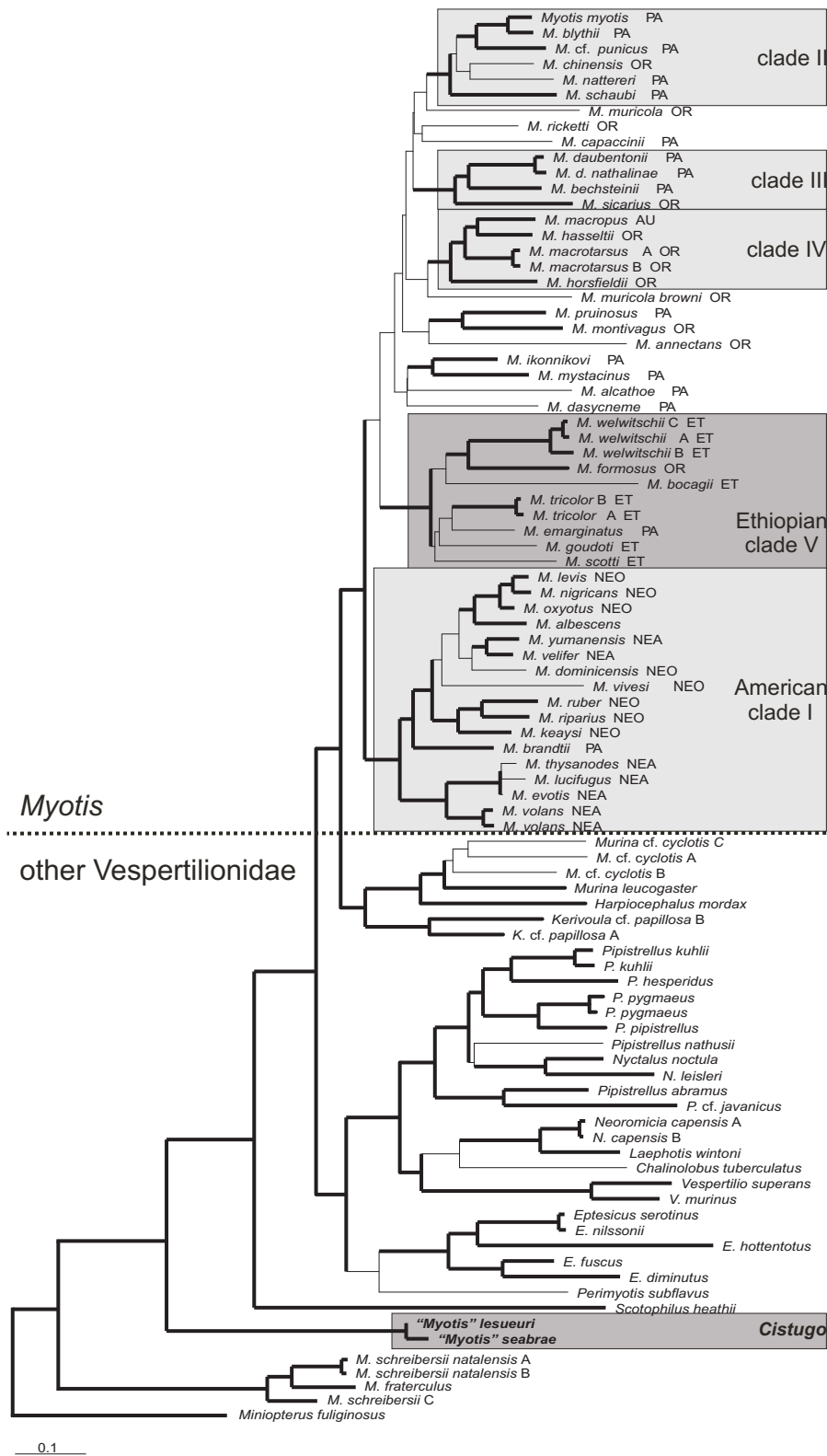
The approximate age of divergence of selected nodes was estimated by the Bayesian relaxed molecular clock approach implemented in the program package MULTIDISTRIBUTE developed by Thorne *et al.* (1998), Kishino *et al.* (2001), and Thorne and Kishino (2002). This software allows the incorporation of multiple constraints from the fossil record, and can take into account both molecular and palaeontological uncertainties to estimate the variance of divergence times. The module ESTBRANCHES first estimates branch lengths and their variance-covariance matrix from the nucleotides data set. ESTBRANCHES needs eight rate category ( $\Gamma_8$ ) values for a gamma distribution, which are estimated with PAML 3.14 (Yang *et al.*, 1998) under the F84 model (Felsenstein, 1984). Given fixed palaeontological constraints (see below), the module MULTIDIVTIME then estimates divergence times between taxa with Markov chains (we used 1 million generations sampled every 100 generations, after a burn-in period of 100,000 cycles). We used the following palaeontological constraints obtained from the fossil record. The maximal age for the root of the *Myotis* radiation is given by its separation from the lineage leading to the Kerivoulinae and Murininae, both of which are currently considered as the closest outgroups to *Myotis* (Horáček *et al.*, 2000; Jones *et al.*, 2002; Hoofer and Van Den Bussche, 2003). According to Horáček (2001), this separation would date back to about 30 million years ago (MYA). We also used two calibration points already described in an earlier paper (Ruedi and Mayer, 2001)

within the *Myotis* tree: *M. daubentonii* and *M. bechsteinii* diverged at least 5 MYA (Topál, 1983) and *M. nattereri* diverged from *M. schaubi* between 5.5 and 6.5 MYA (Horáček and Hanák, 1984). However, to accommodate for the uncertainties associated with the palaeontological age of the fossils, we followed the method suggested by Douady and Douzery to bracket all calibration points with the age of the lower and upper geological layers known to include the fossils i.e. 28–37 MYA for the split between *Myotis* and Kerivoulinae plus Murininae, 3.6–7.1 MYA for the node *nattereri-schaubi* and 3.6–11.2 MYA for the node *bechsteinii-daubentonii*.

## RESULTS

### Cyt B Sequences

Twenty-nine cyt *b* sequences of 1140 bp were obtained for all samples. No insertion or stop codons were noted, suggesting that the sequences came from mtDNA, and are not pseudogenes. These new sequences represent 13 *Myotis* species and 11 other vespertilionid outgroups (see Table 1 and Fig. 1) and are deposited in GenBank under accession numbers AJ841949-AJ841977. Sequences of multiple individuals from the same species were identical or displayed minimal divergence. Two individuals of *M. lesueuri* and two of *M. seabrae*, were almost identical with zero and two substitutions, respectively, even for animals captured in very distinct geographical areas. Two *M. formosus* from Laos differed by eight substitutions from a specimen originating from South Korea. The Ugandan and the Guinean samples of *M. welwitschii* (A and C respectively in Figs. 1 and 2) diverged by 15 substitutions (1.3% sequence divergence) from each other, but were more divergent compared to a sample from South Africa (B in Figs. 1 and 2; 50–51 substitutions or 4.5% sequence divergence). Another high intra-specific divergence concerns *Miniopterus schreibersii*. The European specimen C taken in Spain presents 120 substitutions or 10.5%



sequence divergence with the specimens A and B from South Africa (Figs. 1 and 2). This divergence is similar to the one found between any of the *M. schreibersii* specimens and *M. fraterculus* (123–128 bp). To render phylogenetic analyses more tractable, we kept only haplotypes diverging by at least 1% (or 12 substitutions) from each other, which resulted in 91 distinct *cyt b* sequences in the final alignment.

### Phylogenetic Reconstructions

Phylogenetic relationships are represented by an ML tree of 91 complete *cyt b* sequences of *Myotis* and other vespertilionid bats (Fig. 1). About two-thirds of the inferred nodes received good support (i.e. > 70% bootstrap support). Nodes with lower support are concentrated at the base of the *Myotis* radiation, and are associated especially with Oriental or Palearctic species. Other methods of phylogenetic reconstructions (weighted MP and Bayesian inferences) gave similar tree topologies (not shown), although the statistical support of some nodes depended on which optimisation criterion was used. A striking feature common to all reconstructions is that the two southern African species of the subgenus *Cistugo* were never placed within the otherwise monophyletic *Myotis*-clade. Instead, *M. lesueuri* and *M. seabrae* were placed at a basal position in the tree of Vespertilionids, close to the *Miniopterus* species. The remaining species of *Myotis* formed a monophyletic clade with 71 (MP) to 96% (ML) bootstrap support and a posterior probability of 0.81. The sister group of this *Myotis* clade was Kerivoulinae plus

Murinae in all reconstructions (Fig. 1). There were five distinct clades within the *Myotis*-radiation. The New World species (clade I) were monophyletic and split off early from other species in all reconstructions. Other strongly supported clades included a cluster of large *Myotis* (clade II), and two other clades comprising various Old World species (clades III and IV). Interestingly, the Ethiopian species (except *M. lesueuri* and *M. seabrae*) were grouped together in a monophyletic clade (clade V), which received strong support in all phylogenetic reconstructions (>80% for ML, 92% for MP, and 0.90 posterior probability). Besides the Ethiopian species, this clade also included *M. formosus*, an Oriental species, and *M. emarginatus* from North Africa and Europe. Several other European or Asian species of *Myotis* (e.g., *M. dasycneme*, *M. ricketti*, *M. alcaethoe*) had relatively unstable positions (i.e. low nodal support) within this tree, and their position depended on which phylogenetic method was used (results not shown). Their phylogenetic position should be best considered as unresolved.

Among the outgroups relationships were relatively well resolved. The main features of these outgroups were the paraphyly of the assayed *Pipistrellus* with respect to *Nyctalus* and the sister group relationship of *Neoromicia* and *Laephotis*, two morphologically very divergent taxa.

### Molecular Dating

Estimates of divergence times were obtained with the Bayesian relaxed molecular clock approach (Fig. 2). The relaxed clock



FIG. 1. Phylogenetic relationships of 91 complete cytochrome *b* gene sequences. This maximum likelihood tree was reconstructed by using PHYML (Gascuel and Guindon, 2003) and GTR+G+I as model of nucleotide substitutions. Nodes supported by > 70 % bootstrap values in all ML, MP and Bayesian analyses are indicated as bold lines. Five major clades and the *Cistugo* clade are highlighted in grey. The geographic origin of each *Myotis* species is indicated with the following abbreviations: PA = Palearctic, OR = Oriental, AU = Australian, ET = Ethiopian, NEO = Neotropical, NEA = Nearctic



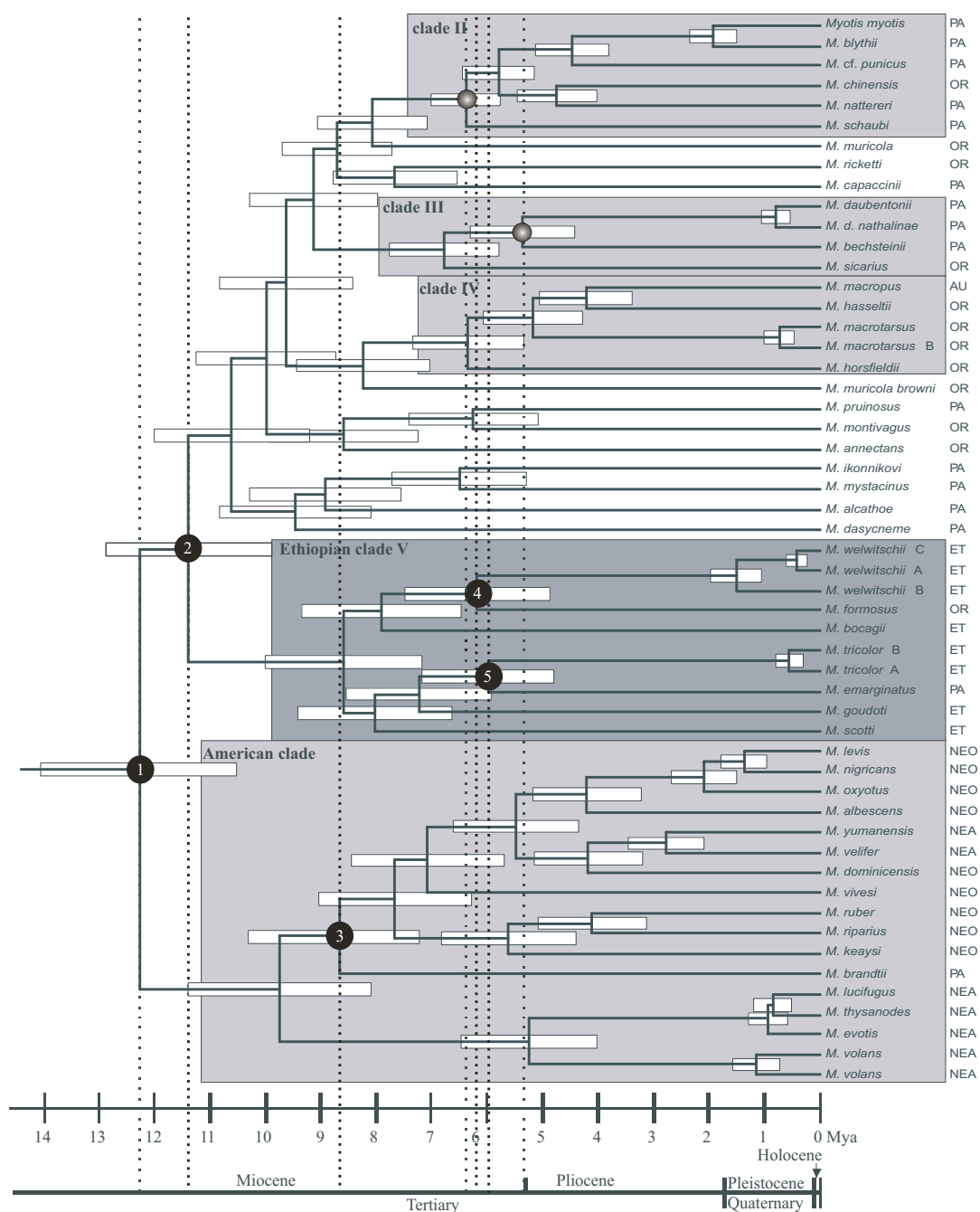


FIG. 2. Chronogram obtained from the cytochrome *b* data set for the *Myotis* ingroup, with ages inferred from the Bayesian rate autocorrelation method using three nodes under palaeontological constraints (grey circles, and the node between *Myotis* and Kerivoulinae plus Murinae, not shown). Horizontal boxes stand for  $\pm 1$  SD around divergence ages. The numbered nodes correspond to (1) the basal split of the genus *Myotis*, (2) the divergence of the Ethiopian clade, (3) the divergence of *M. brandtii* from other American species, (4) the split of the *formosus* and (5) of the *emarginatus* lineages from their respective African ancestors

analyses were run for all available species of *Myotis*, using the Kerivoulinae plus Murinae as the outgroup, and the ML tree (Fig. 1) as input topology. To test the influence of the age of the split between these two groups, the analyses were run with three different ages (37, 33.7, and 28 MYA). However, as these different dates did not influence notably the inferred ages of other, more inclusive nodes, we report only estimates using the more conservative (i.e. the oldest) date (37 MYA) to calibrate the tree. Five nodes of interest were dated with this method (Fig. 2). The first node is the split between the American and the non-American *Myotis* that is estimated at  $12.3 \pm 1.7$  MYA. The second node is the age of divergence of the lineage leading to the Ethiopian clade at about  $11.39 \pm 1.5$  MYA. Node three was estimated at  $8.67 \pm 1.56$  MYA and corresponds to the minimal age of common origin between *M. brandtii* and its American relatives. Similarly, the Oriental *M. formosus* last shared a common ancestor with *M. welwitschii* about  $6.19 \pm 1.32$  MYA (node four). The fifth node of interest is the split between the lineages leading to *M. tricolor* and to *M. emarginatus*. This divergence is dated at about  $5.98 \pm 1.18$  MYA.

## DISCUSSION

The molecular phylogenetic reconstructions based on *cyt b* data suggest a number of surprising relationships amongst species compared to the current, morphology-based systematic arrangement (e.g., Koopman, 1994).

### *Cistugo* are not *Myotis*

The most striking was the position of the two endemic, southern African species *Myotis seabrae* and *M. lesueuri*, which fell outside the *Myotis* radiation. These two

closely related species differed by less than 30 substitutions from each other, and were assumed to form a distinct subgenus, *Cistugo*, within the genus *Myotis* (Koopman, 1994). They were, however, genetically very distinct from any other *Myotis* (>20% uncorrected genetic distance). In fact, all phylogenetic reconstructions concurred in placing these two peculiar species at the base of the vespertilionid diversification. As only representatives of the family Vespertilionidae were considered in the analyses, with *Miniopterus* species as a functional outgroup, it is even possible that these two species actually share closer phylogenetic relationships with other families of bats. Although the exact systematic position of *seabrae* and *lesueuri* within Yangochiroptera (Teeling *et al.*, 2002) has to be further investigated, our molecular data clearly support the original, full generic rank for *Cistugo* as proposed by Thomas (1912), Roberts (1919), and by Rautenbach *et al.* (1993). The same conclusions were reached independently by Bickham *et al.* (2004) who sequenced the cytochrome *b* of *seabrae* from Namibia and *lesueuri* from South Africa. Both karyological (Rautenbach *et al.*, 1993) and molecular data thus imply that *Cistugo* does not have a close phylogenetic relationship with the genus *Myotis*. Morphological similarities shared between these two genera are therefore either plesiomorphic or convergent features.

### *An Ethiopian Clade*

The five other Ethiopian species of *Myotis* analysed here are more widespread within sub-Saharan Africa and represent a morphologically diverse assemblage pertaining to the subgenera *Leuconoe*, *Selysius* and *Myotis* (Koopman, 1994). Despite this apparent morphological heterogeneity, they formed a strongly supported monophyletic clade in all reconstructions (Fig. 1). Results

from other mtDNA markers (16S, 12S, tRNAval) also placed *bocagii* and *welwitschii* in a distinct and strongly supported clade (Hoofer and Van Den Bussche, 2003), although this study included only eight other Old World taxa.

The only Afrotropical species missing in our molecular analyses, *M. morrisi* is known from only two specimens. We anticipate that *M. morrisi* is part of the Ethiopian clade, but a molecular analysis is needed to confirm this.

Contrary to the hypothesis of Godawa Stormark (1998), the molecular analyses did not place *M. goudoti* from Madagascar in a basal position within the *Myotis* tree, but placed it in a derived position, close to *M. scotti* from Ethiopia. The supposedly plesiomorphic dental characteristics of *goudoti* (Godawa Stormark, 1998) are therefore not an ancestral feature, but rather evolved recently from a continental representative.

*Myotis formosus* is currently distributed from Afghanistan to Korea and Indonesia (Koopman, 1994), while *M. emarginatus* is currently distributed from North Africa to Central Asia. Interestingly, all molecular reconstructions placed these two non-Ethiopian species within the African radiation. Their phylogenetic position in this Ethiopian clade (Fig. 1) suggests that they are derived from two African ancestors that secondarily colonized Eurasia. Otherwise, we would have expected a more basal position for these two Eurasiatic species.

*Myotis formosus* and *M. welwitschii* share a unique black and orange coloration of wing membranes (Koopman, 1994), which led Tate (1941) to place them together in a special subgenus *Chrysopteron*. Since these two species were identified as sister taxa in all molecular reconstructions (Fig. 1, and Kawai *et al.*, 2003), this particular coloration probably evolved only once

in the genus *Myotis*. Similarly, phenetic analyses of Findley (1972) based on morphology placed *emarginatus*, *tricolor* and *goudoti* in the same *emarginatus*-group. According to our cytochrome *b* data (Fig. 1), the close phenetic resemblance of this species group corresponds to real phylogenetic relationships. These cases illustrate the difficulties in identifying, a priori, the phylogenetic utility of morphological characters.

### Other Major Clades

All five of the other major clades identified in a previous work (Ruedi and Mayer, 2001) were further supported with the new sequences included here (Table 1). The largest of all *Myotis* species, *M. chinensis*, was included within clade II, which encompassed other large *Myotis* (e.g., *M. myotis*). The Nepalese *M. sicarius* was found at the base of clade III, while the Australian *M. macropus* was closely related to the Indomalayan taxa of clade IV. Our current taxon sampling of Indomalayan and East Palaearctic *Myotis* was, however, too sparse to interpret the phylogenetic relationships of this diverse group in more detail. The American clade, clade I, revealed in previous studies (Ruedi and Mayer, 2001; Hoofer *et al.*, 2003; Stadelmann *et al.*, 2004) was well supported (Fig. 1), which is not surprising as only *M. evotis* had been added here. Furthermore, strong support was given to the pairing of *macropus* and *macrotarsus*, which belong to clade IV in the cyt *b* tree (Fig. 1).

### Dating the Major Biogeographic Events

We included 30 different vespertilionid species from 13 genera as the outgroups because it was not obvious which taxa would provide the best outgroup for the genus *Myotis*, and because increased taxon sampling of outgroups might help to more

firmly establish the position of the root within the *Myotis* radiation (Zwickl and Hillis, 2002). Clearly the *cyt b* supported a sister group relationship between *Myotis* and Murininae plus Kerivoulinae (Fig. 1) as suggested in other molecular-based phylogenies (e.g., Kawai *et al.*, 2002; Hoofer and Van Den Bussche, 2003), or in the chromosomal study of Volleth and Heller (1994). This result also corresponds to modern interpretation of fossil records (e.g., Horáček, 2001), and justifies our use of Kerivoulinae plus Murininae to root the tree of *Myotis* for the clock calibrations. In this respect, the Bayesian relaxed-clock estimates calibrated with the three dated nodes suggest that the genus *Myotis* had a major split in the middle Miocene, some  $12.3 \pm 1.7$  MYA ago (Fig. 2). This split corresponds to the divergence of the American clade from other non-American *Myotis*. The Ethiopian clade diverged at about the same time ( $11.4 \pm 1.5$  MYA; Fig. 2) from these early *Myotis*. Both divergence dates coincide with the middle Miocene climatic transition (Flower *et al.*, 1994; Zachos *et al.*, 2001), which initiated global cooling after the Neogene warmth climax (Flower *et al.*, 1994; Zachos *et al.*, 2001). This major change in global climate might have triggered the evolution of these clades on the American and African continents.

The geographic origins of species mapped onto the phylogenetic tree further indicate that limited faunal interchanges took place during the evolution of *Myotis* assemblages in each major biogeographic region (Fig. 2). Exceptions include *M. brandtii*, which diverged about  $8.7 \pm 1.0$  MYA from its American ancestors and which probably colonised its current Palaearctic range through the Bering Strait. The standard deviation associated with this event is rather large and overlaps with the early divergence of the entire American clade. Again, a more complete sampling

of species in this region is needed to further investigate this biogeographic interpretation. Another exception concerns *M. formosus* and *M. emarginatus* in the Ethiopian clade (Fig. 1). These two independent lineages diverged about 6 MYA from their African ancestors and subsequently colonized their present geographic range in Eurasia.

As our current sampling of *Myotis* species is biased towards West Palaearctic and Ethiopian species, it is possible that some of these dates (especially early radiation) or the origin of these groups might change. We intend in the future to focus on the other regions (East Palaearctic and Oriental) to ascertain the effect of such a potential bias.

### *Taxonomic Considerations*

Besides the elevation of *Cistugo* to full generic rank, our molecular results shed new light on several other systematics issues. *Myotis alcathoe* has been recently described from Europe (von Helversen *et al.*, 2001), and has been found so far in Greece, Hungary, France (Ruedi *et al.*, 2002), Slovakia (Benda *et al.*, 2003), Spain (Agirre-Mendi *et al.*, 2004) and Bulgaria (Schunger *et al.*, In press). The sequence reported here comes from the Jura Mountains, Switzerland, and is the first record of this species in Central Europe. The phylogenetic relationships of this poorly known species are not clear. In preliminary studies based on mtDNA sequences of all European vespertilionids (Mayer and von Helversen, 2001a, 2001b), *M. alcathoe* appeared close to *M. emarginatus*, but without strong bootstrap support. With more intensive taxon sampling (Fig. 1), *M. alcathoe* did not appear to be closely related to *M. emarginatus*. Molecular reconstructions suggest that it belongs to an Eurasiatic assemblage of *Myotis*. However, its precise position within

this assemblage was not well resolved and needs further scrutiny.

The relatively high molecular divergence (4.5% sequence divergence) found between *M. welwitschii* from South Africa (B in Figs. 1 and 2) and those from Uganda and Guinea (A and C, in Figs. 1 and 2) may underlie the large geographic distance separating these allopatric populations (Fahr and Ebigo, 2003), or suggest the existence of additional taxonomic diversity. Further morphological or genetic studies in intervening areas are required to better address these possibilities.

As already shown in other molecular studies (Ruedi and Mayer, 2001; Hoofer and Van Den Bussche, 2003; Kawai *et al.*, 2003; Stadelmann *et al.*, 2004), and in a paper that explored relationships between external morphology and foraging behaviour (Fenton and Bogdanowicz, 2002), the current morphology-based subdivision of the genus *Myotis* into four or more subgenera (e.g., Findley, 1972; Koopman, 1994) does not reflect phylogenetic groupings but represent different adaptive convergences. Since only about half of the extant species have been examined genetically, it is still premature to propose an alternative classification. However, it is rather obvious that the biogeographic origins of species will be an important component in any new systematic arrangement (Hoofer and Van Den Bussche, 2003).

Similarly, species of the rare and enigmatic genus *Laephotis* differ in many traits from species of *Neoromicia* (e.g., very long versus short ears and distinct teeth, Taylor, 2000), yet multiple mtDNA evidence (Fig. 1 and Hoofer and Van Den Bussche, 2003) clearly indicate that they are closely related, suggesting that their morphological differences evolved relatively rapidly. Other contradictory results between the current taxonomy and our molecular results include the paraphyletic position of the

genus *Pipistrellus* with respect to *Nyctalus* (Fig. 1). Again, this result is in line with the recent comprehensive molecular survey of vespertilionids presented by Hoofer and Van Den Bussche (2003) and with cytogenetic results by Volleth (1992) and Volleth and Heller (1994) who already showed this grouping based on banded karyotypes.

Molecular evidence showed that the magnitude of the difference between European *Miniopterus s. schreibersii* (C in Fig. 1) and those from South Africa (A and B, in Fig. 1), usually referred as *M. s. natalensis*, was similar to that between distinct species (e.g., *M. schreibersii* versus *M. fraterculus*, see Fig. 1). This adds further support that European and sub-Saharan *schreibersii* represent at least two distinct species as suggested by a more comprehensive molecular analysis of that group made by Appleton *et al.* (2004). This also supports the recent elevation of the Natal long-fingered bat, *M. natalensis* to full species rank (Simmons, In press).

Finally, specimens identified as *Kerivoula cf. papillosa* and the *Murina cf. cyclotis*, sampled in different parts of Laos (Table 1), proved to be genetically very divergent (Fig. 1). This suggests that these two groups might represent a complex of several species.

### Concluding Remarks

One of the main aims of this study was to understand the phylogenetic and biogeographic origins of the apparently depauperate Ethiopian *Myotis* fauna. The removal of the unique *seabrae* and *lesueuri* species from the genus *Myotis* and their placement in a distinct, independent genus *Cistugo*, leaves only six truly sub-Saharan species in the Ethiopian clade. Molecular dating showed that this clade diverged relatively early during the *Myotis* radiation and the relative paucity of species thus cannot be

attributed to a late colonization of Africa. Rather, the Ethiopian *Myotis* either experienced increased extinction rates compared to other regions (the poor fossil record of bats in Africa precludes any validation of this hypothesis) or could not diversify because of some ecological limitations (e.g., niche saturation). It is also possible that several other cryptic species of *Myotis* exist in Africa, but there is no reason to believe that this 'hidden' diversity is more important in Africa than in other parts of the world. Understanding why *Myotis* were not evolutionary successful in this part of the world is an interesting question. Answers will probably come from an understanding of the radiation of *Myotis* in other biogeographic regions.

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