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Short Communication

Molecular dating of the diversification of Phyllostominae bats based on nuclear and mitochondrial DNA sequences

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ABSTRACT

Times of divergence among the three tribes included within the subfamily Phyllostominae were estimated using a Bayesian approach to infer dates of divergence based on mitochondrial and nuclear sequence data. The subfamily Phyllostominae is particularly attractive for such analysis, as it is one of the few groups of bats to have fossil specimens. Our molecular time analyses suggest that diversification among tribes and genera of phyllostominae bats occurred during the Early to Mid-Miocene, and was coincident with diversification events in two co distributed taxa: Caviomorph rodents and New World monkeys.

1. Introduction

New World leaf-nosed bats (family Phyllostomidae) constitute a large, species-rich group that is the most diverse family of mammals in terms of morphology and feeding strategy; they are a fundamental component of Neotropical communities, and represent one of the most extensively studied bat families (reviewed in Jones et al., 2002). Within the Phyllostomidae, the subfamily Phyllostominae (sensu Baker et al., 2003) includes taxa that vary considerably in feeding strategy, including carnivory (*Chrotopterus, Vampyrum*, *Trachops*), strict insectivory (*Macrophyllum*), and a combination of frugivory and insectivory (*Lophostoma*, *Mimon*, *Phylloderma*, *Phyllostomus*, *Tonatia*). Baker et al. (2003) included 9 genera with 20 species (Simmons, 2005) in the Phyllostominae, and arranged them in 3 tribes: Phyllostomini, including *Lophostoma* (7 species), *Mimon* (2), *Phylloderma* (1), *Phyllostomus* (4), and *Tonatia* (2); Vampyrini including *Vampyrum* (1) and *Chrotopterus* (1); and Macrophyllini including *Macrophyllum* (1) and *Trachops* (1). Despite advances in phyllostomid systematics provided by studies of mitochondrial and nuclear DNA sequences, relationships among the three tribes of phyllostominae bats remain unresolved (Lee et al., 2002; Porter et al., 2003; and Baker et al., 2003).

Although the fossil record of bats is notoriously poor, the subfamily Phyllostominae is among the few bat groups to include fossil representatives. Specimens assigned to phyllostomine genera are known from Mid-Miocene deposits in Colombia, northern South America (Savage, 1951; Czaplewski, 1997). The availability of fossil specimens, in combination with techniques to extract time information from multilocus sequence data, make this subfamily a natural candidate to estimate a time frame for the evolutionary history of the group based on DNA sequence data. The objective of this work is to evaluate divergence times within Neotropical bats in the subfamily Phyllostominae using combined cytochrome-*b*, 12S–16S rRNA and RAG-2 sequence data using multilocus Bayesian dating procedures.

2. Materials and methods

2.1. Specimens examined

We sequenced the complete cytochrome-*b* gene (1140 bp) for 20 specimens representing all genera in the subfamily Phyllostominae (Table 1). To facilitate comparisons with Lee et al. (2002), and Porter et al. (2003), we included specimens representing the subfamilies Desmodontinae, (*Desmodus rotundus*, *Diaemus youngi*, *Diphylla eucaudata*), Micronycterinae (*Micronycterys schmidtorum*,

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Table 1
Specimens examined and their locality information

Taxon	Tissue number	Museum catalog number	Country	GenBank accession number		
				12S–16S rRNA	*RAG2*	*Cyt-b*
Artibeus concolor	TK10378	CMNH63792	Suriname: Commewijne		AF316432	
Artibeus obscurus	TK17080	CMNH68951	Suriname: Nickerie	AY395805		
	TK104001	TTU84773	Ecuador: Pastaza			DQ869392
Anoura caudifer		USNM582796	Peru, Cusco	AY395835		
Anoura geoffroyi		TTU62405	El Salvador: Santa Ana		AF316431	FJ155495
Chrotopterus auritus	TK17104	CMNH68638	Suriname: Saramacca		AF316442	
	TK21039	CMNH 76767	Suriname: Para			FJ15548
	TK70457	MUSM13653	Peru: Cusco	AF411538		
Desmodus rotundusl	TK4764	TTU35582	México: Guerrero	AF263228	AF316444	
	TK40368	TTU 61104	Honduras: Atlantida			FJ155477
Diaemus youngi	TK34625	TTU62792	El Salvador: La Paz	AF411534	AF316445	FJ155475
Diphylla ecaudata	TK13514	TK13514	México: Yucatàn	AF411533	AF316447	
	TK13508	TTU 47509	Mexico: Yucatan			FJ155476
Lonchorhina aurita	TK20560	TTU36531	Mexico, Chiapas	AY395843	AF316457	FJ155494
Lampronycteris brachyotis	TK25238	TCWC55445	Trinidad: Mayaro	AF411536	AF316463	
	TK25239		Trinidad: Mayaro			AY380748
Lophostoma brasiliense	TK18834	AMNH267103	French Guyana	AF411544	AF316489	
	TK49898 (F38605)	ROM106608	Panama			FJ155486
Lophostoma evotis	TK49870	ROM95626	México: Campeche	AF411529		
	TK40341	TTU61070	Honduras: Atlantida		AF442080	
	TK49871	ROM95625	Mexico			FJ155491
Lophostoma schulzi	TK18833	AMNH267106	French Guyana: Paracou		AF442079	
	F38318					FJ155485
	TK49888	ROM101128	Locality Missing	AF411532		
Lophostoma silvicola	TK56716		Paraguay: San Pedro	AF442092	AF442081	FJ155493
Lophostoma silvicola	TK17946	CMNH77174	Suriname: Marowijne	AF263230		
	TK18832	AMNH267107	French Guyana: Paracou		AF442083	
		ROM100949	Guyana			FJ155492
Macrophyllum macrophyllum	TK19119	CMNH78289	Venezuela: Bolivar	AF411540	AF316458	FJ155484
Macrotus waterhousii	TK32030	TTU52481	Cuba: Guantanamo		AF316461	
	TK32021	TTU52478	Cuba: Guantanamo	AF263229		
	TK27889	TTU 71435	Mexico: Morelos			AY380745
Micronycteris schmidtorum	TK70447	MUSM13737	Perú: Camisea	AF411535	AF316470	AY380753
Mimon crenulatum	TK15121	TTU33287	Venezuela: Guarico		AF316472	
	TK25230	CMNH25230	Trinidad and Tobago: Trinidad	AF411534		FJ155478
Phylloderma stenops	TK10201	CMNH63614	Suriname: Saramacc	AF411542	AF316480	
	TK86685		Guyana, Berbice District			FJ155480
Phyllostomus hastatus	TK19289	CMNH19289	Venezuela: Bolivar	AF411541		
	TK19243	CMNH78333	Venezuela: Bolivar		AF316479	FJ155479
Tonatia bidens	TK56633		Paraguay: Dpto. San Pedro	AF442090	AF442087	FJ155489
Tonatia bidens	TK56519	MVZ185673	Brazil: Sao Paulo	AF442091	AF442088	FJ155490
Tonatia saurophila	TK49889	ROM103210	Guyana: Upper Takutu-Upper Essequiba		AF442084	FJ155488
	TK49892	ROM104459	Ecuador: Napo	AF411531		
Tonatia saurophila	TK46028	USNM	Perú: Quebrado		AF442085	
	TK49890	ROM103401	Guyana: Upper Demerara-Berbice	AF411530		
	TK49895	ROM104218	Panama: Canal Zone			FJ155487
Trachops cirrhosus	TK18829	AMNH267129	French Guyana: Paracou	AF411539	AF316490	
	TK19132		Venezuela: Bolivar			FJ155483
Vampyrum spectrum	TK40370	TTU61070	Honduras: Atlántida: Lancitilla	AF411537	AF316495	FJ155482

Specimen number TK49885 is also identified by University of New Mexico voucher number NK30034. Specimens with missing Museum Catalog number have yet to be cataloged.

Lampronycteris brachyotis) and Lonchorhininae (*Lonchorhina aurita*) in our phylogenies. We included samples of *Artibeus* and *Anoura* in the analyses to profit from the timescale of bat diversification estimated by Teeling et al. (2005) and added data from *Macrotus californicus* as the outgroup. We obtained 12S–16S rRNA sequences and RAG-2 sequences used by Lee et al. (2002), Baker et al. (2003), and Porter et al. (2003), as well as those from *Artibeus* and *Anoura* from GenBank. We combined sequences from different individuals within a species, as not all genes were sequenced for the same individuals, and in the cases of *Artibeus* and *Anoura* we combined sequences from closely related species (Table 1).

2.2. Molecular methods

We amplified the complete mitochondrial cytochrome-*b* gene using primers and conditions reported in Hoffmann and Baker (2001), using an additional internal sequencing primer To1L (5′- CTG CCT CTA CCT TCA TGT AGG AC-3′). We sequenced the PCR fragments using BigDye 3.0, followed by electrophoresis in an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, California). We assembled and verified fragments using Sequencer version 3.1.1 (Gene Code Corporation, Ann Arbor, Michigan) and VectorNTI (Informax Inc., Bethesda, Maryland), and performed sequence alignments in CLUSTAL X (Thompson et al., 1997). For rRNA data we followed Hoofer and Van Den Bussche (2003) to delimit ambiguously aligned sites.

2.3. Phylogenetic analyses

Wiens (1998) proposed that when analyses of individual loci produce compatible topologies, data from multiple loci may be concatenated for phylogenetic reconstruction. Trees are considered as compatible if there are no strongly supported conflicting nodes. In this study, we considered nodes to be strongly supported if they

had ⩾95% Bayesian posterior probabilities, or ⩾80% Maximum Likelihood bootstrap support. We performed independent Bayesian and maximum likelihood analyses for each gene and in the absence of topological incongruences, we performed Bayesian and Maximum Likelihood (ML) phylogenetic analyses on concatenated sequences from all genes, as this has been shown to improve tree resolution (Opazo et al., 2006).

2.3.1. Bayesian analyses

Bayesian estimation of phylogenies was done in MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003), running four simultaneous chains for 5×10^6 generations, sampling trees every 1000 generations, and using default priors. We assessed convergence by measuring the standard deviation of the split frequency among parallel chains. Chains were considered to have converged once the average split frequency was lower than 0.01. We summarized results by doing a majority-rule consensus of the 2500 trees collected after convergence was reached, trees collected before chains reached convergence were discarded. In the cases of cytochrome-*b* and RAG2, each codon position was allowed to have an independent GTR + Γ model of nucleotide substitution. When the three genes were combined, we implemented a 7-partition analysis where each codon position in the nuclear RAG2, each codon position in the mitochondrial cytochrome-*b*, and the rDNA fragment had an independent GTR + Γ model of nucleotide substitution.

2.3.2. Maximum likelihood

ML searches were done in Treefinder version June 2007 (Jobb et al., 2004), following a similar strategy to the one used in Bayesian analyses. We selected the best-fitting model of nucleotide substitution for each of the 7-partitions independently using the corrected Akaike Information Criterion (Akaike, 1974; Sugiura, 1978), and performed searches in Treefinder under the selected models. We evaluated support for the nodes with 1000 bootstrap replicates (Felsenstein, 1985).

2.3.3. Hypothesis testing

We compared the likelihood scores of the three different possible taxonomic arrangements among the three tribes of Phyllostominae bats using the Shimodaira–Hasegawa (SH) and Approximately Unbiased (AU) tests (Shimodaira and Hasegawa, 1999), as implemented in Treefinder (Jobb et al., 2004).

2.4. Divergence time estimation

Divergence times within the Phyllostominae were estimated using the multilocus Bayesian Markov Chain Monte Carlo time estimation procedure implemented in *multidivtime* (Thorne and Kishino, 2002). We selected this approach because of its ability to use upper and lower boundaries on nodes, rather than absolute dates as is the case with several ML approaches (see Rannala and Yang (2007) for a more thorough discussion on dating algorithms). These analyses were based on the phylogenetic tree derived from Bayesian and ML analyses pruned to include the subfamily Phyllostominae plus the genera *Anoura* and *Artibeus*. The latter two genera were included in the analyses to take advantage of the framework derived from Teeling et al. (2005). We ran *multidivtime* using the 7-partition scheme used for phylogeny estimation, plus independent analyses for each gene and an additional analysis where we excluded the 3rd codon position from cytochrome-*b*, which is the fastest evolving partition in our data. We first estimated the parameters of an HKY85 model of molecular evolution for each partition using *baseml* (Yang, 1997). Then, we used *estbranches* (Thorne et al., 1998) to estimate branch lengths and their associated variance–covariance matrix for each partition. The resulting matrices were then used by *multidivtime* to estimate times of divergence and their confidence intervals.

Calibration points in these analyses relied on the time framework for Chiropteran diversification proposed by Teeling et al. (2005): the upper and lower boundaries for the node corresponding to the split between the Phyllostominae and the clade containing *Anoura* and *Artibeus* were set to 26 and 17 mya, whereas the upper and lower boundaries for the node corresponding to the split between *Anoura* and *Artibeus* were set to 23 and 13 mya. Based on the results from Teeling et al. (2005), we set a prior on the maximum age of the root of 22 mya, scaled to 2.2. The initial value of the rtrate parameter was set to the median of the distance from the root to the tip of the tree for each gene divided by the scaled estimated age of the root (rttm). We run analyses for 7×10^6 cycles, collecting samples every 100 cycles and discarding the initial 2×10^6 cycles as burn-in.

There are fossil specimens from two genera in the Phyllostominae: *Notonycteris*, in the tribe Vampyrini, and *Lophostoma*, in the tribe Phyllostomini (Savage, 1951; Czaplewski, 1997). All these fossil specimens have been found in deposits dated to be 12–13 mya (Savage, 1951; Czaplewski, 1997; Czaplewski et al., 2003). Fossils in the genus *Lophostoma* provide a callibration point for the divergence within the tribe Phyllostomini, which was constrained to be at least 13 mya, as old as fossil specimens in this tribe. By contrast, fossils assigned to *Notonycteris* are not as easily incorporated into the analyses. In the phylogenetic analyses presented by Czaplewski et al. (2003) the fossil genus *Notonycteris* is the first split in the tribe Vampyrini, implying that divergence between *Notonycteris* and the *Chrotopterus* + *Vampyrum* clade should predate this fossil, as would divergence between the Vampyrini and the two other tribes in the subfamily. The temporal information derived from the *Notonycteris* fossils is redundant with the one provided by the *Lophostoma* fossils.

3. Results

3.1. Description of data

Multiple sequence alignment resulted in 5208 nucleotide positions. Of these, 1140 nucleotides were from the mitochondrial cytochrome-*b*, 1363 from the nuclear RAG2, and 2705 from the mitochondrial 12S–16S rRNA fragment. Alignments for cytochrome-*b* and RAG2 were unequivocal, whereas visual inspection of the 12S–16S rRNA alignment identified 551 positions that were ambiguously aligned, and were therefore excluded from subsequent analyses.

3.2. Phylogenetic analyses

Phylogenetic trees derived from the separate analysis of cytochrome-*b*, RAG2, and mitochondrial 12S–16S rRNA fragment differ in topology, but found no supported conflicting nodes among them (Supplementary Fig. 1), thus, we report results from analyses based on the 7-partition analyses. Maximum likelihood and Bayesian analyses based on the combined sequence data produced identical topologies (Fig. 1). The monophyly of the subfamily Phyllostominae *sensu* Baker et al. (2003) was supported in both Bayesian and ML analyses, as was the monophyly of each of the tribes in the subfamily. The tribes Macrophyllini and Phyllostomini were sister taxa, with Vampyrini as the first divergence within the subfamily, although relationships among the three tribes were not strongly supported in either BA or ML. Within the tribe Phyllostomini, our analyses supported: a clade including *Mimon*, *Phylloderma*, and *Phyllostomus*; monophyly of *Tonatia* and monphyly of *Lophostoma*. Topology tests (SH and AU) did not detect significant differences among the three possible combinations of relationships for the tribes (Supplementary Fig. 2).

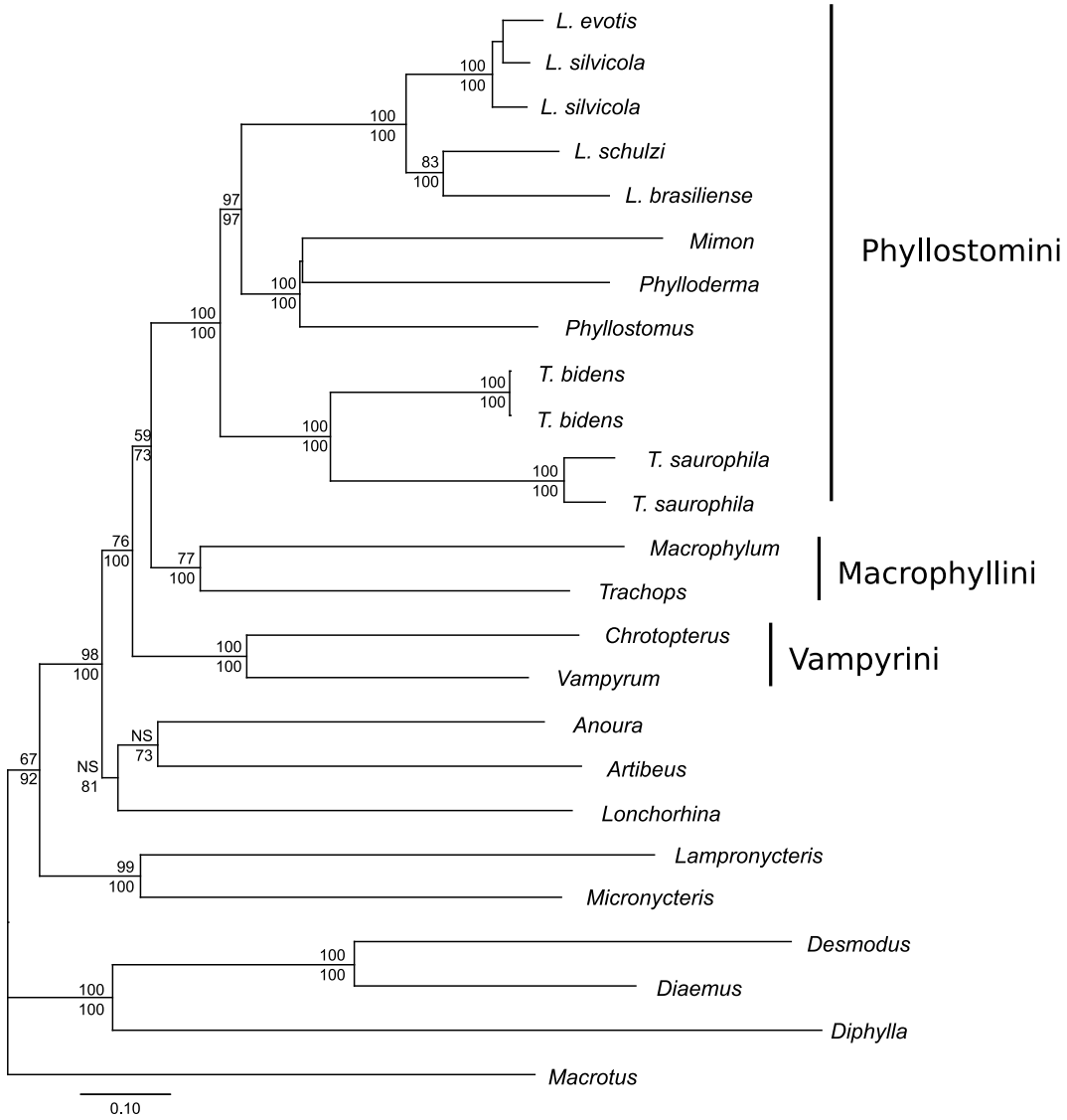

Fig. 1. Maximum likelihood phylogram describing relationships among genera of bats in the subfamily Phyllostominae. We first selected the best-fitting model of nucleotide substitution for each of the 7-partitions independently using the corrected Akaike Information Criterion (Akaike, 1974; Sugiura, 1978), and then performed searches in Treefinder under the selected models. Support for the relevant nodes is presented as bootstrap values (above the nodes) and as Bayesian posterior probabilities (below the nodes). Tribal affiliation of the genera in the subfamily Phyllostominae is indicated by the vertical bars.

3.3. Divergence times

Time estimates from Bayesian MCMC were based on the topology shown in Fig. 1 excluding all non phyllostominae taxa but *Anoura* and *Artibeus*. This analysis dated divergences among tribes to the Early Miocene (18.6–19.5 mya), and divergences among genera to the Early to Mid-Miocene (12.7–16.9 mya). Results were similar in the additional *multidivtime* runs performed (see Supplementary Fig. 1). Time estimates indicate that the different genera within each tribe are relatively old, as the most recent split among genera was estimated to have occurred 12.7 mya, and corresponds to the most recent common ancestor of the genera *Mimon* and *Phylloderma*.

4. Discussion

4.1. Phylogenetic analyses

Phylogenetic relationships within the Phyllostominae have proven difficult to resolve despite efforts using both mitochondrial and nuclear DNA sequences (Baker et al., 2000, 2003; Lee et al., 2002; Porter et al., 2003). Two possible explanations for the apparent lack of resolution are that these genes evolve too slowly to resolve the nodes at the base of the Phyllostominae, or, as proposed by Lee et al. (2002), that rapid contemporaneous diversification among phyllostomine tribes took place. In this study, we analyzed 1.1 Kb of DNA sequence data from the more rapidly evolving mitochondrial cytochrome-*b* gene to explore this hypothesis. Phylogenies from the concatenated data set support the monophyly of all three tribes (Phyllostomini, Vampyrini, Macrophyllini) as recognized in Baker et al. (2003), but lack power to resolve relationships among the three different tribes within this subfamily (Fig. 1).

4.2. Divergence times

According to the molecular time estimates for Chiropteran evolution proposed by Teeling et al. (2005), the split between the genus *Tonatia* (Phyllostominae) and a clade that included the genera *Artibeus* (Stenodermatinae) and *Anoura* (Glossophaginae) was dated to the Early Miocene (22 mya). We used sequence data from the cytochrome-*b*, rDNA and RAG-2 genes, and combined it with

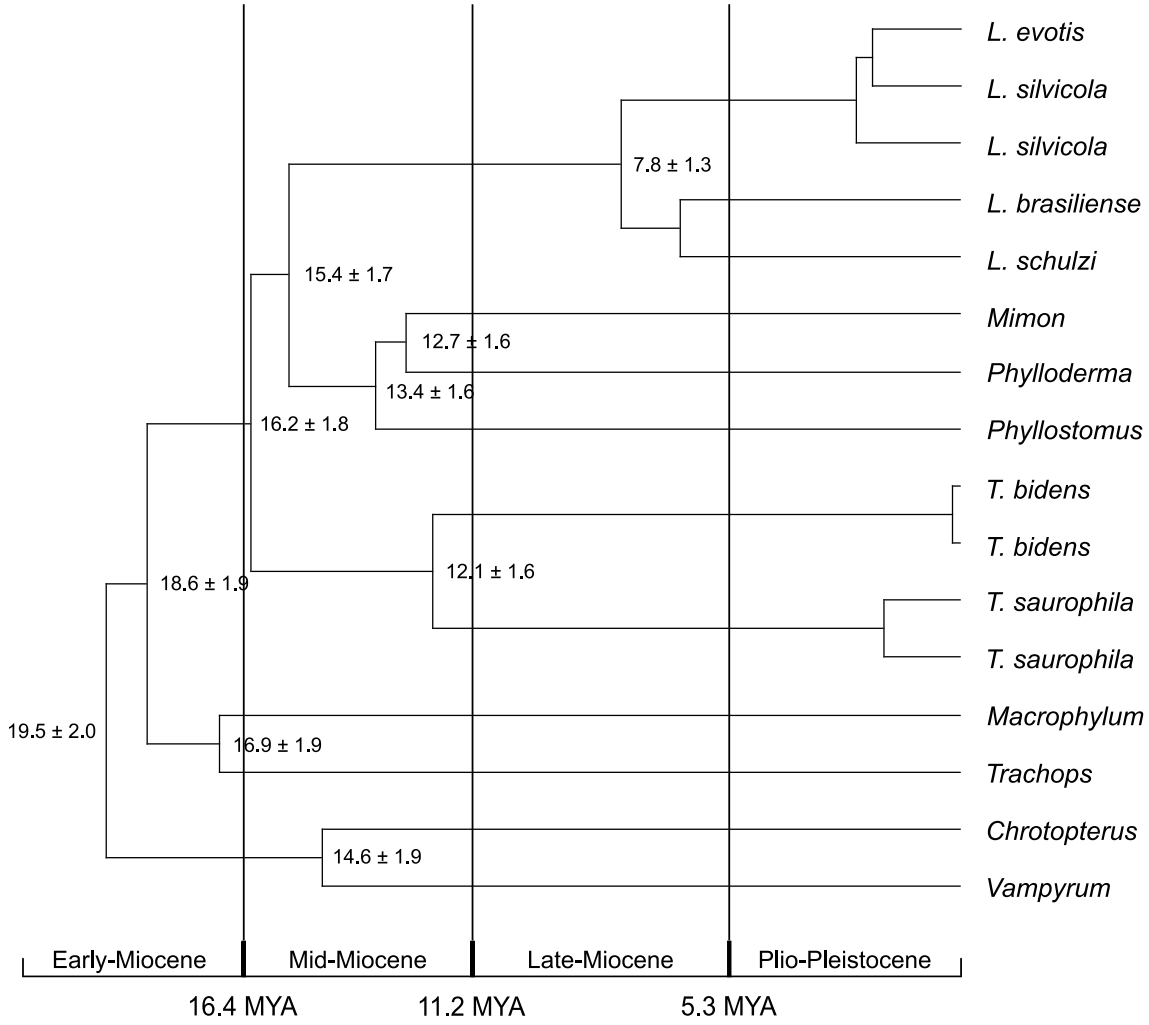

Fig. 2. Divergence time estimates derived from *multidivtime* (Thorne and Kishino, 2002) analyses using the ML topology depicted in Fig. 1, but restricted to taxa in the subfamily Phyllostominae plus the genera *Anoura* and *Artibeus*. The figure only shows the portion of the tree corresponding to the subfamily Phyllostominae. Numbers next to the nodes are the molecular dates in millions of years before present, with the corresponding 95% confidence intervals in parenthesis. Geological times are according to the 1999 geologic timescale of the Geological Society of America (www.geosociety.org/science/timescale/timescl.pdf).

the time framework derived from Teeling et al. (2005) and fossil evidence to estimate dates of divergence within the Phyllostominae. Divergences among the three tribes within the subfamily lie in the Early Miocene, and divergences among genera are estimated to have occurred during the Early to Mid-Miocene (Fig. 2). According to our molecular time estimates divergence among the three Phyllostomine tribes occurred relatively rapidly, within 1.0 million years (Fig. 2), in agreement with the scenario proposed by Lee et al. (2002).

There are fossil specimens from two genera in the Phyllostominae: *Notonycteris* and *Lophostoma* (Savage, 1951; Czaplewski, 1997). The two fossil species of *Notonycteris* have been found in deposits dated to be 12–13 mya (Savage, 1951; Czaplewski, 1997; Czaplewski et al., 2003). In the phylogenetic analyses presented by Czaplewski et al. (2003), *Notonycteris* is the deepest split in the Vampyrini, indicating that the node giving rise to this tribe should be older than 13 mya. Our analyses are consistent with this prediction, estimates of divergence between the two extant genera in the tribe Vampyrini (*Chrotopterus* and *Vampyrum*) are older than 13 mya (see Fig. 2, and Supplementary Fig. 3). Additional records from the same fossil bed include molars that are similar to, but smaller than those of *Lophostoma silvicola* (Czaplewski et al., 2003). Thus, divergence of *Lophostoma* from the remainder of the Phyllostomini must have been at least 12–13 mya, and our estimates of the age of the node giving rise to the genus *Lophostoma* is 15.4 mya (see Fig. 2, and Supplementary Fig. 3).

In all cases, divergences among genera are relatively old, in the Mid-Miocene, as are divergences among tribes, in the Early Miocene. This agrees well with the time-derived classification proposed by Goodman et al. (1998), where the taxonomic ranking of a group is associated with its age. A striking observation is that divergences among several genera of Caviomorph rodents (Opazo, 2005; but see also Poux et al., 2006), New World monkeys (Opazo et al., 2006), and sheath-tailed bats (Lim, 2007), other important components of the fauna of South America, are also dated to the same geological period. Caviomorph rodents and New World monkeys are both hypothesized to have arrived into South America in a single colonization event during the Oligocene (Flynn and Wyss, 1998), coincident with the evolutionary origin of phyllostomid bats inferred by Teeling et al. (2005). The emerging picture for divergence among genera of South American mammals is one of Miocene estimates for nodes giving rise to novel genera (Opazo, 2005; Opazo et al., 2006; Lim, 2007). The Miocene diversification of phyllostominae bats, caviomorph rodents, New World monkeys, and sheath-tailed bats underscores the contribution to present day Neotropical biodiversity of events that preceded the Great American Interchange that occurred after the uplift of the Panama isthmus 5.5 mya (Marshall, 1988).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.08.002.

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