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ScienceDirect
Molecular Phylogenetics and Evolution 43 (2007) 32–48
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular phylogeny of New World *Myotis* (Chiroptera, Vespertilionidae) inferred from mitochondrial and nuclear DNA genes

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Received 20 February 2006; revised 26 May 2006; accepted 20 June 2006
Available online 14 July 2006

Abstract

Recent studies have shown that species in the genus *Myotis* have evolved a number of convergent morphological traits, many of which are more related to their mode of food procurement than to their phylogeny. Surprisingly, the biogeographic origins of these species are a much better predictor of phylogenetic relationships, than their morphology. In particular, a monophyletic clade that includes all New World species was apparent, but only a third of the 38 species have been analysed. In order to better understand the evolution of this clade, we present phylogenetic reconstructions of 17 Nearctic and 13 Neotropical species of *Myotis* compared to a number of Old World congeners. These reconstructions are based on mitochondrial cytochrome *b* (1140 bp), and nuclear Rag 2 genes (1148 bp). Monophyly of the New World clade is strongly supported in all analyses. Two Palaearctic sister species, one from the west (*M. brandtii*) and one from the east (*M. gracilis*), are embedded within the New World clade, suggesting that they either moved across the Bering Strait, or that they descended from the same ancestor that reached the New World. An emerging feature of these phylogenetic reconstructions is that limited faunal exchanges have occurred, including between the North and South American continents, further emphasizing the importance of biogeography in the radiation of *Myotis*. A fossil-calibrated, relaxed molecular-clock model was used to estimate the divergence time of New World lineages to 12.2 ± 2.0 MYA. Early diversification of New World *Myotis* coincides with the sharp global cooling of the Middle Miocene. Radiation of the temperate-adapted *Myotis* may have been triggered by these climatic changes. The relative paucity of species currently found in South America might result from a combination of factors including the early presence of competitors better adapted to tropical habitats.

Keywords: New World *Myotis*; Vespertilionidae; Chiroptera; Nearctic; Neotropical; Cytochrome *b*; Rag 2; Speciation; Molecular dating; Biogeography

1. Introduction

Current mammalian assemblages in the New World have been marked by successive isolation and connections of the North and the South American continents, and by global environmental changes. South American biotas evolved in isolation during most of the Cenozoic era, but a large number of its endemic taxa became extinct when the continent collided with North America in the Pliocene, and allowed faunal exchanges across the Isthmus of Panama (Cox, 2000; Flynn and Wyss, 1998). In turn, North American biotas evolved in close contact with the Eurasian fauna, as the two continents were periodically connected during low sea levels via land bridges over Greenland or the Bering Strait (Cox, 2001). Because they also share the same temperate climate, faunal similarities are greater between North America and Eurasia than with South America (Cox, 2001). North and South American continents were apparently connected three times during the past 70 million years (Marshall and Sempere, 1993), the most recent connection being accomplished by the formation of the

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doi:10.1016/j.ympev.2006.06.019

Panamanian Isthmus about 3.1 million years ago (MYA). Faunal interchanges between North and South America established the background for the present mammalian assemblages in the Nearctic and the Neotropical regions. Land connections between Nearctic and Neotropics, marine transgression (Donato et al., 2003), geological events or climatic changes that could have created barriers to land colonization or promoted speciation, are described in numerous studies on mammals (e.g. Cook et al., 2004; Da Silva and Patton, 1998; Delsuc et al., 2002, 2004; Galewski et al., 2005; Smith and Patton, 1993; Steiner et al., 2005). Most of these evolutionary hypotheses were based on terrestrial mammals. Because active flight renders bats capable of long-distance dispersal, barriers that influenced the evolution of terrestrial mammals in the Nearctic and Neotropical regions might have had a less significant impact on chiropteran diversification.

With about 100 species (Koopman, 1994; Simmons, 2005) distributed throughout the world except in the polar regions, the genus *Myotis* represents one of the most diverse and successful radiations among mammals and offers an exceptional model for investigating speciation and diversification on a worldwide scale. Several molecular studies (Hoofer and Van Den Bussche, 2003; Kawai et al., 2003; Ruedi and Mayer, 2001; Stadelmann et al., 2004a,b), including one that explored relationships between external morphology and foraging behaviour (Fenton and Bogdanowicz, 2002) have shown that 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 rather represents adaptive convergences that produced the same ecomorphs independently through deterministic processes (Losos et al., 1998). Instead, the biogeographic evolution of this genus appears to include strongly imprinted phylogenetic relationships of current species. Indeed molecular studies have demonstrated that morphologically divergent species of *Myotis*, from the same continent tend to group into well-supported clades (Ruedi and Mayer, 2001; Stadelmann et al., 2004a). Such biogeographic clades include one uniting all Ethiopian taxa (Stadelmann et al., 2004a) and another comprised of New World species (Hoofer and Van Den Bussche, 2003; Ruedi and Mayer, 2001). This New World clade thus far includes all *Myotis* from the Nearctic and Neotropical regions tested and one Palaearctic species *Myotis brandtii*, supported by several mitochondrial genes (Hoofer and Van Den Bussche, 2003; Ruedi and Mayer, 2001; Stadelmann et al., 2004b).

However, only 15 of the extant New World species were considered in previous studies, which represent less than half of the 38 species currently known from the Nearctic and Neotropical regions (Simmons, 2005). To further test the validity of the New World clade, we expanded the taxon sampling of North, Central, and South American *Myotis* species using both mitochondrial (Cyt *b*) and nuclear (Rag 2) genes.

With this expanded dataset, we tested the biogeographic hypothesis that all sampled New World species originated from a single common ancestor (i.e. are monophyletic; Stadelmann et al., 2004a). We further explore the biogeographic evolution of New World *Myotis* species in relation to current distributions in the Nearctic and Neotropical regions, using a Bayesian relaxed molecular-clock approach and likelihood reconstruction of ancestral geographic distribution. These analyses provide insight into the pattern and timing of colonization of the New World by bats of the genus *Myotis*.

2. Materials and methods

2.1. Taxon and geographic sampling

According to Simmons (2005), 38 species of *Myotis* are known to occur in the New World. Unless stated explicitly, we follow her taxonomic arrangement in referring to the species analysed. Thirty of the 38 New World species were analysed in the present study (Table 1). Taxa for which no tissue was available include very rare taxa or species with highly restricted distributions (*M. aelleni*, *M. cobanensis*, *M. findleyi*, *M. fortidens*, *M. nesopolus*, *M. peninsularis*, and *M. planiceps*), plus *M. melanorhinus* considered as a subspecies of *M. ciliolabrum* (Holloway and Barclay, 2001). When possible, several individuals from the same species, but from different locations, were analysed to validate taxonomic consistency. However, to keep datasets more tractable, only sequences differing by more that 1% were used in the final analyses. Our sampling was not designed to estimate the extent of intraspecific variability, but was intended to capture major components at the supra-specific level (Ruedi and McCracken, in press). The 30 New World species were compared to 36 other species of *Myotis* from other regions of the world (Table 1). The current distribution of the sampled species was classified based on designations of classical zoogeographical regions (Cox, 2001), as listed in Table 1. For Nearctic and Neotropical regions, we used the limits established by Ortega and Arita (1998). The 36 Old World species of *Myotis* used for comparisons include 20 species from the Palaearctic, 10 species from the Oriental, one from the Oceanian, and five from the Ethiopian regions (Table 1). One sample from Japan was originally referred by Kawai et al. (2003) as *M. mystacinus*, but it is currently recognized as a subspecies of *M. brandtii* (Benda and Tsytsulina, 2000; Simmons, 2005), or as a species of its own, *M. gracilis* (Abe et al., 2005; Horácek et al., 2000). Likewise, *M. muricola browni* from Luzon in the Philippines, mentioned by Ruedi and Mayer (2001), probably does not belong to the species *M. muricola* (L. R. Heaney, pers. comm.) and is thus provisionally referred to as *M.* cf. *browni*. Based on previous genetic evidence from multiple sources, the Kerivoulinae and Murininae are the closest relatives of *Myotis* (Hoofer and Van Den Bussche, 2003; Kawai et al., 2002; Stadelmann et al., 2004b). Thus, *Harpiocephalus mordax* and *Kerivoula* cf. *papillosa* were used to root the tree of *Myotis*.

Table 1
Origin of specimens analysed for the Cyt *b*, and Rag 2

	Biogeographic region	GenBank		Locality	Voucher
Myotis		Cyt *b*	Rag2		
Myotis albescens	Neotropical	Ruedi and Mayer (2001)	AM265661	Tarija, Bolivia	FMNH 162543
Myotis alcathoe	Palaearctic	Stadelmann et al. (2004b)	AM265662	Vaud, Switzerland	MHNG 1828.73
Myotis annectans	Oriental	Stadelmann et al. (2004b)	AM265663	Ban Navang, central Laos	ROM 106376
Myotis atacamensis	Neotropical	AM261882	AM265640	Olmos, Peru	MVZ 168933
Myotis auriculus	Nearctic	AM261884	AM265641	Guanacevi, Mexico	CDR 3288
Myotis austroriparius	Nearctic	AM261885	AM265642	Tennessee, USA	THK 002
Myotis bechsteinii	Palaearctic	Ruedi and Mayer (2001)	AM265643	Vaud, Switzerland	MHNG 1805.24
Myotis blythii blythii	Palaearctic	Ruedi and Mayer (2001)	AM265644	Os, Kirghizstan	MHNG 1805.29
Myotis blythii oxygnathus	Palaearctic	Ruedi and Mayer (2001)	AM265645	Valencia, Spain	EBD 17527*
Myotis bocagii	Ethiopian	Stadelmann et al. (2004a)	AM265646	Agumasta, Ghana	SMF 89673
Myotis brandtii	Palaearctic	AM261886	AM265647	N.-W. Russia	NMP PB 916
Myotis cf. *browni*	Oriental	Ruedi and Mayer (2001)	AM265648	Mindanao, Philippines	FMNH 147067
Myotis californicus	Nearctic	AM261887	AM265649	Durango, Mexico	CDR 3276
Myotis capaccinii	Palaearctic	Ruedi and Mayer (2001)	AM265650	Peloponnese, Greece	No voucher
Myotis chiloensis	Neotropical	AM261888	AM265651	Santiago, Chile	THK I
Myotis ciliolabrum A	Nearctic	AM261889	Ø	Durango, Mexico	CDR 3172
Myotis ciliolabrum B	Nearctic	AM261890	Ø	Canada	No voucher
Myotis dasycneme	Palaearctic	Ruedi and Mayer (2001)	AM265652	Leiden, Holland	MHNG 1805.52
Myotis daubentonii	Palaearctic	Ruedi and Mayer (2001)	AM265653	Vaud, Switzerland	MHNG 1805.53*
Myotis dominicensis	Neotropical	Ruedi and Mayer (2001)	AM265654	St. Joseph's Parish, Dominica	TK 15613
Myotis elegans	Neotropical	AM261891	AM265655	La Selva, Costa Rica	3011
Myotis emarginatus	Palaearctic	Ruedi and Mayer (2001)	AM265656	Macedonia, Greece	MHNG 1807.40*
Myotis evotis	Nearctic	Stadelmann et al. (2004b)	AM265657	Alberta, Canada	No voucher
Myotis formosus	Oriental	Stadelmann et al. (2004b)	AM265658	Dong Amphan, south Laos	ROM 110544
Myotis goudoti	Ethiopian	Stadelmann et al. (2004a)	AM265659	Ambalavao, Madagascar	FMNH 151709
Myotis gracilis	Palaearctic	Kawai et al. (2003)	AM265660	Hokkaido, Japan	KK 0005
Myotis grisescens	Nearctic	AM261892	AM265664	Tennessee, USA	THK 11500
Myotis hasselti	Oriental	Ruedi and Mayer (2001)	AM265665	Selangor, Malaysia	SMF 69345
Myotis horsfieldii	Oriental	Ruedi and Mayer (2001)	AM265666	Malaysia	SMF 87502
Myotis ikonnikovi	Palaearctic	Kawai et al. (2003)	AM265667	Hokkaido, Japan	KK 0065
Myotis keaysi	Neotropical	Ruedi and Mayer (2001)	AM265668	Yucatan, Mexico	TK 13532
Myotis keenii	Nearctic	AM262329	AM265669	Vancouver Island, USA	THK 98-44
Myotis latirostris	Oriental	AM262330	AM265670	Moi-Li Co, Taiwan	MR-608
Myotis leibii	Nearctic	AM262331	AM265671	West Point, NY, USA	THK 2
Myotis levis	Neotropical	Ruedi and Mayer (2001)	AM265672	Sao Paolo, Brazil	FMNH 141600
Myotis lucifugus carrissima	Nearctic	Ruedi and Mayer (2001)	AM265673	Alaska, USA	UAM 22927
Myotis macrodactylus	Palaearctic	Kawai et al. (2003)	Ø	Tushima, Nagasaki, Japan	OCUMS 7243
Myotis macropus	Oceanian	Stadelmann et al. (2004b)	AM265674	Queensland, Australia	No voucher
Myotis macrotarsus	Oriental	Stadelmann et al. (2004b)	AM265675	Madai Cave, Sabah, Malaysia	CMF 960522.46*
Myotis martiniquensis	Neotropical	AM262332	AM265676	Martinique, France DOM	JFM DIREN 2
Myotis montivagus	Oriental	AM262333	AM265677	n. extension, NNT Laos	CMF 960418.2
Myotis cf. *muricola*	Oriental	Stadelmann et al. (2004b)	AM265678	Ban Keng Bit, Laos	SMF 86172
Myotis myotis	Palaearctic	AM261883	AM265679	La Rioja, Spain	EBD 15969
Myotis mystacinus	Palaearctic	Ruedi and Mayer (2001)	AM265680	Vaud, Switzerland	MHNG 1805.81*
Myotis nattereri	Palaearctic	Ruedi and Mayer (2001)	AM265681	Thrace, Greece	MHNG 1807.49*
Myotis nigricans	Neotropical	Ruedi and Mayer (2001)	AM265682	Paraiba, Brazil	MVZ 185681*
Myotis occultus	Nearctic	AM262334	AM265683	New Mexico, USA	THK 25
Myotis oxyotus	Neotropical	Ruedi and Mayer (2001)	AM265684	Lima, Peru	FMNH 129208
Myotis punicus	Palaearctic	Ruedi and Mayer (2001)	AM265685	Ghar Tabouda, Tunisia	MHNG 1807.66*
Myotis pruinosus	Palaearctic	Kawai et al. (2003)	Ø	Ishikawa, Japan	OCUMS 7495
Myotis ricketti	Oriental	Stadelmann et al. (2004a)	AM265686	Guillin, Guangxi, China	No voucher*
Myotis riparius	Neotropical	Ruedi and Mayer (2001)	AM265687	Pernambuco, Brasil	MVZ 185996
Myotis ruber	Neotropical	Ruedi and Mayer (2001)	AM265688	Salesopolis, Brazil	MVZ 185999
Myotis schaubi	Palaearctic	Ruedi and Mayer (2001)	AM265689	Choplu, Iran	NMP PB 1278
Myotis scotti	Ethiopian	Stadelmann et al. (2004b)	Ø	Coccia, Ethiopia	ZMMU 167226
Myotis septentrionalis	Nearctic	AM262335	AM265690	New Hampshire, USA	THK 1
Myotis sicarius	Oriental	Stadelmann et al. (2004b)	Ø	Annapurna, Nepal	ZMMU 164493
Myotis simus	Neotropical	AM262336	AM265691	Brasil	THK Mys-ET3
Myotis sodalis	Nearctic	AM262337	AM265692	Vermont, USA	THK 2002-JK-01
Myotis thysanodes	Nearctic	Ruedi and Mayer (2001)	AM265693	Texas, USA	TK 78796
Myotis tricolor	Ethiopian	Stadelmann et al. (2004b)	AM265694	Transvaal, South Africa	TM 40300
Myotis velifer	Nearctic	Ruedi and Mayer (2001)	AM265695	Sonora, Mexico	MVZ 146766

Table 1 (*continued*)

	Biogeographic region	GenBank		Locality	Voucher
Myotis vivesi	Nearctic	Stadelmann et al. (2004a)	AM265696	Baja California, Mexico	No voucher
Myotis volans A	Nearctic	Ruedi and Mayer (2001)	Ø	Texas, USA	TK 78980
Myotis volans B	Nearctic	Ruedi and Mayer (2001)	AM265697	Texas, USA	TK 78925
Myotis welwitschii A	Ethiopian	Ruedi and Mayer (2001)	AM265698	Transvaal, South Africa	TM 39421
Myotis welwitschii B	Ethiopian	Stadelmann et al. (2004b)	AM265699	Simandou Range, Guinea	SMF DNA 105
Myotis yanbarensis	Palaearctic	Kawai et al. (2003)	Ø	Okinawa, Japan	SM 7365
Myotis yumanensis	Nearctic	Ruedi and Mayer (2001)	AM265700	California, USA	MVZ 155853
Outgroups					
Subfamily Kerivoulinae					
Kerivoula cf. *papillosa*	Oriental	Stadelmann et al. (2004b)	Hoofer et al. (2003)	Dong Nai, Vietnam	ROM 110850*
Subfamily Murininae					
Harpiocephalus mordax	Oriental	Stadelmann et al. (2004b)	AM265701	Nam Et, north Laos	CMF 980322.73

The specimens newly sequenced are indicated by their GenBank number. Vouchers are deposited in the following institutions or belong to personal collections: Senckenberg Museum of Frankfurt (SMF), Transvaal Museum, South Africa (TM), Natural History Museum of Geneva (MHNG), and the Royal Ontario Museum (ROM), C. M. Francis, National Wildlife Research, Ottawa (CMF), Museum of Texas Tech University (TK), Field Museum of Natural History in Chicago (FMNH), Kunico Kawai (KK), Sumiko Matsumura (SM), Osaka City University Graduate School of Medicine (OCUMS), National Museum Prague (NMP), Estacion Biologica de Doñana (EBD), Instituto Politécnico Nacional in Mexico (CDR), Museum of Vertebrate Zoology at Berkeley (MVZ), University of Alaska Museum (UAM), Zoological Museum of Moscow State University (ZMMU), Jean-François Maillard, DIREN, Martinique (JFM DIREN), and uncatalogued specimens of T.H. Kunz (THK). Samples without vouchers were from bats released following capture. For those species marked with an asterisk, the two genes were not sequenced in the same individuals.

2.2. DNA extraction

All samples consisted of ethanol-preserved tissues collected with appropriate permits or obtained as loans from various institutions (Table 1). Prior to DNA extraction, about 10–30 mg of tissue was soaked for 1–2 h in sterile water. Total genomic DNA was then isolated following a salting out protocol developed by Miller et al. (1988), as detailed in Castella et al. (2001). The final extraction product was rediluted into 50 µl of low TE buffer and stored at −20 °C until further analyses.

2.3. Amplification, sequencing and alignment

2.3.1. Cytochrome b

Depending on samples, various primer pairs were used for the amplification by polymerase chain reactions (PCRs) of the complete cytochrome *b* gene (Cyt *b*), as described in Stadelmann et al. (2004a,b). PCR cocktails (50 µl reaction volume) included 2–10 µl of DNA extract, 0.2 µM of each primer, 2.5–4 mM of $MgCl_2$, 0.2 mM each of 4 dNTPs, 1 unit of *Taq* DNA polymerase (QIAgen, Inc., Switzerland) with appropriate buffer and ddH_2O. Thermal profiles of amplifications started with three minutes of 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). To amplify the entire Cyt *b*, the preferred primer pair was L14724 (Irwin et al., 1991)–BSVES268H (Stadelmann et al., 2004b). However, few samples needed a re-amplification step to obtain sufficient material for sequencing. In the latter case 1 µl of the initial PCR product was taken and amplified to obtain two overlapping PCR fragments: the 5′ part of the Cyt *b* was obtained with L14724–MVZ16 (Irwin et al., 1991), while the 3′ end was obtained with L15162 (Smith and Patton, 1991)–BSves268H (see Stadelmann et al., 2004b). For these re-amplifications, only 19–25 cycles including an annealing temperature set between 54 and 56 °C were used. All final PCR products were purified using a QIAgen PCR Purification Kit and sequenced directly (ABI Prism 377 automated DNA sequencer) in one direction using primers L14724 and BSves268H. The two smaller, overlapping PCR fragments were assembled manually, edited and aligned with the software BioEdit (Hall, 1999). The overlap of about 300 bp of these two fragments made it possible to check for consistency. Sequences were translated into amino acids to check for reading frame.

2.3.2. Recombination activating gene 2

Because the history of mitochondrial genes do not necessarily reflect the phylogenetic history of the organisms bearing them (Avise, 1994), a nuclear gene was also sequenced for a subset of taxa. Although known to be more conservative than Cyt *b*, the Recombination Activating Gene 2 (Rag 2) proved to be useful to infer the phylogenetic evolution of mormoopids and noctilionids (Lewis-Oritt et al., 2001), and thus was chosen to resolve the *Myotis* radiation.

Previous primers used to amplify the Rag 2 in bats (Hoofer et al., 2003; Lewis-Orrit et al., 2001) failed to amplify several samples of our *Myotis* or yielded multiple bands, so we redesigned a new primer pair based on an alignment of human, mouse and two *Myotis* species (*M. riparius* and *M. velifer*) sequenced by Hoofer et al. (2003). The new primer pair is: 179F (5′- CAGTTTTCT CTAAGGAYTCCTGC-3′), and 1458R (5′- TTGCTATC TTCACATGCTCATTGC-3′). PCR cocktails and thermal profiles were identical to Cyt *b*, except for the annealing temperature fixed at 60 °C. In cases where re-amplification

was necessary to obtain sufficient PCR product, two other internal primers were designed: 968R (5′-CCCATGTTGC TTCCAAACCATA-3′) coupled with 179F, and 428F (5′-ATGTGGTATATAGTCGAGGGAAGAGC-3′) used with 1458R. In these situations, the PCR profile only consisted of 19–25 cycles, and the annealing temperature was increased to 63 °C. Purifications, sequencing and edition of the final products were accomplished as described above. The 1148 bp of the Rag 2 obtained in this dataset correspond to homologous positions 241–1388 in the human sequence (Ichihara et al., 1992).

2.4. Phylogenetic analyses

All phylogenetic analyses initially were conducted separately on the Cyt *b* and on the Rag 2 datasets. Because there are few variable characters in the Rag 2 partition, conflict between estimates of phylogeny was minimal and non-significant from the partition-homogeneity test implemented in PAUP* (Swofford, 2001). Subsequent reconstructions also were performed on the combined datasets. Probabilistic methods were used to reconstruct phylogenetic trees with Maximum Likelihood (ML; implemented in PHYML version 2.4.4, Guindon and Gascuel, 2003), and Bayesian inferences (implemented in MRBAYES 3.0b4, Ronquist and Huelsenbeck, 2003). Hierarchical-likelihood ratio tests performed with Modeltest 3.04 (Posada and Crandall, 1998) determined that a General Time Reversible model with rate variation among sites and a proportion of invariable sites (GTR + Γ_8 + I; Lanave et al., 1984; Rodriguez et al., 1990; Yang, 1994) represent the best fit model of nucleotide substitution for the Cyt *b*, and the combined datasets. The simpler HKY + Γ_8 + I was more appropriate for the Rag 2 dataset. However, to simplify all model-based analyses in the different partitions, the more parameter-rich GTR + Γ_8 + I model was chosen for all phylogenetic reconstructions. In the PHYML procedure, the starting tree was obtained with Bionj (Gascuel, 1997), which is an improved version of the neighbour-joining algorithm of Saitou and Nei (1987). The parameters of the GTR + Γ_8 + I model were estimated and optimized during the search. Reliability of nodes was assessed with 300 non-parametric bootstraps replicates (Felsenstein, 1985) using PHYML. Bayesian posterior probabilities were calculated using a Metropolis-coupled, Markov chain, Monte Carlo (MCMCMC) sampling approach, as implemented in MRBAYES 3.0b4 (Huelsenbeck and Ronquist, 2001) and using the same GTR + Γ_8 + I model. Four simultaneous Markov chains were run for 1,000,000 generations with trees sampled every 10 generations. After about 20,000 generations, the log-likelihoods of trees reached an asymptote. These initial trees were discarded as burn-in. Posterior probabilities were subsequently computed from the consensus of the remaining 98,000 sampled trees. MRBAYES was set to estimate model parameters independently and simultaneously for each gene partition. We adopted this strategy with the combined dataset.

Cladistic analyses also were performed to reconstruct phylogenetic trees, using the principle of maximum parsimony (MP) implemented in PAUP* version 4.0b10 (Swofford, 2001). Because the unweighted parsimony method performs poorly when multiple substitutions are likely to affect nucleotides (Huelsenbeck and Lander, 2003), we used a stepmatrix to weight tranversions 13 and 11 times over transitions, respectively, to the Cyt *b* and combined datasets. These ratios were empirically determined from the datasets with PAUP*. The most parsimonious tree was estimated using a heuristic search with 100 random additions of taxa, and complete tree-bisection-reconnection branch swapping for each iteration (Swofford et al., 1996). MP was not carried out with Rag 2 dataset alone because the few variable characters resulted in multiple, equally parsimonious solutions impeding analyses to complete heuristic searches in a reasonable time. For the Cyt *b* and combined datasets, nodal support under MP framework was assessed by bootstrap analyses with 1000 replicates, each consisting of 15 random additions of taxa, and complete tree-bisection-reconnection branch swapping.

Comparison of tree topologies with and without topological constraints was investigated under a likelihood framework, using the Kishino and Hasegawa (1989) (KH), the Shimodaira and Hasegawa (1999) (SH) tests implemented in PAUP*, and the approximately unbiased (AU) test of Shimodaira (2002). Topological tests were applied on the combined dataset only.

2.5. Molecular dating

Divergence age of selected nodes was estimated by the Bayesian relaxed molecular-clock approach implemented in the program MULTIDISTRIBUTE, developed by Thorne et al. (1998), Kishino et al. (2001), and Thorne and Kishino (2002). Calculations were performed on the ML tree obtained from the combined dataset. This method relaxes the molecular-clock assumption along the branches of the optimal tree, and allows the incorporation of multiple constraints from the fossil record, while taking into account both molecular and palaeontological uncertainties to estimate the variance of divergence times. The dating procedure consists in two steps. The module ESTBRANCHES first estimates branch lengths and their variance–covariance matrix from the nucleotide dataset. *Harpiocephalus mordax* is used as an outgroup. The eight-rate category (Γ_8) values for a gamma distribution needed in ESTBRANCHES were estimated with PAML 3.14 (Yang et al., 1997) under the F84 model (Felsenstein, 1984). Given fixed palaeontological constraints (see below), the second module MULTIDIVTIME estimates the mean divergence times between taxa, with standard deviation, and 95% confidence intervals. Markov chains Monte Carlo was sampled every 100 generation over 1 million generations, after a burn-in period of 100,000 cycles. We used the following priors of Gamma distributions for the model of rate autocorrelation: a mean 30 million years ago (MYA) for the expected time

units between root and tips (rttm), corresponding to the separation between *Myotis* and the lineage leading to the Kerivoulinae and Murininae, 0.004898 substitution per site per million years for the rate at the root node (rtrate), and 0.03508 for Brownian motion constant that describes the degree of rate autocorrelation along the descending branches of the tree (brownmean). MULTIDIVTIME needs a parameter called "bigtime" that represents the greatest amount of time between root and tips. This date was set to 65 MYA, the inferred date of divergence of bats from other mammalian ancestors (Teeling et al., 2005). Following Ruedi and Mayer (2001), we used two internal calibration points within the *Myotis* tree: first, *M. daubentonii* and *M. bechsteinii* were constrained to have diverged at least 5 MYA (Topál, 1983), and second, *M. nattereri* was constrained to have diverged from *M. schaubi* between 5.5 and 6.5 MYA (Horácek and Hanák, 1983–84). To accommodate for uncertainties associated with the palaeontological age of fossils, we followed the method suggested by Douady and Douzery (2003) to bracket these calibration points with the age of the lower and upper geological layers known to include the fossils (i.e. 3.6–11.2 MYA for the *bechsteinii–daubentonii* node and 3.6–7.1 MYA for the *nattereri–schaubi* node).

2.6. Reconstruction of biogeographic origin

To infer the possible biogeographic origin of the New World *Myotis*, we used a maximum likelihood method implemented in Mesquite version 1.05 developed by Maddison and Maddison (2004). This program can trace character history on a tree following a probabilistic approach and calculation, and reconstruct the ancestral state of a character (here biogeographic origin) by giving probabilities of each state at each node. Moreover, it takes into account that a change is more likely to occur along a long branch, than in shorter ones. The current geographic distribution of extant species was coded based on classical zoogeographic regions (Table 1). The model of character evolution was a simple stochastic model (Mk1), which incorporates a symmetrical single rate of change between any two states. This rate of change is estimated based on the character distribution and the tree topology.

3. Results

3.1. Phylogenetic analyses

3.1.1. Cyt b tree

Twenty-one complete sequences of the Cyt *b* were newly obtained, which represent 15 New World, 2 Palaearctic, and 3 Oriental species of *Myotis* (Table 1). These new sequences are deposited in the GenBank Accession Nos. AM261882–AM261892 and AM262329–AM262337. The final alignment of Cyt *b* sequences thus consisted in 1140 positions, of which 567 were variable. Within the genus *Myotis*, 560 positions were variable, 134 of which were first codon position, 56 second codon position, and 370 third position. Saturation curves indicate that beyond about 15% raw sequence divergence, substitution at the Cyt *b* are underestimated (Fig. 1). The mean genetic distance (corrected for multiple hits with GTR + Γ_8 + I) is 38% within the genus *Myotis*, and 79% when *Myotis* species are compared to the outgroup. The ML tree of Fig. 2a presents the phylogenetic relationships of all 71 sequences of Cyt *b* representing 68 species. Tree topologies obtained with MRBAYES and MP topologies were similar except for the position of some basal nodes within the Old World *Myotis*. Bootstrap supports of nodes estimated with the three methods are shown on Fig. 2a. All sequenced New World species of *Myotis* are grouped in a strongly supported monophyletic clade in all reconstructions. This clade includes two Palaearctic lineages, represented by the Eurasian species *M. brandtii* and the Eastern Palaearctic *M. gracilis*. We designated this pair of sister species the *brandtii* lineage. Besides the *brandtii* lineage, the New World clade is further subdivided into two major subclades, one containing 12 Nearctic species, while the other is mostly Neotropical. Thus, we designated them as the Nearctic and Neotropical subclades, respectively. The latter includes all 12 sequenced Neotropical species, *M. vivesi* from Baja California, and four North American species (*Myotis yumanensis*, *M. velifer*, *M. austroriparius*, and *M. grisescens*). Several other major clades have moderate to strong support within the Old World *Myotis*. These clades are named with Roman numerals (Fig. 2) and include the Ethiopian clade of a previous paper (Stadelmann et al., 2004a). Although the ML tree of Fig. 2a suggests a common origin for these Old World species, low or no statistical support was provided by the resampling procedures.

Another major feature emerging from the Cyt *b* tree is the basal position of *M. latirostris*. Posterior probabilities for this basal position exceeded 0.81, but weak (36% bootstrap in ML) or no (MP) bootstrap support was found in other phylogenetic reconstructions. Four distinct specimens of *M. latirostris* from different locations in Taiwan have been sequenced for the complete Cyt *b* and all haplotypes

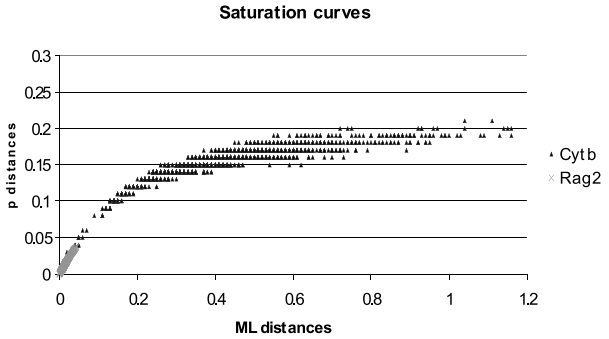

Fig. 1. Uncorrected (p-distance) pairwise distances plotted against ML distance (corrected with GTR + Γ_8 + I) measured for the Cyt *b* (black triangle) and Rag 2 (light grey cross) datasets. This saturation curve indicates that Cyt *b* substitutions reached a plateau beyond about a p-distance of 0.15.

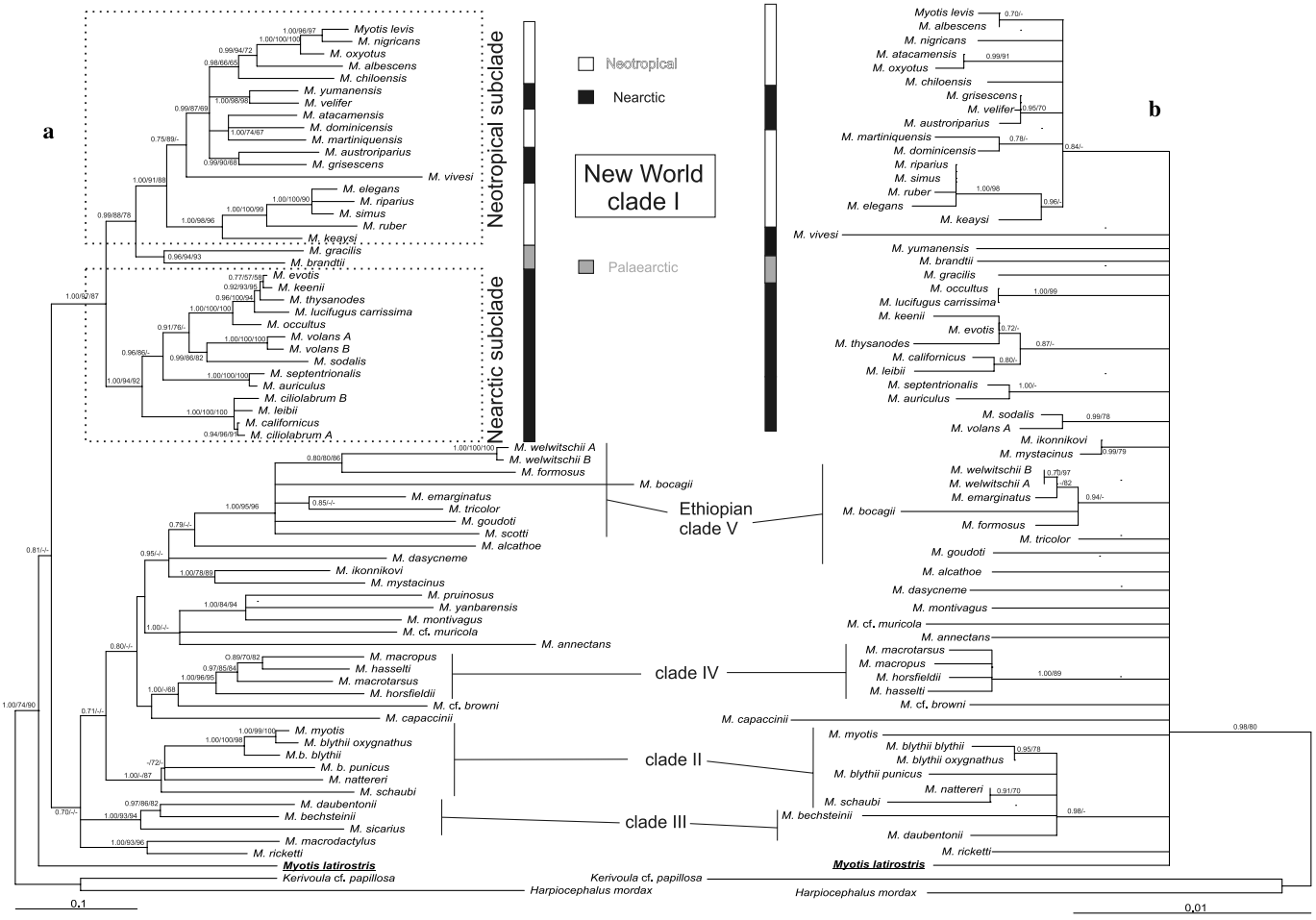

Fig. 2. Phylogenetic relationships of *Myotis* species and two outgroup taxa obtained from DNA datasets analysed separately. (a) Phylogenetic relationships of 71 complete Cyt *b* gene sequences analysed with maximum likelihood criteria implemented in PHYML (Guindon and Gascuel, 2003) and GTR + Γ_8 + I as model of nucleotide substitutions. Nodal support (Bayesian analyses, ML, and MP) is indicated throughout the entire New World clade. For the remaining parts of the tree, nodal support was only indicated if bootstrap or posterior probabilities exceeded 70%. When none of the three methods reached this level of support, the node was collapsed. Five major clades are indicated within the Old World species. Within the New World clade, the thick white line indicates Neotropical species, the black line Nearctic species, and the grey line Palaearctic species. (b) Phylogenetic relationships based on 63 Rag 2 sequences. This maximum likelihood tree was reconstructed by using PHYML (Guindon and Gascuel, 2003) and GTR + Γ_8 + I as model of nucleotide substitutions.

were within 0–3 mutations of the sequence deposited in GenBank (Table 1). Thus, alternative phylogenetic reconstructions based on any of these haplotypes resulted in a basal position of *M. latirostris*.

3.1.2. Rag 2 tree

We ensured that the same individual was sequenced for the Cyt *b* and Rag 2 genes, but this was not possible for all species (see asterisk in Table 1). Moreover, because tissue samples were not available for all species used in the Cyt *b* dataset (e.g. those taken only from GenBank), the Rag 2 dataset was slightly reduced (Table 1). The final Rag 2 dataset consists of 63 partial sequences (1148 bp), representing 61 species (Table 1). These new sequences are deposited in GenBank Accession Nos. AM265640–AM265701. The alignment of Rag 2 sequences consisted of 1148 positions, of which 192 were variable. In contrast to Cyt *b*, Rag 2 sequences do not show evidence of saturation (Fig. 1). Within the genus *Myotis*, 162 positions were variable, 22 of which were first codon positions, 19 second codon positions, and 121 third positions. The mean genetic distance at the Rag 2 (corrected for multiple hits with HKY + Γ_8 + I) is 1.2% within the genus *Myotis*, and 3.2% when only the distances between the outgroup and the *Myotis* are considered. The mean genetic distances among *Myotis* species is about 30 times smaller with the Rag 2 than with the Cyt *b*. For example, species pairs that are morphologically and genetically distinct with the Cyt *b* gene (*M. riparius* and *M. simus*, or *M. lucifugus carrissima* and *M. occultus*) do not differ over the 1148 bp of the Rag 2. Given the high number of taxa considered and the modest number of variable positions, the resulting phylogenetic trees based on Rag 2 only are much less resolved than those based on Cyt *b* (Fig. 2). Only 22 nodes are supported by more than 70% ML bootstrap or more than 0.7 posterior probabilities in Bayesian reconstructions (Fig. 2b). Because

of the multitude of equally parsimonious solutions held at each step of heuristic searches, parsimony analyses could not be performed on this dataset alone. Yet several major features of phylogenetic trees obtained with the Rag 2 dataset corroborate those issued from the Cyt *b* dataset. The Neotropical subclade found with the Cyt *b* is present with Rag 2 except that *M. vivesi* and *M. yumanensis* are not part of it. The Nearctic subgroups are described but cannot be linked together in a strongly supported subclade as with Cyt *b*. The clade IV, including three Oriental and one Oceanian species, was strongly supported by both datasets although the branching order within this clade vary among datasets (Figs. 2a and b). The Ethiopian clade V was highly supported by the Cyt *b* dataset ($\geqslant 95\%$ bootstrap or posterior probabilities in all reconstructions, Fig. 2a), but is only partly supported by the Rag 2 dataset (Fig. 2b). Indeed, *M. goudoti* and *M. tricolor* are placed in an undetermined position in the latter phylogenetic reconstructions. *M. latirostris* appears at the base of the *Myotis* radiation, as in the Cyt *b* dataset, but again this position is not supported statistically (i.e $\leqslant 70\%$) and thus is not included in Fig. 2b. The divergence between *Myotis* and the outgroup is strongly supported with Rag 2. If poor phylogenetic resolution appears from the Rag 2 reconstructions, no strong contradiction emerges when compared to the Cyt *b* tree.

3.1.3. Combined analyses

ML topology resulting from the analysis on the combined nuclear (Rag 2) and mitochondrial (Cyt *b*) datasets are shown in Fig. 3. The combined dataset comprises 63 lineages and has the same taxon sampling as for Rag 2 alone. The three methods of reconstruction (MRBAYES, ML, MP) produced congruent topologies (Fig. 3), which are very similar to the tree based on Cyt *b* dataset (Fig. 2a). Exceptions include the position of *M. occultus*, which appears sister to *M. lucifugus carrissima* in the combined tree, while it was placed at the basis of a clade containing *M. evotis*, *M. keenii*, and *M. thysanodes* in the Cyt *b* tree. This is not surprising since *M. occultus* and *M. l. carrissima* share exactly the same sequence of Rag 2. Other incongruent phylogenetic positions between Cyt *b* and combined datasets include "floating" species like *M. capaccinii*, *M.* cf. *muricola*, or *M. dasycneme*. These individual species have an unstable position (i.e. no strong support) in any reconstruction (Figs. 2 and 3).

The monophyly of the New World clade and further subdivision within this clade are supported with increasing bootstrap and posterior probability values compared to Cyt *b* or Rag 2 datasets alone. The basal position of *M. latirostris* also receives a higher posterior probability for the combined dataset.

3.1.4. Alternative tree topologies

The precise position of the Eurasian *brandtii* lineage within the New World clade has important implications for biogeographic reconstructions. Thus, we tested whether this lineage could be placed in alternative phylogenetic positions without altering significantly the likelihood of the optimal tree. KH, SH, and AU tests rejected (p-value $\leqslant 0.02$) the possibility that the *brandtii* lineage could be more derived (i.e. embedded) within the Neotropical subclade. Tree topologies forcing the *brandtii* lineage to be within the Nearctic subclades were rejected by the three tests (p-value $\leqslant 0.015$), as were the constrained topologies placing the *brandtii* lineage within the Old World lineage (p-value $\leqslant 0.002$). Tree topologies constraining the *brandtii* lineage to be sister to the Nearctic subclade were not clearly rejected by the three tests (p-value $\leqslant 0.09$). The last alternative topology explored was to force *brandtii* to be the sister lineage to all remaining New World species. This alternative topology was marginally rejected by the KH and SH tests with a p-value $\leqslant 0.07$, but more so by the AU test (p-value $\leqslant 0.04$). At least part of these last two indecisive results could be attributed to the unstable position of "floating" species from Eurasia. Indeed, if *M. dasycneme*, *M. alcathoe*, or *M. capaccinii* are removed from the dataset, the possible sister-group position of *brandtii* relative to New World species is rejected by all tests (p-value $\leqslant 0.05$ for KH and SH; p-value $\leqslant 0.025$ for AU).

Another aspect investigated by topological constraints was the possibility that all Neotropical and all Nearctic species are reciprocally monophyletic, unlike on the optimal trees obtained in Figs. 2 or 3. Again, KH, SH, and AU tests rejected these alternative phylogenetic hypotheses as significantly worse than the optimal trees (p-value $\leqslant 0.0017$). *M. yumanensis*, *M. velifer*, *M. austroriparius*, *M. grisescens*, and *M. vivesi* are clearly part of the Neotropical subclade according to reconstructions based on the combined dataset.

3.2. Molecular dating

The divergence time of all *Myotis* from the *Kerivoula* lineage obtained with Bayesian relaxed molecular clock on the combined dataset and ML tree (Fig. 3) is 16.2 ± 2.9 MYA (Middle Miocene). *M. latirostris* split off about 13.0 ± 2.2 MYA, while the New World clade diverged about 12.2 ± 2.0 MYA. Within the New World, the Nearctic subclade started diverging about 9.9 MYA ± 1.7. The *brandtii* lineage then split from the Neotropical subclade 8.7 ± 1.6 MYA. Within the latter, the two pairs, *M. austroriparius–M. grisescens* and *M. velifer–M. yumanensis* separated from their Neotropical sister species 6.2 ± 1.7 MYA, and 6.0 ± 1.6 MYA, respectively. Estimates of divergence time are reported in Fig. 4.

To investigate the effect of the two internal calibration points on these estimates, MULTIDIVTIME was also run on the same dataset but without internal constraint. The resulting estimates of node ages increased by a mean of 1.8 MYA, and associate standard deviations increased by a mean of 1.9 MYA. The *M. daubentonii–M. bechsteinii* split was inferred to be 5.8 ± 1.1 MYA, falling within the interval fixed between 3.6 and 11.2 MYA from the fossils. Similarly, the *M. schaubi– M.nattereri* split was inferred to be 6.3 ± 1.2 MYA, using the unconstrained estimates. This result is again similar to the interval delimitated from the fossil record (between 3.6 and 7.1 MYA).

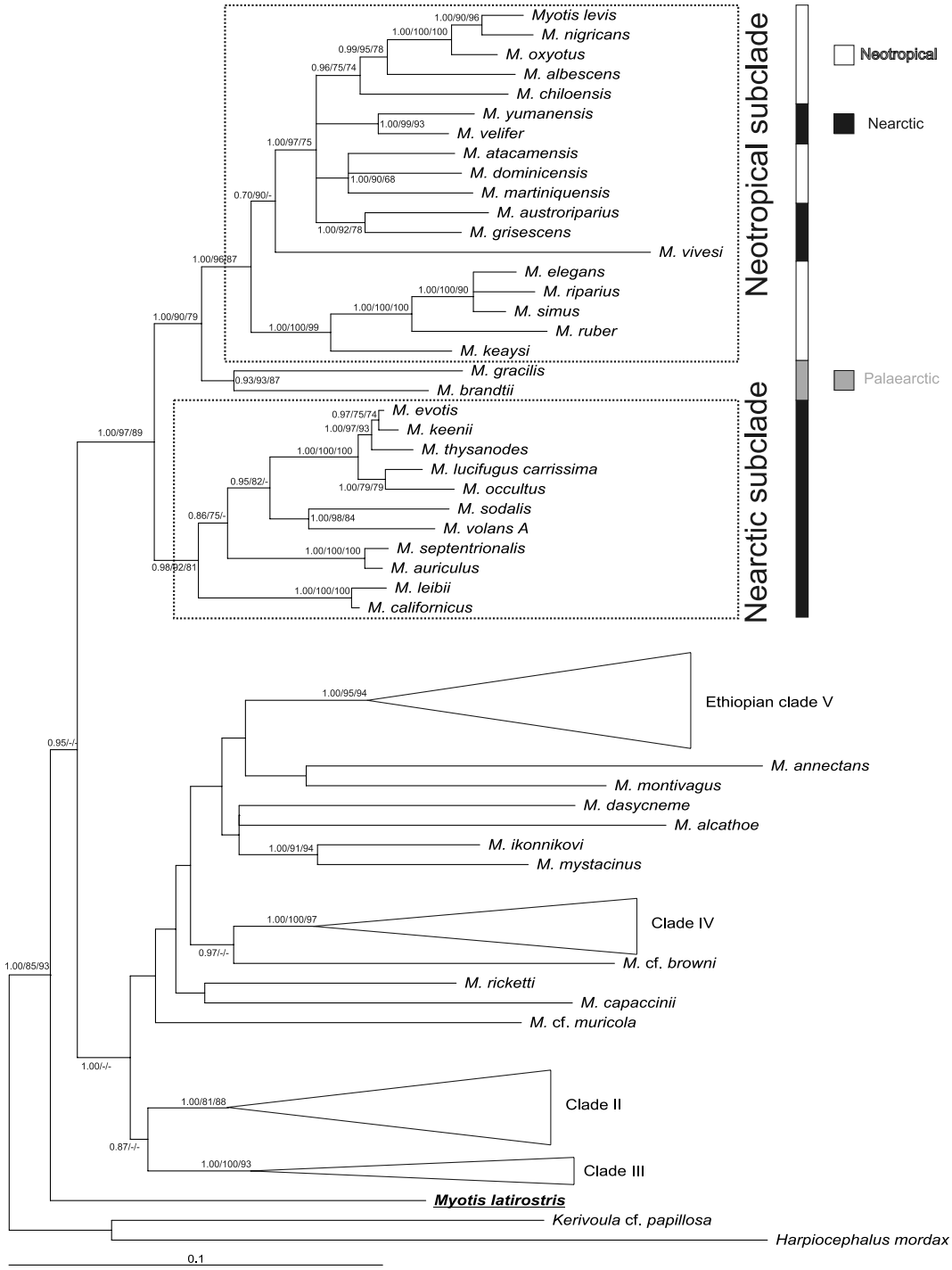

Fig. 3. Phylogenetic relationships of 63 combined sequences of Cyt *b* (1140 bp) and Rag 2 (1148 bp) genes. This maximum likelihood tree was reconstructed using PHYML (Guindon and Gascuel, 2003) and a GTR + Γ_8 + I as model of nucleotide substitutions. Nodal supports follow the same conventions as for Fig. 2. To focus on the New World species, four highly supported, congruent Old World clades (II–V) are symbolized by triangles. Within the New World clade, a solid vertical bar indicates the position of Nearctic species, a hollow bar Neotropical species, and a grayed bar the Palaearctic species.

3.3. Biogeographic reconstructions

Because the main focus of this study is on New World *Myotis* and because the taxonomic sampling of Old World species is still too fragmentary, the maximum likelihood method implemented in Mesquite was restricted to the New World clade to infer possible biogeographic changes along branches of the tree in Fig. 3. The Old World species were only used as outgroups to polarize the ML tree. Notice that the very short internodes among four lineages within the Neotropical subclade received poor statistical support and translate into multifurcation in Fig. 3. Although they appear fully resolved in Figs. 4 and 5, biogeographic interpretations will account for

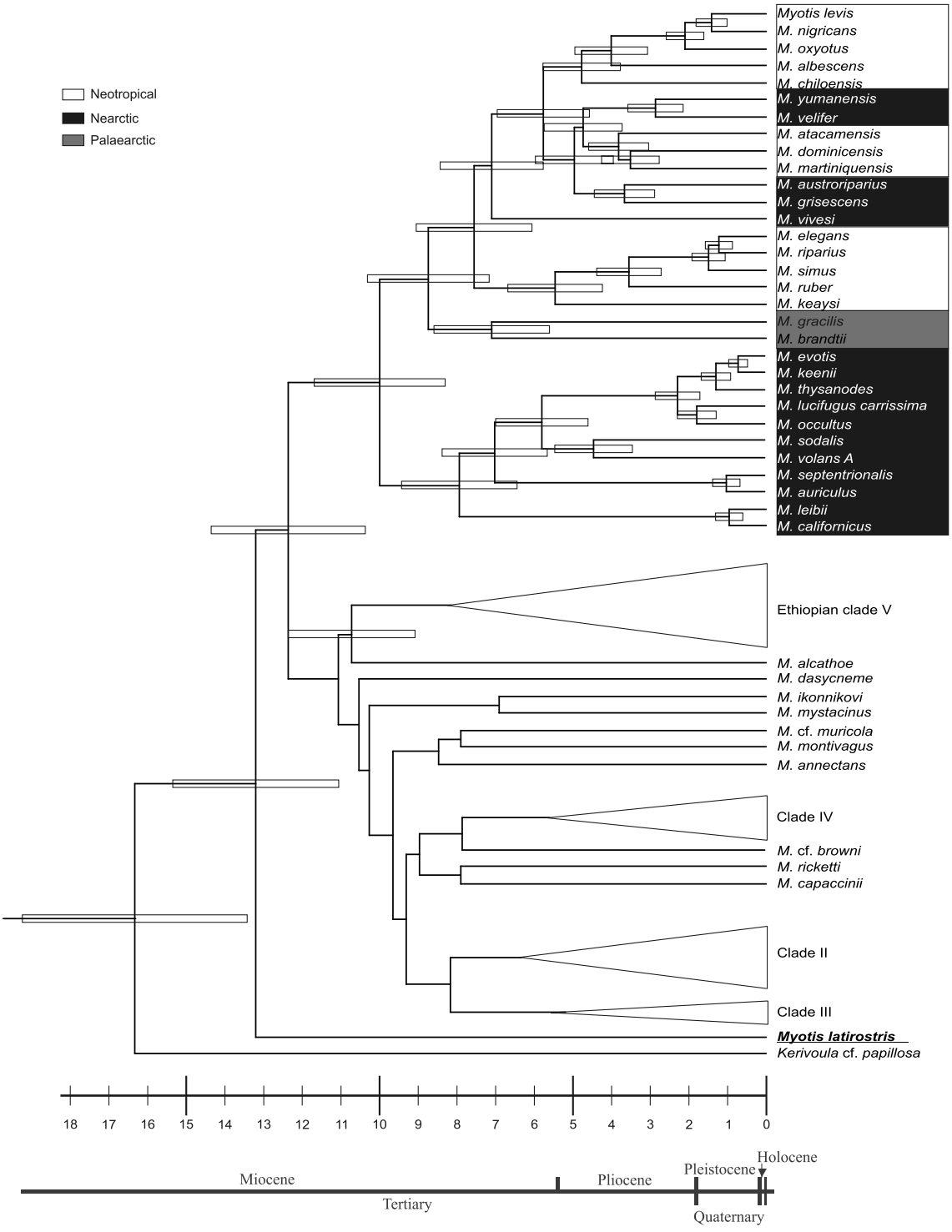

Fig. 4. Chronogram obtained from the combined Cyt *b* and Rag 2 datasets. Ages are inferred from the Bayesian-rate autocorrelation method using three nodes with palaeontological constraints. Horizontal boxes stand for ±one standard deviation around divergence ages, and are fully illustrated only for New World clade, and a few other nodes of interest.

these uncertainties. The likelihood of each biogeographic state for each node of the New World clade is shown in Fig. 5. According to this analysis, the common ancestor of the New World clade and the Old World *Myotis* indicate a probability of 96% of being Palaearctic, 4% of being Nearctic, and 0% of being Neotropical (Fig. 5). The basal node of the New World radiation of *Myotis* has a probability of 52% of being Palaearctic, 41% of being Nearctic, and 4% of being Neotropical (Fig. 5). In other words, the most recent common ancestor of all New World species most likely lived in the Palaearctic. The second most basal node, at the origins of the *brandtii* lineage and the

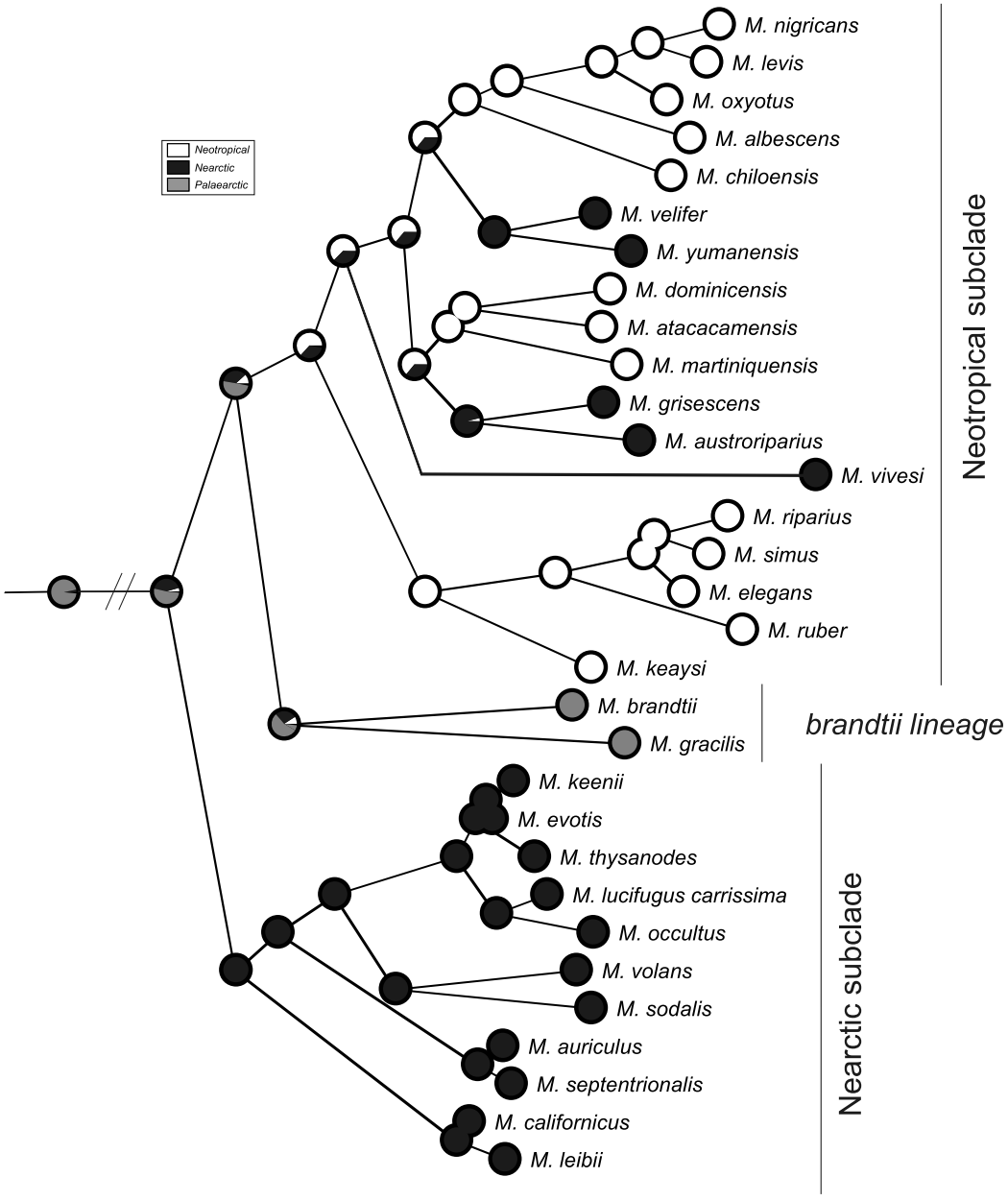

Fig. 5. Likelihood reconstruction of possible biogeographic origins of *Myotis* ancestors of the New World clade. This analysis is based on the ML tree of Fig. 3. Probability of each state (Neotropical in white, Nearctic in black, and Palaearctic in grey) is given for all nodes.

Neotropical subclade, has a 54% probability of being Palaearctic, and 46% of being New World (i.e. Nearctic or Neotropical). The common ancestor of *M. brandtii* and *M. gracilis* has a probability of 55% of being Palaearctic, and 45% of being New World. The two pairs of North American species found in the Neotropical subclade (*M. austroriparius–M. grisescens*, and *M. velifer–M. yumanensis*) have a probability of 66% to have descended from a Neotropical ancestor (Fig. 5), and 34% from a Nearctic one. The same probabilities are found with respect to the origin of *M. vivesi*. Finally, as all species of the Nearctic subclade currently reside in that biogeographic region, likelihood reconstructions identify with 100% probability that they originated in the Nearctic region.

4. Discussion

Traditional biogeographic analyses of the New World mammalian fauna emphasize the distinction of terrestrial mammals versus bats, because the latter generally are more vagile (Fleming and Eby, 2003). Although it is indisputable that bats are more wide ranging than small terrestrial rodents, several phylogeographic studies have shown that some groups of bats can have geographically highly-structured populations as well (see reviews in Carstens et al., 2004; Ruedi and McCracken, in press). Likewise, some narrow stretches of open water (>14 km) can be absolute barriers for *Myotis* bats, which otherwise are wide ranging over terrestrial lansdcapes (e.g. Castella et al., 2000). On a

continental scale, our phylogenetic analyses of mitochondrial and nuclear DNA sequences (Figs. 4 and 5) demonstrate that New World species of *Myotis* have only rarely dispersed across biogeographic regions, despite their ability to fly long distances (Fleming and Eby, 2003).

Unlike all previous systematic and taxonomic accounts based exclusively on morphological characters (e.g. Findley, 1972; Koopman, 1994; La Val, 1973; Tate, 1941a), molecular reconstructions demonstrate that ecologically and/or morphologically similar species found in different continents, for example, Palaearctic *M. daubentonii* and the Nearctic *M. lucifugus* (Gaisler and Zukal, 2004) or *M. nattereri* and *M. thysanodes*, are not closely related (Ruedi and Mayer, 2001). Rather, our analyses suggest a long history of independent evolution and parallel or convergent evolution of morphological characters. This evidence of convergent or parallel evolution of characters, and of the strong influence of geographic origins of samples in shaping phylogenetic relationships is now supported by growing number of molecular studies (Hoofer and Van Den Bussche, 2003; Kaessmann et al., 1999; Kawai et al., 2003; Matveev et al., 2005; Ruedi and Mayer, 2001; Stadelmann et al., 2004a,b). An additional emergent feature of these studies is the particular place occupied by the New World species of *Myotis*, which is the main focus of this paper.

4.1. Phylogenetic relationships and taxonomy

4.1.1. Phylogenetic evidence of a New World clade

Ruedi and Mayer (2001) and Hoofer and Van Den Bussche (2003) analysed 17 of the 38 species of New World *Myotis* with various mitochondrial DNA genes and suggested that these taxa apparently evolved from a common ancestor distinct from most Old World species. By increasing the taxon sampling to about 30 species and by using nuclear DNA sequences as well, the monophyly of the New World clade is further supported (Figs. 2–4). Combined analyses of Cyt *b* and Rag 2 datasets support with high values (87–100%) this monophyletic group, regardless of the method used to reconstruct phylogenetic trees (Fig. 3). Another common feature of our molecular reconstructions is that a single lineage comprised of two Palaearctic species (*M. brandtii* and *M. gracilis*) is also included in this New World clade. Alternative topologies placing the *brandtii* lineage outside the New World clade are all rejected by topological tests. These two species are sometimes considered as only subspecifically distinct (e.g. Benda and Tsytsulina, 2000; Koopman, 1994) and are distinctive as being distributed in the northern limits of temperate bat species (Horácek et al., 2000). *Myotis gracilis* is distributed in Japan and the Russian Far East, and thus lives in close proximity to the western side of the Bering Strait (Horácek et al., 2000). Other species of *Myotis* living at the eastern border of the Palaearctic region (e.g. *M. ikonnikovi*, *M. frater*) have all been sequenced for the Cyt *b* gene (Kawai et al., 2003), but none are closely related to the *brandtii* lineage or to other members of the New World clade (Fig. 2a). Thus, species derived from the *brandtii* lineage appear to be the only Old World representatives closely linked and embedded in the New World clade.

Two alternative hypotheses emerge from the different analyses concerning the origin of the *brandtii* lineage. The first is derived from the topological constraints and divergence times (Fig. 4), which suggest that the *brandtii* lineage diverged after the separation of the New World clade. This would imply that it colonized the Palaearctic region secondarily, probably via the Bering Strait. Conversely, maximum likelihood reconstruction of ancestral areas (Fig. 5) suggest that the *brandtii* lineage has a slightly higher probability of having a Palaearctic ancestor, instead of a New World one (54% versus 46%). This alternative hypothesis implies that the *brandtii* lineage never crossed the Bering Strait to reach the New World, but that it is a Palaearctic descendant of the same ancestral bat that crossed the Bering Strait and lead to the New World radiation. The current datasets do not permit a clear distinction between these two historical-based hypotheses.

4.1.2. Relationships within the New World clade

Besides the firm position of the *brandtii* lineage within the New World clade, a further subdivision highly correlated with geography is strongly supported by the Cyt *b* and combined datasets in all reconstructions (Figs. 2a, 3). This subdivision groups all sampled Neotropical species of *Myotis* into a monophyletic unit, which also comprises the Baja Californian *M. vivesi*, and two species pairs currently distributed in North America (*M. yumanensis–M. velifer* and *M. austroriparius–M. grisescens*). Two species found in the Antilles, *M. dominicensis* and *M. martiniquensis*, are also part of this monophyletic unit. All remaining species of this Neotropical subclade are distributed in Central and South America. The nuclear dataset analysed alone (Fig. 2b) is also consistent with this arrangement, except that *M. vivesi* and *M. yumanensis* are placed in an undetermined position.

The remaining New World species are all currently distributed in the Nearctic region (see Table 1) and is therefore referred to as the Nearctic subclade (Figs. 2a, 3 and 4). Owing to low levels of variation at the Rag 2 dataset, phylogenetic reconstructions based on this gene alone were inconclusive (Fig. 2b), but Cyt *b* and combined datasets strongly supported this Nearctic subclade. Previous genetic data based on other mitochondrial genes and on a smaller taxon sampling also supported this subdivision (Hoofer and Van Den Bussche, 2003). Thus, according to multiple molecular evidence, species as distinct morphologically and ecologically as *M. elegans* (small-footed, aerial feeder) and *M. ruber* (large-footed, water gleaner) are phylogenetically close relatives within the Neotropical subclade. Likewise, North American species traditionally viewed as belonging to different groups (e.g. *M. thysanodes* and *M. evotis*) appear closely related in the molecular tree (Figs. 2 and 3). Conversely, species phenetically similar, such as *M. californicus* and *M. yumanensis* emerge in completely distinct

clades in all molecular reconstructions. This again confirms that many cases of similarity in external morphology in the genus *Myotis* represent phylogenetically unrelated ecomorphs (Fenton and Bogdanowicz, 2002; Ruedi and Mayer, 2001), as was suggested by Findley (1972, p. 44).

Although our current sampling of New World taxa of *Myotis* represents more than 80% of all species recognised in recent taxonomic accounts, not all extant species have been analysed. It is difficult to estimate the impact of the eight missing species (*M. aelleni*, *M. cobanensis*, *M. findleyi*, *M. fortidens*, *M. melanorhinus*, *M. nesopolus*, *M. peninsularis*, and *M. planiceps*) on the phylogenetic trees presented here. Except perhaps for *M. planiceps*, which has both a very distinctive morphology among *Myotis* species and lives in a transition zone between typical Nearctic and Neotropical biotas (Ortega and Arita, 1998), we hypothesize that all other species not yet included in our analyses will fall in the subclade corresponding to their current distribution. *M. aelleni* occurs exclusively in Argentina and is usually considered close to or even conspecific with *M. chiloensis* (Barquez et al., 1999), thus there is little doubt that it would appear in the Neotropical subclade. The same holds for *M. cobanensis*, inhabiting Guatemala, *M. nesopolus* from the Lesser Antilles, and *M. fortidens* from southern Mexico and Guatemala. The latter has been sampled and sequenced for several genes by Hoofer and Van Den Bussche (2003) and indeed appears close to other Neotropical species. *Myotis melanorhinus* from Canada to Mexico is treated as a subspecies of *M. ciliolabrum* by various authors (e.g. Holloway and Barclay, 2001) and should appear in the Nearctic subclade. *Myotis peninsularis* from southern Baja California (Mexico), and *M. findleyi* endemic to the Tres Marias Islands (western Mexico), are rare and endangered species more difficult to assign to a biogeographic group.

Of course, there are also a number of cryptic taxa, which still need to be investigated, notably within the *M. lucifugus* or *M. nigricans* species complexes (Piaggio et al., 2002). The same uncertainty concerning the detailed relationships within problematic groups such as the *leibii–ciliolabrum–californicus* (Rodriguez and Ammerman, 2004) or the *evotis–thysanodes–keenii* complexes are not resolved by our analyses, because they would require a larger sample of individuals to appropriately address these questions (Ruedi and McCracken, in press). At least, the four individuals of *M. evotis* from different locations sequenced in the present study (only one is reported in Table 1 and figures, because they diverged by less than 1% from each other) are not part of the *leibii* group, but are clearly associated to *M. keenii* and *M. thysanodes* (Figs. 2 and 3), as suggested in earlier studies based on allozymes and chromosomes (Reduker et al., 1983). This contradicts the surprising results of Rodriguez and Ammerman (2004) and suggests a misidentification of the single *M. evotis* sequenced in that study. But again, we do not expect that these uncertainties would alter the general biogeographic pattern reported here.

4.2. Origins, dating and biogeography of the Nearctic and Neotropical radiation

The most recent interpretation of the fossil record supports a late Oligocene origin of the genus *Myotis* in Eurasia (Horácek, 2001). This early appearance of the genus was followed in the Early Miocene by a burst of diversification, as indicated by many fossils of *Myotis* found in Europe, but was not apparent in North America before the Middle-Late Miocene (Czaplewski, 1991; Czaplewski et al., in press). To date, no fossil remains of *Myotis* have been found in Miocene (or earlier) deposits from South America (Czaplewski et al., 2003b).

Our molecular reconstructions corroborate the fossil record and imply that the New World clade was separated early from the Old World taxa during the evolution of *Myotis* (Figs. 2 and 3). Indeed, estimates of divergence time from Bayesian inferences (Fig. 4) suggest that the New World clade diverged in the Middle Miocene (12.2 ± 2.0 MYA), only shortly after the initial split of the *latirostris* lineage from the remaining species of *Myotis* (about 13.0 ± 2.2 MYA). Biogeographic reconstructions (Fig. 5) also support a Palaearctic origin for the ancestors of the New World clade. The initial trans-Bering colonization coincides with the sharp climatic transition episode of the Middle Miocene (Zachos et al., 2001; Böhme, 2003), which involved global cooling and lowering of sea levels, and the development of more temperate habitats (Flower and Kennett, 1994). This global decline in temperature and drop in sea levels correspond to the first major periods of mammalian immigrations via the Bering land connection between Siberia and Alaska in the Middle Miocene (Wolfe, 1994).

Further cooling and aridification of habitats probably occurred during the Late Miocene (Cerling et al., 1997; Fox, 2000; White et al., 1997), which may have triggered the early diversification of New World bats, as suggested by La Val (1973). The South American continent might have been reached during this epoch, as suggested by our estimates of divergence times (about 7–10 MYA; Fig. 4). These dates largely predate the closure of the Panamanian Isthmus, about 3–4 MYA (Collins et al., 1996), but intervening islands might have functioned as stepping-stones to facilitate over-water colonization. It is also during the cool period of the Late Miocene that the ancestors of the *brandtii* lineage split from the New World species, about 8.6 ± 1.6 MYA. By the Late Miocene, Nearctic and Neotropical *Myotis* apparently began to diversify independently on each subcontinent (Figs. 4 and 5). Indeed, likelihood reconstruction (Fig. 5) suggests that a maximum of three Neotropical lineages crossed the Isthmus of Panama northwards. These are the ancestors of *M. vivesi* and of the two pairs of species (*M. yumanensis–M. velifer* and *M. grisescens–M. austroriparius*) currently distributed in North America. However, the lack of phylogenetic resolution within this group (Fig. 3) and their current distribution suggest these could be the descendant of a single widely distributed, circum-Caribbean

ancestor. This alternative scenario would imply even fewer trans-continental migrations.

4.3. Evolutionary success

The Neotropics is a hotspot of biodiversity offering an evolutionary cornucopia for many groups of animals, including for one quarter of the World's mammalian fauna (Nowak, 1999). With regard to the genus *Myotis*, which is the second largest and successful mammal genus (more than 103 species distributed worldwide), this region currently harbours only 18 species. From these, several are insular endemics (e.g. *M. dominicensis*, *M. martiniquensis*) or restricted to Central America. Thus, only about 10 species are specific to the continental Neotropical region south of Panama. This is low compared to *Myotis* assemblages found in the Northern Hemisphere (Simmons, 2005), or in other groups of bats in South America (e.g. the fruit and nectar-feeding Phyllostomidae).

In his seminal paper on the evolution and taxonomy of Neotropical *Myotis*, La Val (1973) hypothesized two factors that may have acted to limit the number of ancestral species that reached or diversified in South America. La Val (1973) suggested the existence of large marine transgressions into southern Central America and South America throughout most of the Tertiary (Donato et al., 2003), represented physical barriers for immigrant species arriving via Central America. Our molecular reconstructions indeed support surprisingly few faunal interchanges between North and South America, but estimates of divergence times suggest that the early *Myotis* lineages reached South America before the formation of the Isthmus of Panama (Fig. 4). Phylogenetic reconstructions (Figs. 2–4) further suggest that at least two species (*M. dominicensis* and *M. martiniquensis*) reached West Indian islands from continental South America by over-water dispersal. Thus, open water is not impassable to *Myotis* dispersal and alone cannot account for the fewer species found today in South America.

La Val (1973, p. 48) proposed that the South American species may not have diversified because they were of fairly recent origin, having arrived during the late Tertiary, or perhaps during the Pleistocene. Molecular data suggest a more ancient origin for the Neotropical subclade (Pliocene or older). Thus, the failure to diversify extensively in the southern continent is not uniquely due to a shorter evolutionary history for *Myotis* in South America.

Another factor hypothesized by La Val (1973) was possible higher competition of *Myotis* species to survive in temperate habitats. This "pre-adaptation" hypothesis is exemplified by the higher diversity of species found today in the Northern Hemisphere. Tropical forests were established in the Amazon lowlands by the Middle Miocene (Colinvaux and De Oliveira, 2001), and thus might have been a barrier to further expansion and radiation in temperate South America. Indeed, when the ancestors of the Neotropical subclade reached South America, other small insectivorous bats already flourished in the forests of the Neotropics, which might have impeded the diversification of *Myotis*. Estimates of divergence dates based on molecular data (Teeling et al., 2005) and the fossil record (Czaplewski and Morgan, 2003a) support the hypothesis that insectivorous bats of the families Thyropteridae, Furipteridae, or Molossidae were already present in the Neotropics, at least since the Miocene. Early *Myotis* bats might have been better competitors in more temperate habitats, such as in mountain areas or in the periphery of the Amazonian basin, where most Neotropical species occur at the present time (La Val, 1973).

Finally, it is also possible that the relative paucity of Neotropical *Myotis* partly reflects our poor current knowledge of their taxonomy. Indeed the majority of the Neotropical species have large geographic ranges, spanning different habitats (e.g. *M. nigricans*, *M. levis*). Thus, it is possible that denser geographic sampling and further genetic analyses might reveal more cryptic diversity. Such additional information is needed to test the hypothesis of a decreased rate of diversification in Neotropical versus Nearctic species.

4.4. Taxonomic implications

Given the poor correlation between the morphology-based subdivision of species of *Myotis* and of their evolutionary history, Ruedi and Mayer (2001) and Hoofer and Van Den Bussche (2003) proposed to abandon the classical subgeneric subdivision proposed by Tate (1941b) or by Findley (1972). The new taxonomy of *Myotis* should rather reflect their biogeographic origins, which better represent their evolutionary history. However, molecular reconstructions also identified the Asian species *M. latirostris* as the most basal to all species of *Myotis* sequenced to date (Figs. 2–4) would make any further subdivision of the genus paraphyletic. The surprising position of *latirostris* prompted the reexamination of voucher specimens from which sequences were derived, to exclude a problem of mislabeling or misidentification. The examination of this material confirmed that it corresponds to *M. latirostris* described by Kishida (1932). It is characterized by a very small size, an unusually flattened braincase and a suite of other typical morphological characters. Furthermore, careful examination of the dental characteristics of *M. latirostris* revealed that unlike in all other recent and fossil species of *Myotis* (see Menu, 1987), its lower molars are nyctalodont, not myotodont. According to this unique character and to the molecular reconstructions, the *latirostris* lineage should be given generic rank. By excluding the *latirostris* lineage from other *Myotis* species, it is then consistent to further subdivide the remaining species into two genera, or subgenera, as proposed by Hoofer and Van Den Bussche (2003). The appropriate name for all New World species plus *M. brandtii* and *M. gracilis* would be *Aeorestes*, which was given originally by Fitzinger in 1870 to *M. albescens*, *M. levis*, *M. nigricans*, and *M. villosissimus*. The remaining Old World species,

exclusive of the *latirostris* lineage, would represent the true *Myotis*, as this was originally used by Kaup (1829) to designate European species.

Of course, before a more comprehensive sampling of *Myotis* from the Old World is analysed, it is possible that either taxa might appear at the basis of the tree, or in an unexpected phylogenetic position. Thus, it is our aim to complete the sampling, especially in Asia, to be able to propose a global evolutionary history of the genus *Myotis*.

Acknowledgments

We are deeply indebted to those individuals and institutions who generously donated tissues or specimens. These samples are the fundamentals of such broad taxonomic surveys and would not be possible without their help. These individuals are: Loren Ammerman (San Angelo State University), Petr Benda (Natural History Museum, Prague), Francisco Bozinovic (Pontificia Universidad Católica de Chile), Douglas W. Burles (Victoria, Canada), Susan Campbell (University of Melbourne), Chris Conroy (Museum of Vertebrate Zoology, Berkeley), Joseph Cook (formerly at Alaska Museum), C.M. Francis (Canadian Wildlife Service, Ontario), Larry R. Heaney and William Stanley (Field Museum of Natural History, Chicago), Gareth Jones (University of Bristol), Serguei Kruskop and Alex V. Borissenko (Zoological Museum of Moscow State University), Nicky Ladkin (Museum of Texas Tech University), Cori Lausen (University of Calgary), Celia Lopez-Gonzalez (Instituto Politecnico Nacional, Durango), Jie Ma (Beijing, China), Vitaliy Matveev (Moscow Lomonosov State University), Jean-François Maillard (Office National de la Chasse et de la Faune Sauvage, Fort de France), R. Scott Reynolds (St. Paul's School, USA), Ronald A. Van Den Bussche (Oklahoma State University), Otto von Helversen and Frieder Mayer (University of Erlangen), and Maarten Vonhof (Western Michigan University).

We also are grateful to José Fahrni for help with sequencing, to Jan Pawlowski, Juan Montoya, Xavier Pochon, and Jackie Guiard for advice and discussion. This research was supported by grants from the Swiss National Funds for Scientific Research to MR (3100A0-105588) and from Bat Conservation International.

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