

A Nuclear DNA Phylogenetic Perspective on the Evolution of Echolocation and Historical Biogeography of Extant Bats (Chiroptera)

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Bats (Order Chiroptera), the only mammals capable of powered flight and sophisticated laryngeal echolocation, represent one of the most species-rich and ubiquitous orders of mammals. However, phylogenetic relationships within this group are poorly resolved. A robust evolutionary tree of Chiroptera is essential for evaluating the phylogeny of echolocation within Chiroptera, as well as for understanding their biogeographical history. We generated 4 kb of sequence data from portions of four novel nuclear intron markers for multiple representatives of 17 of the 18 recognized extant bat families, as well as the putative bat family Miniopteridae. Three echolocation-call characters were examined by mapping them onto the combined topology: (1) high-duty cycle versus low-duty cycle, (2) high-intensity versus low-intensity call emission, and (3) oral versus nasal emission. Echolocation seems to be highly convergent, and the mapping of echolocation-call design onto our phylogeny does not appear to resolve the question of whether echolocation had a single or two origins. Fossil taxa may also provide insight into the evolution of bats; we therefore evaluate 195 morphological characters in light of our nuclear DNA phylogeny. All but 24 of the morphological characters were found to be homoplasious when mapped onto the supermatrix topology, while the remaining characters provided insufficient information to reconstruct the placement of the fossil bat taxa with respect to extant families. However, a morphological synapomorphy characterizing the Rhinolophoidea was identified and is suggestive of a separate origin of echolocation in this clade. Dispersal-Vicariance analysis together with a relaxed Bayesian clock were used to evaluate possible biogeographic scenarios that could account for the current distribution pattern of extant bat families. Africa was reconstructed as the center of origin of modern-day bat families.

Introduction

Bat systematics has experienced great upheaval in recent years with the advent of large-scale molecular studies and application of explicit phylogenetic methodologies. First, the superorder Archonta (Novacek and Wyss 1986; Novacek 1992) comprising Chiroptera (bats), Dermoptera (flying lemurs), Primata (primates), and Scandentia (tree shrews) is erroneous. Several molecular studies have shown that Chiroptera belong to the Laurasiatheria (represented by carnivores, pangolins, cetartiodactyls, eulipotyphlans, and perissodactyls) and are only distantly related to dermopterans, scandentians, and primates (Nikaido et al. 2000; Lin and Penny 2001; Madsen et al. 2001; Murphy et al. 2001a, 2001b; Van Den Bussche and Hoofer 2004). Second, molecular studies have challenged the notion, based largely on morphological characters, that bats are diphyletic with megabats being more closely related to primates than to microbats (Smith and Madkour 1980; Hill and Smith 1984; Pettigrew 1986; Pettigrew et al. 1989). Instead, these studies have consistently shown high support for a monophyletic Chiroptera (Nikaido et al. 2000; Murphy et al. 2001a, 2001b; Volleth et al. 2002; Van Den Bussche and Hoofer 2004). Finally, the division of the Chiroptera into two suborders, the Megachiroptera (nonecholocating bats) and Microchiroptera (echolocating bats) (Gray 1821 *vide* Miller 1907) has been challenged by some molecular studies that place Old World

fruit bats and rhinolophoid microbats (excluding nycterids) in one clade, with all other microbats in another (Hutcheon, Kirsch, and Pettigrew 1998; Teeling et al. 2000, 2002, 2003, 2005; Springer et al. 2001; Hutcheon and Kirsch 2004; Van Den Bussche and Hoofer 2004).

Given the disparity between molecular and non-molecular phylogenies, acceptance of microchiropteran diphyly has not been widespread (Schintzler, Kalko, and Denzinger 2004; Simmons and Conway 2003) with authors citing the need for more comprehensive and robust molecular phylogenies based on increased sampling at the family level (Teeling et al. 2002; Simmons and Conway 2004). Within the framework of monophyly of Chiroptera, paraphyly of microbats either requires loss of echolocation along the pteropodid lineage, only to reevolve in *Rousettus* albeit in a more primitive form (Holland, Waters, and Rayner 2004), or two independent origins of echolocation within the chiropteran lineage. Thus, more evidence for or against the paraphyly of microbats is essential for evaluating the evolution of echolocation within the monophyletic Chiroptera.

By using increased taxon sampling (Pollock and Bruno 2000; Pollock et al. 2002; Zwickl and Hillis 2002), we were interested in exploring the utility of novel nuclear intron sequences for recovering higher level systematic relationships among extant chiropteran families. This study was prompted by the availability of a suite of unique nuclear DNA intron markers useful for recovering phylogenetic information at several different taxonomic levels (Matthee and Davis 2001; Matthee et al. 2001, 2004; Willows-Munro, Robinson, and Matthee 2005).

Our aims were fourfold. First, to provide an independent assessment of evolutionary relationships within Chiroptera based on comprehensive taxon sampling and phylogenetic analyses of a nuclear intron supermatrix. Second, we were

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interested in examining the evolution of echolocation in bats using the phylogenetic framework developed in this study. Third, we aimed to reevaluate the morphological characters identified by Simmons and Geisler (1998) in terms of our molecular phylogeny to assess their utility for reconstructing relationships of fossil bat taxa to extant groups. This aided our interpretation of the evolution of echolocation. Last, given the complex biogeographical distribution as well as the scant paleontological record of bats, we were interested in providing a timescale for the radiation of extant chiropteran families using a relaxed Bayesian clock. This allowed us to evaluate possible biogeographical scenarios that explain the current distribution of bats.

Materials and Methods

Taxonomic Sampling

Multiple representatives of 17 of the 18 currently recognized extant bat families (excluding the monotypic Crasonycteridae) (Simmons, in press) were included in this study (table 1). We also included species representatives of the putative bat family Miniopteridae (Mein and Tupinier 1977; Gopalakrishna and Chari 1983; Pierson 1986; Tiunov 1989; Kawai et al. 2002; Van Den Bussche and Hoofer 2004; Hutcheon and Kirsch 2004). The two species comprising the vespertilionid genus *Cistugo* Thomas 1912 were included as this taxon may be sufficiently different to warrant separate family-level status (M. Ruedi, personal communication). We included only three representatives of Laurasiatheria (Nikaido et al. 2000; Lin and Penny 2001; Madsen et al. 2001; Murphy et al. 2001a, 2001b; Van Den Bussche and Hoofer 2004), namely the horse (Order Perissodactyla), genet (Order Carnivora), and pangolin (Order Pholidota) as outgroups (table 1) because recent comprehensive mammalian phylogenies have unambiguously demonstrated the monophyly of Chiroptera (Nikaido et al. 2000; Lin and Penny 2001; Madsen et al. 2001; Murphy et al. 2001a, 2001b; Van Den Bussche and Hoofer 2004).

Data Collection

Total genomic DNA was extracted from 95% ethanol or dimethyl sulfoxide-preserved tissue using a phenol-chloroform-isoamyl procedure (Sambrook, Fritsch, and Maniatis 1989). Introns from four nuclear genes (PRKC1, SPTBN, STAT5A, and THY) were targeted using primers designed previously (Matthee et al. 2001, 2004). To increase the success of amplification across all taxa included, chiropteran-specific primers were designed (supplementary table 1, Supplementary Material online), and used in various combinations with previously published primers. Sequence data for the horse, *Equus caballus*, was obtained from GenBank (Matthee et al. 2001; table 1), whereas intron sequence data for the other two out-group taxa were generated in this study. The cycling profile and subsequent purification and automated sequencing followed protocols outlined in Matthee et al. (2004). In most instances, both strands were sequenced to ensure accuracy of the sequence data, and the sequence identity was checked by GenBank BlastN searches. Heterozygous changes occurred in a limited number of nuclear fragments, and these were assigned

an IUBMB ambiguity code. Nuclear intron sequences and alignments generated in this study have been deposited in EMBL (STAT5A: AJ865389–AJ865445, ALIGN_000804; THY: AJ865634–AJ865690, ALIGN_000805; PRKC1: AJ866286–AJ86636, ALIGN_000806; SPTBN: AJ866337–AJ86639, ALIGN_000807).

Sequence Alignment

Sequences were initially aligned using the multiple alignment program T-COFFEE (Notredame, Higgins, and Heringa 2000) and thereafter optimized manually in MacClade 4.0 (W. P. Maddison and D. R. Maddison 1989), using the conserved exon sequences on either the 5' or 3' end of the sequence to anchor the alignments. Insertion deletion (indel) events were observed among taxa, and gaps were introduced (by T-COFFEE or manually) to maintain the alignment. Although there were some areas where alternative alignments were likely, our investigations showed that minor changes to the alignment in these regions did not significantly alter relationships among taxa (data not presented). Two regions within the SPTBN intron (region 796–815 and region 832–1190; ALIGN_000807) could not be aligned, and these were therefore excluded in all analyses. All alignment gaps were treated as missing characters in phylogenetic analyses. Following the suggestions of Matthee et al. (2001) only indels longer than 2 bp were mapped onto the tree obtained from analysis of the supermatrix.

Data Set Characterization and Phylogenetic Analyses

Base composition was estimated using MEGA v2.1 (<http://www.megasoftware.net>), and base frequency stationarity was evaluated using a χ^2 test implemented in PAUP* v4.0b10 (Swofford 2002). Incongruence between data sets was evaluated using the recommendations of De Queiroz (1993) by determining whether there were any consistently strongly ($\geq 70\%$ bootstrap support, ≥ 0.95 Bayesian posterior probability) supported nodes in one data set that conflicted with strongly supported nodes in another. As no such incongruent nodes were present, the four introns were concatenated to form a supermatrix. The genes were analyzed separately as well as combined using maximum parsimony (MP) and maximum likelihood (ML) in PAUP* v4.0b10 (Swofford 2002), and Bayesian inference (BI) as implemented by MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). For MP searches, trees were generated using equal weighting and the heuristic search option with tree-bisection-reconnection (TBR) branch swapping and stepwise addition of taxa using 1,000 random sequence addition replicates, with one tree retained per stepwise addition replicate. For ML analyses, nucleotide substitution models were selected using Modeltest v 3.06 (Posada and Crandall 1998), and searches were performed under both likelihood ratio test (LRT) and Akaike Information Criterion (AIC) optimal models if these were different. In all ML analyses, starting trees were obtained by neighbor-joining followed by TBR branch swapping. Nodal support for the MP analyses was assessed from 1,000 nonparametric bootstrap replicates (full heuristic search; two random stepwise addition of taxa). Nodal support for individual intron ML

Table 1
Chiropteran and Out-Group Taxa Included in this Study

Order	Family	Species	Source	Accession Number
Chiroptera	Emballonuridae	<i>Pteropteryx kappleri</i>	Francois Catzeflies	T3852
		<i>Rhynconycteris naso</i>	David S. Jacobs, UCT	DSJ120
		<i>Saccopteryx bilineata</i>	Francois Catzeflies	T3631
		<i>Taphozous mauritanus</i>	David S. Jacobs, UCT	DSJ118
	Furipteridae	<i>Furipterus horrens</i>	AMNH	AMNH109523
	Hipposideridae	<i>Anthops ornatus</i>	Australian Museum	EBU23999
		<i>Cloeotis percivali</i>	David S. Jacobs, UCT	17.12.02C1p(SDC)
		<i>Hipposideros caffer</i>	David S. Jacobs, UCT	01.08.02Hc2(PP)
		<i>Hipposideros commersoni</i>	David S. Jacobs, UCT	DSJZM26
	Megadermatidae	<i>Cardioderma cor</i>	FMNH	FMNH158010
		<i>Megaderma lyra</i>	TK	TK21292
		<i>Megaderma spasma</i>	FMNH	FMNH168890
		<i>Miniopterus australis</i>	Australian Museum	EBU9208
	Miniopteridae	<i>Miniopterus macrocneme</i>	Australian Museum	EBU29472
		<i>Miniopterus fraterculus</i>	David S. Jacobs, UCT	10.04.02Mf2(CDH)
		<i>Miniopterus inflatus</i>	Northern Flagship Institution	TM41802
		<i>Miniopterus natalensis</i>	David S. Jacobs, UCT	08.04.02Ms16(CDH)
	Molossidae	<i>Chaerephon ansorgei</i>	F.W.C. Cotterill	FWC4783
		<i>Mormopterus petrophilus</i>	David S. Jacobs, UCT	06.10.02Sp1(AED)
		<i>Otomops martiensseni</i>	David S. Jacobs, UCT	DSJ116
		<i>Tadarida aegyptiaca</i>	David S. Jacobs, UCT	06.10.02Ta1(AED)
	Mormoopidae	<i>Mormoops megalophylla</i>	Angelo State Natural History Collection	ASK6045
		<i>Pteronotus parnellii</i>	Francois Catzeflies	T3408
		<i>Pteronotus personatus</i>	Francois Catzeflies	T2586
		<i>Mystacina tuberculata</i>	Brian Lloyd	DSJ225
	Mystacinidae	<i>Myzopoda aurita</i>	FMNH	FMNH165454
	Myzopodidae	<i>Natalus major</i>	AMNH	AMNH103028
		<i>Natalus micropus</i>	AMNH	AMNH102717
	Natalidae	<i>Noctilio albiventris</i>	AMNH	AMNH109543
		<i>Noctilio leporinus</i>	Robert Timm	DSJ224
	Nycteridae	<i>Nycteris grandis</i>	Northern Flagship Institution	TM41863
		<i>Nycteris javanica</i>	ROM	ROM101970
		<i>Nycteris macrotis</i>	Northern Flagship Institution	TM41763
		<i>Nycteris thebaica</i>	David S. Jacobs, UCT	04.08.02Nt1(LA)
	Phyllostomidae	<i>Artibeus jamaicensis</i>	David S. Jacobs, UCT	DSJ136
		<i>Desmodus rotundus</i>	David S. Jacobs, UCT	DSJ123
		<i>Glossophaga soricina</i>	David S. Jacobs, UCT	DSJ138
		<i>Trachops cirrhosus</i>	Francois Catzeflies	T3642
	Pteropodidae	<i>Cynopterus sphinx</i>	Francois Catzeflies	T0830
		<i>Epomophorus minor</i>	Northern Flagship Institution	TM41781
		<i>Epomophorus wahlbergii</i>	David S. Jacobs, UCT	DSJ82
		<i>Rousettus aegyptiacus</i>	David S. Jacobs, UCT	13.09.02RA(BCT)
	Rhinolophidae	<i>Rhinolophus capensis</i>	David S. Jacobs, UCT	DSJ5
		<i>Rhinolophus darlingi</i>	David S. Jacobs, UCT	DSJ9
		<i>Rhinolophus fumigatus</i>	David S. Jacobs, UCT	31.7.02Rf(Th)
		<i>Rhinolophus hildebrandtii</i>	David S. Jacobs, UCT	4.08.02Rh1(LG)
	Rhinopomatidae	<i>Rhinopoma hardwickei</i>	TK	TK25640
	Thyropteridae	<i>Thyroptera laval</i>	ROM	ROM104026
		<i>Thyroptera tricolor</i>	Francois Catzeflies	T3679
	Vespertilionidae	<i>Eptesicus hottentotus</i>	David S. Jacobs, UCT	06.10.02Eh1(AD)
		<i>Myotis tricolor</i>	David S. Jacobs, UCT	4.10.02Mt4(DHC)
		<i>Scotophilus dinganii</i>	David S. Jacobs, UCT	23.12.02Sd1(AED)
		<i>Nycticeinops schlieffeni</i>	David S. Jacobs, UCT	21.12.02Nys1(KWP)
		<i>Cistugo lesueuri</i>	David S. Jacobs, UCT	8.10.02M11(A)
		<i>Cistugo seabrai</i>	Manuel Ruedi	M977
Carnivora	Viverridae	<i>Genetta genetta</i>	Evolutionary Genomics Group, US	SUN1161
Perrisodactyla	Equidae	<i>Equus caballus</i>	GenBank	AF165597, AF165598, AF165599, AF165601
Pholidota	Manidae	<i>Manis temmincki</i>	Evolutionary Genomics Group, US	SUN106

NOTE.—AMNH, American Museum of Natural History; FMNH, Field Museum of Natural History, Chicago; TK, Museum of Texas Tech University; UCT, University of Cape Town; US, University of Stellenbosch. ROM, Royal Ontario Museum.

analyses was assessed from 100 ML bootstrap replicates using NNI as the branch-swapping algorithm. Due to computational demands, ML bootstrap runs for single nuclear introns were performed with a constraint topology

in which all families with more than one taxon representative were constrained to be monophyletic (as supported in Murphy et al. 2001b). For the supermatrix, ML bootstrap support was determined from 75 replicates with

TBR branch swapping and no phylogenetic constraints imposed. BI was implemented setting the prior model to that specified by Modeltest for each data set. If the model suggested by AIC and LRT differed, two separate runs were performed. The supermatrix was analyzed using both a single model and in a partitioned manner to allow the selection of different optimal parameters for each partition (Huelsenbeck and Ronquist 2001). A random tree generated by MrBayes was used as a starting tree for each Markov chain. Four Markov chains were run for 1 million generations, comprising one cold chain and three incrementally heated chains. Tree sampling was performed every 50 generations, thereby generating 20,000 sample points. The sump command was used to generate plots of generation number versus the log probability of observing the data, and samples taken during the first 25,000 cycles of the chain were discarded as “burn-in” (Huelsenbeck 2002). Posterior probabilities were based on the remaining 19,500 trees. Three independent Bayesian runs with different random starting trees were performed to ensure convergence on the same topology (Huelsenbeck and Ronquist 2001). Nodes that received $\geq 70\%$ bootstrap support or those with ≥ 0.95 Bayesian posterior probability were considered well supported. To investigate the amount of phylogenetic signal in the data sets, the number of unique topologies in the 95% posterior interval was estimated for all data sets (Buckley et al. 2002). Alternative tree topologies were compared with the optimal ML tree topology using the approximately unbiased (AU) test (Shimodaira 2002) implemented in Consel V1.0g (Shimodaira and Hasegawa 2001).

Mapping of Echolocation Characters

MacClade version 4.0 (W. P. Maddison and D. R. Maddison 1989) was used to optimize characters related to echolocation behavior on the supermatrix topology using parsimony as an optimality criterion. Reconstruction of characters was examined using both delayed transformations (Deltran) and accelerated transformations (Actran) optimization, but no differences in the reconstructions for the ancestral nodes of interest were observed. Echolocation characters were evaluated under two scenarios as the current gene trees suggest that echolocation of extant chiropteran families have either two independent origins or one origin and one loss of this character in the Old World fruit bat lineage. The three echolocation characters mapped onto the phylogeny were (1) high-duty versus low-duty cycle echolocation calls (Fenton et al. 1995), (2) low-intensity versus high-intensity echolocation calls (Arita and Fenton 1997; DeBaeremaker and Fenton 2003), and (3) nasal echolocation versus oral echolocation (Pederson 1993, 1995, 1998). Two families of bats, the Hipposideridae and Rhinolophidae, as well as the mormoopid species *Pteronotus parnellii* were classified as high-duty cycle echolocators (Fenton 1999; Jones 1999). Five families of bats were classified as comprising bats producing mainly low-intensity echolocation calls, namely Thyropteridae (Fenton et al. 1999a), Nycteridae (Aldridge et al. 1990), Megadermatidae, Phyllostomidae (Schintzler and Kalko 2001), and Furipteridae (Fenton et al. 1999b). Vespertilionidae was coded as having both low-intensity and high-intensity echolocat-

ing bats as some vesper species produce low-intensity echolocation calls, for example, *Myotis emarginatus* (Schumm, Krull, and Neuweiler 1991) and *Plecotus auritus* (Waters and Jones 1995). Six families of bats were classified as nasal echolocators, namely Rhinolophidae, Hipposideridae, Megadermatidae, Nycteridae, Phyllostomidae, and Rhinopomatidae. Although it is uncertain whether rhinopomatids emit their echolocation calls through their mouths or nostrils, Pederson (1993) classified *Rhinopoma muscatellum* as a nasal echolocator based on cephalometric characters. Similarly Göbbel (2002) reports similarities between *Rhinopoma hardwickei*, rhinolophids and megadermatids (the latter two both unambiguous nasal echolocators) in the external nasal cartilage. Although some vespertilionids possess rudimentary nose leaves that are often associated with nasal emission, for example, *Nyctophilus*, *Pharotis*, and *Antrozous* (Nowak 1999), it is unclear if these bats emit echolocation signals orally or nasally; therefore vespertilionids were not coded as polymorphic for oral-nasal echolocation.

Morphology, Molecular Scaffolds, and Fossil Taxa

The 195 morphological characters identified by Simmons and Geisler (1998) were reevaluated in light of the paraphyly of microbats using the “trace character” option in MacClade. Morphological characters were characterized as either homoplasious or nonhomoplasious by mapping them on the gene tree comprising two clades—one containing the fruit bats and rhinolophoid microbats, and the other comprising all remaining bat families. Relationships among families within these two clades were collapsed to polytomies. *Craseonycteris* was placed within the fruit bat rhinolophoid lineage on the basis of morphological and molecular evidence suggesting a close affiliation with this group (Simmons and Geisler 1998; Hulva and Horacek 2002). Only morphological characters in congruence with the gene tree and scored for at least one fossil taxon were used to reconstruct the relationships of the fossil bats to the extant taxa. Although character polarity is dependent on the choice of out-groups (in this instance representatives of Scandentia and Dermoptera), the aim of this exercise was merely to reexamine the placement of the fossil taxa as suggested by Springer et al. (2001) because we argue that the exclusion of a significant amount of homoplasious characters can potentially alter the conclusions reached by these authors. The molecular scaffold used by Springer et al. (2001) was used as one of two backbone constraints in MP analyses with selected morphological characters and all the taxa included in the data set of Simmons and Geisler (1998) and was as follows: (Scandentia, Dermoptera, (Pteropodidae (Hipposideridae, Megadermatidae)), (Molossinae, Emballonuridae, Phyllostomidae)). This scaffold was congruent with the intron supermatrix topology generated in our study. The second scaffold used constrained Kerivoulinae, Murinae, Myotinae, Antrozoidae, Tomopotinae, and Vespertilionidae to form a monophyletic group (Teeling et al. 2002; Hofer and Van Den Bussche 2003; Hutcheon and Kirsch 2004) as well as members of the superfamily Noctilionoidea (Phyllostomidae, Mormoopidae, Furipteridae, Mystacinidae, Noctilionidae, and Thyropteridae) and the Old World fruit bat—rhinolophoid

Table 2
Characteristics of the Nuclear Introns Used in This Study

Gene	Total Number of Taxa	Total Length of Alignment	Variable Characters	Parsimony Informative Characters	MP Tree Length	RI Values	Number of Equally Parsimonious Trees	Number of Trees in 0.95 Posterior Interval
PRKC1	52	940	374	279	774	0.74	439	13,014
SPTBN	54	1,432	649	528	2,045	0.66	4	11,975
STAT5A	58	583	439	360	1,620	0.62	158	11,965
THY	58	1,047	470	373	1,327	0.67	360	11,479
Supermatrix	58	4,002	1,932	1,540	5,768	0.66	1	690 ^a

NOTE.—The maximum parsimony (MP) tree length refers to the length of the strict consensus topology. Retention index (RI) values and MP tree lengths are given with uninformative characters excluded. The number of trees in the 0.95 interval is the average from three independent Bayesian runs under a hLRT model.

^a Number of trees in the 0.95 posterior interval under a mixed model, that is, each intron partition in the supermatrix allowed to evolve according to its optimal model.

microbat clade (Pteropodidae, Rhinolophidae, Hipposideridae, Megadermatidae, Rhinopomatidae, and Craseonycteridae) based on consensus between the results from this and other studies (Teeling et al. 2000, 2002, 2003, 2005; Hooper and Van Den Bussche 2003; Van Den Bussche and Hooper 2004). Unweighted parsimony analysis with 1,000 random addition sequence replicates was performed, setting the maximum number of trees saved to 20,000. Nonparametric bootstrap estimates were based on 1,000 replicates, with a maximum of 1,000 trees saved per replicate.

Molecular Clock

The relaxed Bayesian clock method (Thorne, Kishino, and Painter 1998; Thorne and Kishino 2002) following the methodology outlined in Matthee et al. (2004) was used to date the evolution of the various chiropteran lineages. As priors we used 65 Myr (standard deviation [SD] = 65 Myr) between the tip and the root and 0.003 (SD = 0.003) substitutions per site per Myr for the rate at the root node. The conservative prior by 65 million years was chosen based on a strict interpretation of the Explosive model of placental diversification, which places extant placental ordinal diversification in the early Paleocene (Archibald and Deutschman 2001). The value of the substitutions per site at the root rate was determined by using a median amount of evolution (substitutions per site) among genes separating roots and tips; this value was divided by the 65 million years that was believed to be a reasonable age for the diversification of the Chiroptera. The value of the rate of evolution at the root node was varied, and it was found that even large changes to the root rate had little influence on clock estimates. In addition, large differences between the prior and posterior time estimates were observed, tending to support the notion that most of the molecular dating information was based on the concatenated DNA markers and not the priors. *Equus caballus* was designated as the out-group, and to obtain reasonably narrow posterior distributions for divergence times, six time constraints were incorporated from the fossil record. The first pair of constraints was a minimum of 34 MYA and a maximum of 55 MYA for the split between *Megaderma* and *Rhinopoma* (McKenna and Bell 1997; Teeling et al. 2003). The second pair of constraints was a minimum of 37 MYA and a maximum of 55 MYA for the split between the hipposiderids and rhinolophids (McKenna and Bell

1997; Teeling et al. 2003). A minimum of 63 MYA was used for the carnivore pangolin split based on fossil data (McKenna and Bell 1997), and a minimum of 55 MYA was used for the appearance of bats based on the oldest bat fossils (*Icaronycteris* and *Australonycteris*) discovered to date (Jepsen 1966; Hand et al. 1994). The highest possible divergence for the in-group was set at 100 Myr. Analyses were repeated removing one constraint per run to estimate the sensitivity of the molecular clock to any one particular constraint. To examine the “clock-like” signal within each intron, divergence estimates were also estimated for each intron separately.

The International Union of Geological Sciences International Stratigraphic Chart (available at <http://www.iugs.org/iugs/pubs/intstratchart.htm>) was used in conjunction with McKenna and Bell (1997) for delineating epochs.

Biogeographic Analyses

Dispersal-vicariance analysis (Diva) (Ronquist 1997), as implemented in the computer program DIVA v. 1.1 (Ronquist 1996), was used to reconstruct ancestral distributions of extant Chiroptera. The supermatrix phylogeny with families as terminal taxa was used, and seven biogeographical areas were recognized based on continental designations: Africa (A), Asia (B), Australia (C), Europe (D), North America (E), South America (F), and New Zealand (G). Families were coded for their current distributions based on distribution tables from Walker's Mammals of the World (Nowak 1999). Although *Myzopoda* currently only occurs in Madagascar, early Pleistocene fossil records have been found in East Africa (McKenna and Bell 1997), hence this family was coded as present in Africa. The analysis was run with no constraint on the number of “maxareas.”

Results

Sequence Data

The combined intron supermatrix comprised 4,002 alignment positions for 58 taxa (table 2). Despite multiple amplification attempts, the SPTBN region did not produce homologous sequences to the targeted intron for *Tadarida aegyptiaca*, *Mormopterus petrophilus*, *Chaerephon ansorgei*, and *Cistugo seabrai*. Likewise, no PRKC1 data could

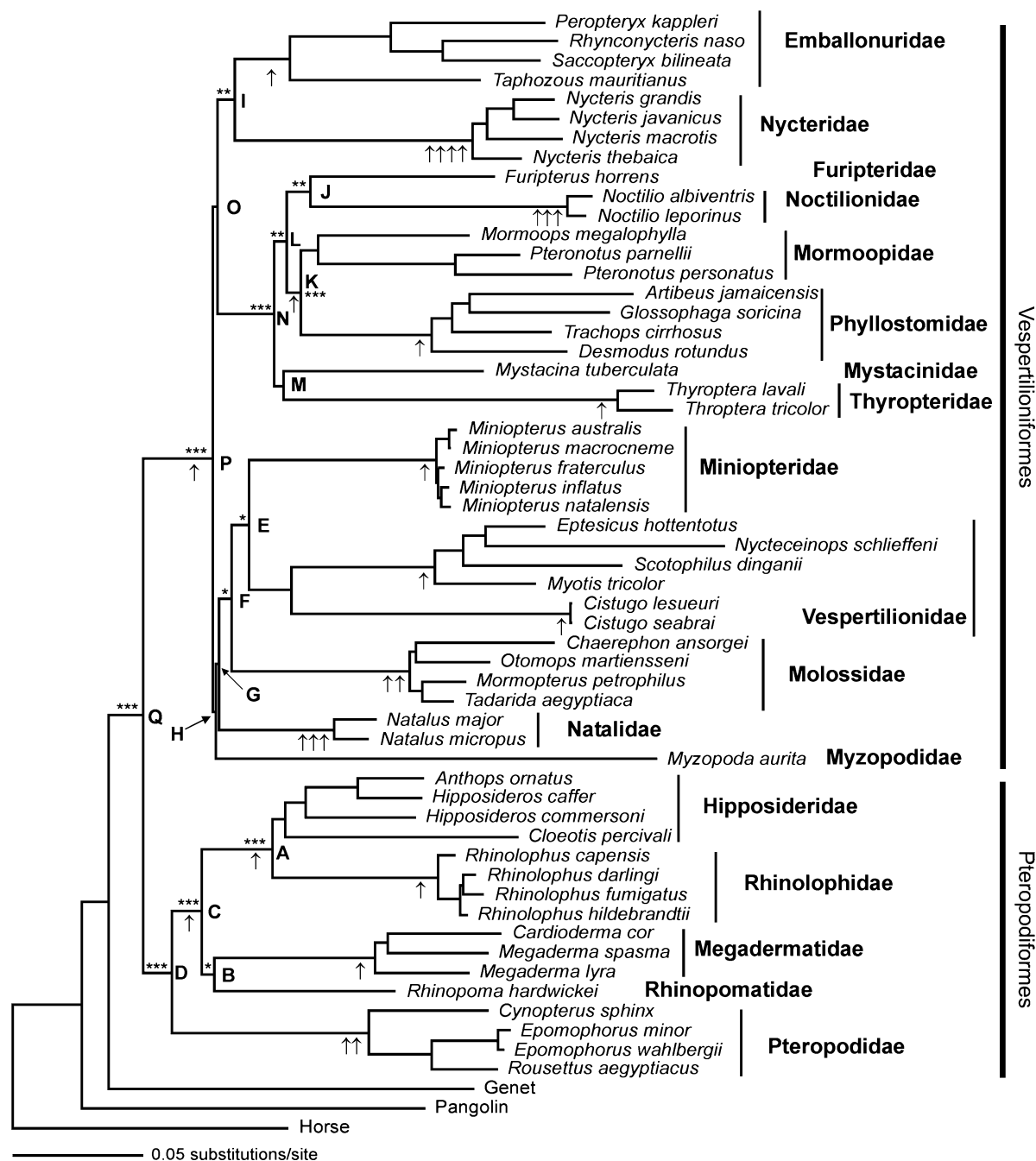


FIG. 1.—Single ML tree ($-\ln$ likelihood = 36,114.20) recovered from analysis of the intron supermatrix using a GTR + I + Γ model of nucleotide evolution. Nodes labeled A–Q correspond to those in table 3. Vertical arrows indicate unique indel events supporting phylogenetic associations. The number of asterisks indicate whether labeled nodes were recovered with $\geq 70\%$ bootstrap or ≥ 0.95 Bayesian posterior probability by all three (***), two (**), or only one (*) of the three methods of phylogenetic inference used. Absolute values for the nodes can be viewed in supplementary table 3 (Supplementary Material online). Branch lengths are proportional to the number of substitutions as indicated by the scale bar.

be generated for *Thyroptera lavalii*, *Thyroptera tricolor*, *Myzopoda aurita*, *Otomops martiensseni*, *Hipposideros commersoni*, and *Cynopterus sphinx*. Retention index (RI) values were relatively high (>0.6), indicating low levels of homoplasy in the intron data sets. Base frequencies did not deviate from stationarity across all lineages for all taxa for three of the four nuclear introns used in this study (PRKC1: $\chi^2 = 82.3$, $df = 153$, $P = 0.99$; STAT5A: $\chi^2 = 181$, $df = 171$, $P = 0.29$; THY: $\chi^2 = 95.1$, $df = 171$,

$P = 0.99$). For SPTBN, there was seemingly significant deviation from stationarity for all taxa ($\chi^2 = 204.13$, $df = 159$, $P = 0.009$), although this appears to be due to a paucity of adenines in the SPTBN intron of *Cistugo lesueuri*. When the latter taxon was removed the result became nonsignificant ($\chi^2 = 158.11$, $df = 156$, $P = 0.44$). Despite this, *C. lesueuri* was not excluded from the analyses as its phylogenetic placement was invariant across all introns and all types of analyses.

Table 3
Congruence Between the Topologies Derived from Analysis of Each Intron Compared to the Topology Derived from the Intron Supermatrix (fig. 1)

Gene	Analyses Type	Node																% Congruence	
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P		Q
PRKCI	MP	✓ ⁸⁸	X	✓ ⁸⁶	X	X	X	X	✓ ⁵⁰	✓ ⁵⁰	✓ ⁵⁰	X	✓ ⁸³	??	✓ ⁷⁸	✓ ⁵⁰	✓ ⁸²	X	50
	ML	✓ ⁹⁸	✓ ⁵⁰	✓ ⁹⁴	✓ ⁵⁰	✓ ⁵⁰	X	X	✓ ⁵⁰	✓ ⁵⁰	✓ ⁵⁰	X	✓ ⁸⁹	??	✓ ⁹²	X	✓ ¹⁰⁰	X	63
	BI	✓ ¹⁰⁰	✓ ⁵⁰	✓ ¹⁰⁰	✓ ⁵⁰	✓ ⁵⁰	X	X	X	✓ ⁵⁰	✓ ⁵⁰	X	✓ ¹⁰⁰	??	✓ ¹⁰⁰	✓ ⁵⁰	✓ ¹⁰⁰	X	69
SPTBN	MP	✓ ⁹⁰	✓ ⁸⁷	✓ ⁶⁸	✓ ⁷⁴	✓ ⁵⁰	X	X	X	X	✓ ⁵⁰	✓ ⁵⁰	✓ ¹⁰⁰	??	✓ ⁹⁶	X	✓ ⁶⁸	X	59
	ML	✓ ⁹⁸	✓ ⁸⁹	✓ ⁸³	✓ ⁹²	✓ ⁵⁰	✓ ⁵⁰	X	X	✓ ⁵⁰	✓ ⁵⁰	✓ ⁶⁹	✓ ⁵⁰	✓ ⁵⁰	✓ ⁹³	X	✓ ¹⁰⁰	X	76
	BI	✓ ¹⁰⁰	✓ ⁹⁹	✓ ⁹⁸	✓ ¹⁰⁰	✓ ⁵⁰	✓ ⁵⁰	X	X	✓ ⁵⁰	✓ ⁵⁰	✓ ⁹³	✓ ⁶¹	✓ ⁵⁰	✓ ¹⁰⁰	✓ ⁵⁰	✓ ¹⁰⁰	X	82
STAT5A	MP	✓ ⁹⁸	✓ ⁵⁰	X	✓ ⁶²	✓ ⁵⁰	✓ ⁵⁰	X	X	✓ ⁵⁰	✓ ⁵⁴	X	X	X	✓ ⁵⁰	✓ ⁵⁰	✓ ⁶⁹	✓ ⁷⁷	65
	ML	✓ ¹⁰⁰	✓ ⁵⁵	✓ ⁵⁰	✓ ⁷¹	✓ ⁵⁰	✓ ⁵⁰	✓ ⁵⁰	✓ ⁵⁰	✓ ⁵⁰	✓ ⁶⁹	✓ ⁵⁰	X	X	✓ ⁵⁴	✓ ⁵⁰	✓ ⁸⁸	✓ ⁷⁵	88
	BI	✓ ¹⁰⁰	✓ ⁵¹	✓ ⁵⁰	✓ ⁹⁹	✓ ⁶⁷	✓ ⁸⁶	✓ ⁵⁰	✓ ⁵⁰	✓ ⁷²	✓ ⁷⁸	✓ ⁵⁰	X	X	✓ ¹⁰⁰	✓ ⁵⁰	✓ ¹⁰⁰	✓ ¹⁰⁰	88
THY	MP	✓ ⁹⁸	✓ ⁶⁷	✓ ⁹⁶	✓ ⁷⁶	X	X	✓ ⁵⁰	✓ ⁵⁰	✓ ⁵⁰	✓ ⁸⁰	✓ ⁵⁰	X	X	✓ ⁷⁶	X	✓ ⁷⁹	✓ ⁸⁹	71
	ML	✓ ⁹⁷	✓ ⁷²	✓ ⁹⁹	✓ ⁸¹	X	✓ ⁵⁰	✓ ⁵²	✓ ⁵⁰	✓ ⁶⁴	✓ ⁸⁸	✓ ⁸⁴	✓ ⁵⁰	X	✓ ⁹⁰	✓ ⁵⁰	✓ ⁸²	✓ ⁹¹	88
	BI	✓ ¹⁰⁰	✓ ⁵⁸	✓ ¹⁰⁰	✓ ⁹⁸	✓ ⁵⁰	✓ ⁵⁰	✓ ⁵⁰	✓ ⁸⁹	✓ ¹⁰⁰	✓ ¹⁰⁰	✓ ¹⁰⁰	✓ ⁵⁰	X	✓ ¹⁰⁰	✓ ⁵⁰	✓ ⁹⁹	✓ ¹⁰⁰	94
% Obtained		100	92	92	92	75	58	42	42	92	92	67	67	33	100	0	100	50	
% Supported		100	33	67	67	0	0	0	0	8	25	17	25	0	83	0	83	50	

NOTE.—Nodes A–Q correspond to those labeled on the topology in fig. 1. “X” indicates the node was not recovered, and “??” indicates missing data for Thyropteridae for the PRKC1 intron (see text for details). “✓” indicates the presence of a node, and the values indicated above this sign correspond to bootstrap or posterior Bayesian probability values. The percentage of times a node was supported ($\geq 70\%$ bootstrap, $\geq 95\%$ posterior probability), as well as the percentage congruence with the supermatrix topology is also indicated.

Insertions and Deletions

A total of 34 potentially phylogenetically informative indels were identified in the four intron data sets. Of these, 22 indels provided confirmation for the monophyly of several bat families, while only four supported associations at the higher taxonomic level (fig. 1). The eight remaining indels were homoplasious when mapped onto the supermatrix topology and are not shown. Large autapomorphic insertions (>100 bp) were present in the introns of some taxa, and BlastN searches of these revealed no significant homology to any annotated gene sequences available in GenBank. The only exception was the 199-bp insertion in *T. aegyptiaca*, which showed significant homology to a published *Myotis myotis* microsatellite (Castella and Ruedi 2000).

Model Selection

For two of the five data sets (STAT5A and the supermatrix) the same optimal model was chosen under hierarchical likelihood ratio test (hLRT) and AIC criteria (supplementary table 2, Supplementary Material online). For PRKC1, SPTBN, and THY analyses, analyses were performed twice using both models, respectively. These alternative models did not influence any of the topologies, and for consistency, only the ML and Bayesian topologies based on hLRT will be presented.

Separate Analysis of Nuclear Introns

All nodes defining the monophyly of chiropteran families and also those defining the associations among chiropteran families were labeled A through to Q (fig. 1). Only three nodes (fig. 1, A, N, and P) were consistently obtained when each intron was analyzed separately (table 3). However, no incongruent nodes recovered by any of the analytical methods were supported by high bootstrap or posterior probability values. The STAT5A and THY introns showed the most congruence with the supermatrix topology, with 65%–88% of the nodes present recovered for the former and 71%–94% of the nodes recovered for the latter (table 3). The lack of resolving power when each intron is analyzed separately is emphasized by the large number of trees ($>11,000$ trees) present in the 95% confidence interval of each intron (table 2).

Combining Data

Combining the introns into a supermatrix dramatically improved the resolving power of the data. Single MP and ML trees were recovered, and the number of trees in the 0.95 Bayesian confidence interval decreased by approximately 16-fold compared with the analysis of each intron separately (table 2). Whereas only one node (Node A) received robust support across all analyses when introns were analyzed separately (table 3), seven nodes received significant support using all three methods of analysis when the data were combined, and an additional three nodes received significant support from two of the three phylogenetic analyses (fig. 1). Bayesian analyses under a single model or

Table 4
Approximate Unbiased (AU) *P* values for the Best ML Tree and Alternative *A Priori* and *A Posteriori* Phylogenetic Hypotheses

Hypothesis	AU
Best ML tree	0.740
Microbats monophyletic	0.003*
Rhinolophoidae <i>sensu</i> Simmons and Geisler (1998)	<0.001*
Nataloidea <i>sensu</i> Simmons and Geisler (1998)	<0.001*
Noctilionoidae (fig. 1, Node N) not monophyletic	<0.001*
Nycterids + Emballonuridae not sister taxa	0.033*
Noctilionidae + Furipteridae not sister taxa	<0.001*
Miniopteridae + Vespertilionidae not sister taxa	0.301
Miniopteridae + Molossidae sister taxa	0.054
Cistugo + Vespertilionidae not monophyletic	<0.001*
Myzopoda + Emballonuridae + Nycteridae (fig. 1, Node I)	0.366
Myzopoda + Noctilionoidea (fig. 1, Node N)	0.381
Myzopoda + Emballonuridae + Nycteridae + Noctilionoidea (fig. 1, Node O)	0.641

* Significance at $P < 0.05$.

using a partitioned model resulted in identical topologies, and Bayesian posterior probability values for nodes differed by 0.02–0.03 at most. The number of trees found in the 95% posterior interval was slightly lower under a partitioned model than under a combined model.

Intron Supermatrix and Chiropteran Phylogenetics

The single ML tree and Bayesian consensus topology recovered from analysis of the intron supermatrix were identical (fig. 1). The MP tree differed from this topology in only one respect: *M. aurita* was placed as sister taxon to Miniopteridae, rather than basal to Miniopteridae, Vespertilionidae, Molossidae, and Natalidae.

Nuclear intron data support paraphyly of the microbats (Hutcheon, Kirsch, and Pettigrew 1998; Teeling et al. 2000, 2002, 2003, 2005; Hutcheon and Kirsch 2004; Van Den Bussche and Hofer 2004). The association between Old World fruit bats (Pteropodidae) and rhinolophids, hipposiderids, megadermatids, and rhinopomatids (hereafter referred to as “rhinolophoid microbats”) received significant statistical support from all three methods of phylogenetic analyses (table 4). Furthermore, a phylogenetic hypothesis in which the microbats are monophyletic was rejected at the 95% confidence level by the AU test (table 4). The remaining microbats grouped within a clade that received significant bootstrap support from all three methods of phylogenetic analysis and was supported by a 15-bp deletion in the STAT5A intron. This corresponds to the suborder Yangochiroptera of Teeling et al. (2002, 2003, 2005), and if this holds, our data suggest that the families Furipteridae, Myzopodidae, Thyropteridae, and Miniopteridae, which were not included in previous studies, can now also be included in this subordinal rank. Following the recommendations of Hutcheon and Kirsch (2004), we refer to the two suborders of chiropterans as “Pteropodiformes” (comprising the Pteropodidae, Rhinolophidae,

Hipposideridae, Megadermatidae, and Rhinopomatidae) and “Vespertilioniformes” (remaining microbat families). The association of the families Noctilionidae, Furipteridae, Phyllostomidae, Mormoopidae, Mystacinidae, and Thyropteridae in a single, robustly supported clade (fig. 1) corresponds to the expanded Noctilionoidea of Van Den Bussche and Hofer (2004). Within this clade, the sister taxon relationship between Mormoopidae and Phyllostomidae is supported both by robust bootstrap and Bayesian posterior probability values as well as a unique indel in THY (Kirsch et al. 1998; Van Den Bussche and Hofer 2000, 2001, 2004; Teeling et al. 2003, 2005). Noctilionoidae and Furipteridae are sister taxa, based on nodal support values as well as tests of alternative topologies (fig. 1; table 4), as supported by the mitochondrial-based studies of Van Den Bussche and Hofer (2001, 2004) and Hofer et al. (2003). Nycteridae and Emballonuridae are sister taxa as supported by Teeling et al. (2002, 2003, 2005), with the intron data rejecting a hypothesis in which these two groups are separated from each other (table 4). Miniopterids appear most closely related to the vespertilionids, with the molossids sister taxon appearing most closely related to the miniopterids and vespers (fig. 1). The grouping of vespertilionids, miniopterids, molossids, and natalids corresponds to the Superfamily Vespertilionoidea of Teeling et al. (2002, 2005), although they included no miniopterids in their study. In our study, there is a lack of strong statistical support for this group in contrast to the high bootstrap and Bayesian support (>90%) found by Teeling et al. (2002, 2003, 2005). Alternative hypotheses that miniopterids and vespertilionids are not sister taxa and that miniopterids and molossid are more closely related could not be rejected by the intron data (table 4). *Myzopoda aurita*, characterized by a relatively long branch, was placed basal to the Vespertilionoidea. However, this finding should be treated with caution as there was no significant nodal support for this placement. Furthermore, evaluation of alternative topologies in which *M. aurita* was associated with the nycterids and emballonurids (fig. 1, Node I), Noctilionoidea (fig. 1, Node N) or a clade comprising nycterids, emballonurids and the Noctilionoidae (fig. 1, Node O) could not be rejected by the data (table 4).

Within the Pteropodiformes, phylogenetic relationships were congruent with those found in previous studies, with rhinolophids and hipposiderids sister taxa and megadermatids and rhinopomatids sister taxa (Teeling et al. 2002, 2003). Unfortunately, because *Craseonycteris thonglongyai* was not included in this study, the hypothesis that this family is allied to the rhinolophoid microbats (Hulva and Horacek 2002) could not be evaluated. The concept of a clade Rhinolophoidea *sensu* Simmons and Geisler (1998) comprising rhinolophids, hipposiderids, megadermatids, rhinopomatids, and nycterids based on morphological characters was rejected by the intron data (table 4). Similarly, Nataloidea *sensu* Simmons and Geisler (1998) (comprising Furipteridae, Natalidae, Thyropteridae, and Myzopodidae), again a clade strongly supported by morphological characters, was also rejected by the intron data (table 4).

The genus *Cistugo* appears distinct from other vespertilionids. Whereas all other vespers are characterized by

a unique deletion in SPTBN, the two species of *Cistugo* lack this indel character and are instead characterized by a unique insertion in PRKC1, which in turn is not present in the other vespertilionids sequenced (fig. 1). This observation indicates that there may be some validity to the hypothesis that *Cistugo* is a distinct family. However, more comprehensive taxonomic sampling within the Vespertilionidae needs to be conducted before this question can be adequately addressed.

Evolution of Echolocation

The following discussion is based on the assumption that there was no change in echolocation characters between their time of origin and their present state in extant families. Based on mapping of echolocation characters on the supermatrix topology, if one origin of echolocation in the bat lineage is assumed, the ancestral crown group protobat probably produced echolocation calls of low-duty cycle and high intensity. Whether the ancestral bat was an oral or nasal emitter is equivocal. If two separate origins of echolocation are inferred, the ancestor of Vespertilioniformes was likely an oral emitter producing low-duty cycle, high-intensity calls. The ancestor of the Rhinolophidae, on the other hand, was probably a nasal emitter producing calls of high intensity. Whether this ancestor produced high-duty or low-duty cycle calls is equivocal because of the sister taxa status of the rhinolophid-hipposiderid and megadermatid-rhinopomatid clades. Regardless of whether echolocation evolved once or twice in the bat lineage, high-duty cycle echolocation has evolved at least twice, at least once in the Pteropodiformes, and once in the Vespertilioniformes. In the latter lineage, it is a derived form of echolocation evolving from a low-duty cycle ancestor.

Low-intensity echolocation calls have evolved independently at least six times in the bat lineage, with high-intensity echolocation pulses the ancestral condition. Similarly, there may have been up to three independent origins of nasal echolocation in Chiroptera—once in the rhinolophid lineage, once in the nycterids, and once in the phyllostomids, assuming two origins of echolocation or one origin of echolocation with an oral-emitting protobat. There have been at least two independent transitions from oral to nasal echolocation within the Vespertilioniformes lineage: once in the nycterids and once in the lineage leading to the phyllostomids.

Reevaluation of Morphological Characters and Placement of Fossil Bat Taxa

Fifty-nine nonhomoplastic morphological characters were identified that did not contradict microbat paraphyly. These represent 31 soft tissue and 28 hard tissue characters. Of these, a total of 24 were scored for at least one fossil taxon and were used in subsequent analyses. Regardless of the backbone molecular scaffold used, the strict consensus topology was unresolved with respect to placement of the fossil taxa relative to extant taxa (supplementary fig. 1, Supplementary Material online). Reevaluating the morphological characters in light of the intron supermatrix

Table 5
Prior and Posterior Estimates of Divergence Dates (MYA) for Selected Nodes from fig. 1

Node	Prior Distribution			Posterior Distribution		
	Age	SE	95% Credibility Intervals	Age	SE	95% Credibility Intervals
A	45	5	37–54	41	3	37–47
B	44	6	35–54	50	3	44–54
C	54	8	42–73	54	3	48–60
D	63	9	48–83	58	3	52–65
E	39	12	17–62	45	4	39–53
F	46	12	22–69	48	4	42–56
G	53	11	29–75	50	4	44–58
H	60	11	38–80	52	4	46–59
I	47	13	20–72	50	4	43–57
J	29	14	5–59	39	3	33–46
K	35	13	12–63	39	3	33–46
L	44	13	20–69	41	3	35–48
M	34	15	5–65	42	3	36–49
N	51	13	26–75	44	3	37–51
O	59	11	35–81	52	4	46–60
P	66	10	47–86	53	4	47–61
Q	71	9	55–90	62	4	56–70
<i>Cistugo/</i>						
Vespertilionidae	32	12	10–56	41	4	34–48

topology, character 82 of Simmons and Geisler (1998) was found to be a synapomorphy for Rhinolophoidea as defined in this study. Rhinolophids, hipposiderids, megadermatids, rhinopomatids, and *Craseonycteris* are all characterized by ossification of their first costal cartilage, which is fused to the manubrium as well as to the first rib, whereas all other bat families do not have this character. Another synapomorphy of the Rhinolophoidea is the presence of one pair of pubic nipples in females (character 176 of Simmons and Geisler 1998). The presence of a triangular flange on the anteromedial edge of the scapula was a morphological synapomorphy for the clade comprising vespers, miniopterids, molossids, natalids, and *M. aurita*. No projections or flanges are present on the anteromedial edge of the scapula of other bat families.

Molecular Clock and Biogeography

The molecular clock hypothesis was rejected for the combined DNA data set by a LRT under the general time reversible (GTR) + model with invariant sites + Γ model: $\delta = 2 \times (\ln L \text{ unconstrained} - \ln L \text{ clock}) = 2 \times [-36 114.20 - (-36 356.72)] = 242.52$; $df = 56$; $P < 0.0001$. This indicates that there is extensive rate variation among lineages that precludes the application of the linearized tree method (Takezaki, Rzhetsky, and Nei 1995). Thus, the use of a relaxed molecular clock approach designed to accommodate rate variation is preferable for estimating divergence ages with this data set.

Independent Markov chains initiated from different starting points converged on the same divergence times. Estimates of divergence dates based on posterior estimates were characterized by much smaller SDs and narrower credibility intervals than the prior estimates (table 5). Allowing genes to evolve with independent rates or

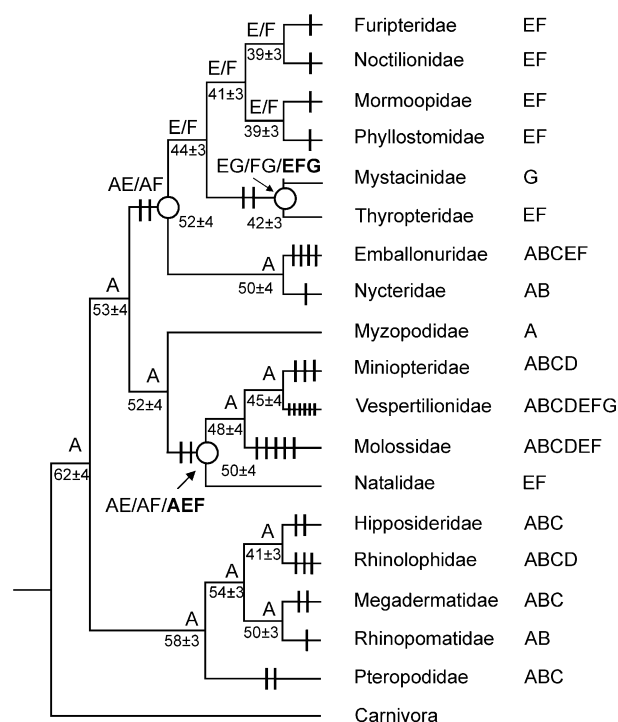


FIG. 2.—Summary of the optimal reconstructions of ancestral distributions of extant chiropteran families using dispersal-vicariance analysis (Diva). At each node, the optimal distribution is given with alternative equally optimal distributions separated with a forward slash. The optimal reconstruction required 39 dispersal events. All possible ancestral distributions are indicated above the branches at each node, with the most likely explanation in bold text. Symbols: circle, vicariance event; cross bar, dispersal event. The unit areas correspond to Africa = A, Asia = B, Australia = C, Europe = D, North America = E, South America = F, New Zealand = G. Estimates of divergence times from a Relaxed Bayesian clock are indicated below or adjacent to nodes (also see table 5).

allowing them to evolve with correlated rates resulted in almost identical divergence estimates. Estimates of divergence time were also remarkably robust to removal of single time constraints. The largest change observed was when removing the constraint on the upper divergence of *Megaderma* and *Rhinopoma*, which resulted in a systematic increase in divergence date estimates, although the values were still well within the 95% credibility intervals obtained using all six time constraints. Divergence estimates based on individual introns displayed far wider credibility intervals and larger SDs than the divergence estimates based on the supermatrix (data not shown). Interestingly, in all 11 independent runs based on the complete intron supermatrix, the null hypothesis that STAT5A versus SPTBN, and STAT5A versus PRKC1 evolve independently was rejected.

The molecular clock applied herein suggests that the first divergence among chiropterans dates back to approximately 62 MYA \pm 4 MYA (table 5). It also suggests that by 33 MYA at least 17 of the 18 extant chiropteran families were present (table 5).

Diva analysis suggests an African origin for the ancestor of extant Chiroptera, with 39 dispersal events and three vicariant events required to explain the current distribution of chiropteran families (fig. 2).

Discussion

Resolving Power of Nuclear Intron Sequences

The limitation of using single markers for resolving family-level chiropteran relationships was highlighted by the results obtained from analyses of individual nuclear intron fragments (table 3). The large number of equally parsimonious trees found for PRKC1, STAT, and THY and the large number of trees present in the 95% posterior probability interval further emphasizes the poor resolving power of individual nuclear markers (table 2). In short, these results agree with previous studies that emphasize the importance of a supermatrix approach for improving phylogenetic resolution (Baker and DeSalle 1997; Murphy et al. 2001a, 2001b; Buckley et al. 2002), particularly when the radiation was rapid resulting in short internal branches (Matthee et al. 2001, 2004; Willows-Munro, Robinson, and Matthee 2005).

Chiropteran Phylogeny

Despite different degrees of taxonomic sampling, both within and among families (Hutcheon, Kirsch, and Pettigrew 1998; Teeling et al. 2000, 2002, 2003, 2005; Hutcheon and Kirsch 2004; Van Den Bussche and Hofer 2004), the paraphyly of the microbats is a consistent finding across studies, separating bats into the superordinal clades Pteropodiformes and Vespertilioniformes.

Another area of agreement among studies is the superfamily Noctilionoidea (Springer et al. 2001; Teeling et al. 2003, 2005; Van Den Bussche and Hofer 2004). Within this superfamily, the sister taxon relationship between Phyllostomidae and Mormoopidae (Kirsch et al. 1998; Van Den Bussche and Hofer 2000, 2001, 2004; Teeling et al. 2003) and Furipteridae and Noctilionidae (Van Den Bussche and Hofer 2001, 2004; Hofer et al. 2003, Teeling et al. 2005) are well supported. Similarly, the sister taxon status of the emballonurids and nycterids reported by others (Teeling et al. 2000, 2002, 2003, 2005) and the superfamily Vespertilionoidea (Van Den Bussche 2004, miniopterids) is also supported by our study. Within the Pteropodiformes, there is widespread support for a clade comprising rhinolophids, hipposiderids, megadermatids, and rhinopomatids (Rhinolophoidea) based on both molecular (Teeling et al. 2000, 2002, 2003, 2005) as well as two morphological synapomorphies, namely ossification of the first costal cartilage which is fused to the manubrium as well as to the first rib and the presence of one pair of pubic nipples in the female. Furthermore, the rhinolophid-hipposiderid + megadermatid-rhinopomatid sister taxon relationship is well supported by this and other studies (Teeling et al. 2000, 2002, 2003, 2005), with the Old World fruit bat lineage consistently reconstructed as the sister taxon to this rhinolophoid microbat clade, indicating microbat diphyly. Thus, microbats are paraphyletic.

Areas of phylogenetic uncertainty include branching patterns within the superfamily Vespertilionoidea, with the association of the miniopterids with the vespers or molossids being uncertain. However, the basal position of the natalids to these three families, presented in this study, also receives support from previous studies (Hofer

et al. 2003; Teeling et al. 2003). A clade comprising the nycterids-emballonurids sister to the superfamily Noctilionoidea received moderate nodal support in our study, in agreement with Teeling et al. (2002, 2003, 2005), but is absent from other studies, indicating that this node may not be completely stable. The precise placement of Mystacinidae within the superfamily Noctilionoidea, as well as the branching order of the families within this clade requires further investigation. A further area of uncertainty concerns the precise placement of *M. aurita*. This taxon is characterized by a long branch and was placed basal to the superfamily Vespertilionoidea in this study. The association of *M. aurita* with this clade in the intron supermatrix, although not well supported by molecular characters, is also supported by the synapomorphic triangular anteromedial flange on the anteromedial edge of their scapula. A possible association between Myzopodidae and Natalidae was previously suggested based on the resemblance of the myzopodid skeletal structure (Miller 1907). However, in a study based on Rag 2 nuclear data (Hoofer et al. 2003), *M. aurita* was basal to all Vespertilioniformes, and in a study by Teeling et al. (2005) *M. aurita* was associated with the Noctilionoidea. Additional taxonomic sampling of vespertilionids and the inclusion of different types of data, such as SINE insertions (Kawai et al. 2002; Murphy, Pevzner, and O'Brien 2004; Pecon-Slattery et al. 2004), are clearly needed to resolve this issue.

Although Springer et al. (2001) and Teeling et al. (2005) found phylogenetic resolution among extinct and extant bat taxa, our analyses suggest that much of this resolution was obtained from homoplasious characters. While we recognize the importance of incorporating morphological data into phylogenetic reconstructions, especially for fossil data which can provide vital information (Wiens 2004), the high level of parallel evolution when using morphological characters is problematic. This convergence most likely results from the constraints imposed by the key innovations of flight coupled to echolocation, as well as adaptation to particular ecological niches (Ruedi and Mayer 2001).

Evolution of Echolocation

The unresolved position of the fossil taxa casts doubt on explanations of the evolution of echolocation that are based on the basal position of these fossil bat taxa (Springer et al. 2001). Although chiropteran monophyly provides strong evidence that flight has evolved only once (Simmons and Geisler 1998), microbat paraphyly makes the evolution of echolocation less clear. The molecular phylogeny presented here is consistent with either two independent origins of echolocation within the chiropteran lineage or a single origin (Teeling et al. 2000, 2002, 2003, 2005). A single origin of echolocation requires that echolocation was secondarily lost in the pteropid lineage and regained in rudimentary form in *Rousettus*. Two independent origins of echolocation propose that it arose in the lineage leading to the Rhinolophoidea, as well as in the ancestor of the Vespertilioniformes.

The mapping of echolocation-call design onto our phylogeny does not appear to resolve the question of

whether echolocation had one or two origins. Although the echolocation-call design and processing in rhinolophids and hipposiderids, who use high-duty cycle echolocation calls and Doppler-shift compensation along with an acoustic fovea to separate call and echo in frequency rather than time, is fundamentally different from the low-duty cycle calls used in all Vespertilioniformes (except one), rhinomatids and megadermatids also use low-duty cycle echolocation. High-duty cycle echolocation is not therefore a synapomorphy for the Rhinolophoidea, which would have provided strong support for two independent origins of echolocation.

Much like morphological characters, echolocation seems to be highly convergent. For example, high-duty cycle echolocation and the associated mechanical and neurological tuning required to exploit Doppler-shifted echoes has evolved convergently in the mormoopid *P. parnelli* (Kossel et al. 1999). Similarly, low-intensity echolocation calls, often associated with gleaners (Faure, Fullard, and Barclay 1990; Miller and Treat 1993), have evolved independently at least six times in both Vespertilioniformes and Pteropodiformes lineages (i.e., in the Phyllostomidae, Thyropteridae, Nycteridae, Furipteridae, Vespertilionidae, and Megadermatidae). The nasal-oral emitting dichotomy also shows no phylogenetic pattern, with at least two switches from oral emission to nasal emission within the Vespertilioniformes (Nycteridae and Phyllostomidae) and three independent origins of nasal echolocation within the Chiroptera. The latter is supported by morphological data. Whereas phyllostomid skulls lack resonating chambers and instead are characterized by large olfactory fossae and a well-developed voremonasal complex, nycterid skulls possess resonating chambers situated external to the bony nasal cavity. This in turn is different to the skull morphology of megadermatids, rhinolophids, and hipposiderids, where resonating chambers are formed by the nasal cavities and lie within the skull. The olfactory fossae and voremonasal complex are also distinctly smaller in the rhinolophoids and nycterids compared to the phyllostomids (Pederson 1993, 1995, 1998). Convergent evolution is also evident in the co-occurrence of low-intensity echolocation calls and nasal emission in three lineages—Megadermatidae, Nycteridae, and Phyllostomidae. This might indicate some association between nasal emission and the inability to produce echolocation calls of high intensity (Pederson 1993, 1995), which in turn may constrain foraging options. This may hold true particularly for the phyllostomids—they possess neither resonating chambers nor a “tuned” rostrum, so their emission is muffled in the nasal passages, resulting in low-intensity echolocation calls (Pederson 1998). Further investigation is required, however, to evaluate if the low-intensity echolocation calls produced by nycterids and megadermatids, both of which possess resonating chambers, can be linked to distinctive morphological features of their pharynx and skulls.

It is perhaps unsurprising that there appears to be no phylogenetic patterning to any of the three dichotomies summarizing echolocation behavior in bats. Echolocation-call design in bats arises as a result of strong selective pressures intimately linked to the ecological and environmental conditions bats are exposed to when navigating

or searching for food (Harbesetter 1981; Schintzler and Kalko 2001; Schintzler, Moss, and Denzinger 2003; Von Helversen and von Helversen 2003). This results in remarkable congruence in echolocation behavior and call design among bats foraging in similar habitat types (e.g., uncluttered space, background-cluttered space, or highly cluttered space) independent of phylogenetic associations (Surlykke et al. 1993; Schintzler and Kalko 2001; Denzinger, Kalko, and Jones 2004; Schintzler, Kalko, and Denzinger 2004).

Although the three echolocation characters discussed above do not lend unambiguous support for two independent origins of echolocation, one of the morphological synapomorphies characterizing the Rhinolophoidea suggests a separate origin of echolocation in this clade. All rhinolophoids have an ossified first costal cartilage fused to the manubrium and first rib. This may be an adaptation for decreasing the energetic costs associated with echolocation from a stationary position (Speakman, Anderson, and Racey 1989; Speakman and Racey 1991; Speakman et al. 2004), which strongly suggests that in this clade, echolocation may have developed in a perch-hunting, gleaning ancestor. The protorhinolophoid was likely a perch-hunting, flying, nocturnal or crepuscular small mammal which used passive cues such as prey-generated sound and vision to localize and detect prey on the substrate and flight to get from branch to branch. Ossification of the first costal cartilage and fusion of this to the rib and manubrium would have allowed energetically inexpensive production of echolocation calls while stationary (Speakman et al. 2004). Rhinolophoids (rhinolophids, hipposiderids, megadermatids) that echolocate while stationary also share modifications with other bats such as nycterids, which also echolocate while stationary; for example, they all possess a first rib at least twice the width of other ribs (character 81 Simmons and Geisler 1998) and have a second rib that articulates with the manubrium with no contact between the rib and mesosternum (character 83 Simmons and Geisler 1998), both of which presumably play some role in decreasing the costs of echolocation while stationary. Given that the two groups are not closely related, these modifications have evolved convergently in the nycterids and gleaning rhinolophoids. This is in contrast to character 82 of Simmons and Geisler (1998). This character is absent in nycterids (and other Vespertilioniformes families) but present in all rhinolophoid microbats. Thus, the presence of this character in all rhinolophoid families and the absence in all Vespertilioniformes indicate that echolocation may have had two independent origins within Chiroptera.

In conclusion, although the echolocation characteristics evaluated on the intron supermatrix do not provide clear support for two independent origins of echolocation in bats, the presence of a unique synapomorphy linked to echolocation in rhinolophoid microbats suggests that there may have been two independent origins of echolocation in bats. Furthermore, the advantages that echolocation confers upon an organism makes it unlikely that echolocation, once evolved, would have been lost in the pteropodids, only to be regained by *Rousettus*, as required by a single origin of echolocation (Arita and Fenton 1997; Speakman 1999, 2001). Assuming parallel evolution of echolocation in

the Vespertilioniformes and rhinolophoid microbats, the numerous similarities in echolocation in these two groups are presumably due to homologous developmental pathways underlying the ability to echolocate.

Biogeography and a Molecular Clock

On the basis of their current distribution patterns and thermoregulatory abilities, it has been hypothesized that bats originated somewhere in the Old World tropics (Legendre 1980; Hand 1984; Hall 1989; Hall and Woodside 1989; Hand et al. 1994), and paleontological evidence suggests that bats had their origins within the tropical forests of Laurasia (Cracraft 1973; Hand 1984). Sige (1991), however, hypothesized that modern bat groups evolved from isolated immigrant archaic groups somewhere in the Southern Hemisphere. Unfortunately, the bat fossil record is depauperate (Hand 1984) and is biased towards Europe and to a lesser extent North America, with very few early Asian, African, and South American examples (Savage and Russell 1983; McKenna and Bell 1997). Teeling et al. (2005) recently proposed that bats originated in Laurasia based on a phylogeny including fossil bats and extant taxa. However, we found little phylogenetic resolution between fossil taxa and extant bats using a similar approach where we limited our choice of morphological characters to those that did not contradict microbat paraphyly. Therefore in this study, we focused on evaluating biogeographic hypotheses for extant taxa only.

The Diva reconstruction of Africa as the center of origin of modern-day bat families is in accord with a Southern Hemisphere origin (fig. 2). The relaxed date estimates from our study are congruent with those of previous studies that place the diversification of extant Chiroptera at the Cretaceous-Tertiary boundary approximately 65 MYA (Springer et al. 2003; Teeling et al. 2003; Delsuc, Vizcaino, and Douzery 2004). This was rapidly followed by diversification of the superfamilies, and from our analyses it seems evident that extant bat families appear to have radiated fairly rapidly, with all families having evolved before the late Eocene. This deep, rapid radiation is supported by short internal branches near the base of the radiation (fig. 1).

The timing of diversification in the late Paleocene–early Eocene coincides both with the late Paleocene thermal maximum and early Eocene climate optimum, that is, the late Paleocene–early Eocene global warming interval (Zachos et al. 2001). During this period, lowland tropical rainforest covered large parts of Africa (Axelrod and Raven 1978), and there was an increase in insect herbivore diversity (Wilf and Labandeira 1999), perhaps also reflecting a general increase in insect diversity which may have provided the trigger for the initial diversification of the modern chiropteran ancestor into Pteropodiformes and Vespertilioniformes. There was a progressive cooling and drying trend in the middle Eocene, with tropical rainforests on the decline around the world (Prothero 1994). The middle/late Eocene transition in North America and Asia (37 MYA) was characterized by a severe extinction event, resulting in the elimination of many arboreal and archaic mammals typical of early and middle Eocene forests. However, there was no major extinction event in Europe, and it is unknown

what occurred in Africa or South America during this period due to the poor fossil record of these two continents (Janis 1993; Prothero 1994). Jacobs and Herendeen (2004) found that at least during the middle Eocene, tropical rain forests did not extend across the whole of tropical Africa but were replaced in some areas with woodland vegetation similar to Miombo woodlands. Thus, the diversification of the Rhinolophidae and Hipposideridae in Africa during the middle Eocene–late Eocene boundary as indicated by Diva analysis and the diversification of the noctilionoids in North or South America around this time (fig. 2) may have resulted from vegetation shifts and concordant changes in food abundance due to the effects of increased aridity and cooler temperatures.

Dispersal appears to have played the major role in shaping the distribution patterns of extant bat families (39 dispersal events vs. three vicariant events required to explain their modern-day distribution; fig. 2). Two intercontinental dispersal events, from Africa to either North America or South America in the early Eocene, followed shortly thereafter by cessation of gene flow explain the presence of the ancestor of the superfamily Noctilionoidea and family Natalidae in the Americas (fig. 2). South America and Africa had split from each other between 100–84 MYA and were separated by the South Atlantic Ocean. North America and South America were separated at the end of the Jurassic, but a reconnection was established in the Late Cretaceous across the proto-Caribbean archipelago, with a connection between South and North America likely present throughout the Eocene (Parrish 1993; McLoughlin 2001; Sanmartin and Ronquist 2004). Given this background, two scenarios could account for dispersal of bats from Africa to South America or North America.

The first scenario would require a direct transatlantic dispersal from Africa to South America. The latest direct connection between South America and Africa is likely to have been in the Central South Atlantic, along the Rio Grande Rise and Walvis Ridge. However, this connection was severed around the mid-Early Cretaceous, and after this time, island hopping across widening water barriers when sea levels were low or rafting would have been the only possible modes of dispersal between these two areas (Parrish 1993). Upon arrival in South America, dispersal to North America could then have occurred via the proto-Caribbean archipelago, which connected North America and South America from 100 to about 49 MYA, by means of island hopping (Sanmartin and Ronquist 2004) or direct flight. New World or Platyrrhine monkeys are thought to have used rafting to reach South America from Africa some time in the late Eocene or early Oligocene (Aiello 1993; Schrago and Russo 2003). Similarly, caviomorph rodents appear to have reached South America from Africa by a transatlantic crossing (George 1993; Wyss et al. 1993), indicating that dispersal between Africa and South America was possible across a large water barrier, even for terrestrial mammals.

The second possible migration route to explain the dispersal of bats from Africa to South America in the Eocene would involve a northwards dispersal to Eurasia across the Tethys sea, entry into North America via Beringia or three

possible transatlantic land bridges, and finally dispersal into South America via the Caribbean archipelago (Janis 1993; Sanmartin, Enghoff, and Ronquist 2001). Intermittent exchange across the Tethys seas has been hypothesized based on similarities in fauna on either side (Prothero 1994); there appears to have been dispersal of some mammals between Europe and Africa during the early Tertiary, extending into the early Eocene (Gheerbrant 1987, 1990), suggesting the presence of some kind of filter or sweepstakes route. Chances of Tethys crossing via a sweepstakes route are thought to have increased throughout the early Tertiary as the African plate rotated northwards (Savage and Russell 1983). The early Eocene was a time of maximal fauna interchange between Europe and North America, suggesting use of a North Atlantic route (Janis 1993; Prothero 1994). North America and Asia were also linked intermittently throughout the Cenozoic by Beringia (Janis 1993; Prothero 1994; Sanmartin, Enghoff, and Ronquist 2001), and terrestrial connections between Europe and North America persisted along various North Atlantic land bridges until at least the early Eocene (50 MYA) (Sanmartin, Enghoff, and Ronquist 2001). During the Eocene, the landmass of Eurasia was largely or completely split down the middle by a combination of the Western Siberian Obik Sea to the north and the Turgai Straits to the South, which is thought to have acted as a barrier to dispersal between Asia and Europe. However, some authors have invoked migration along the coasts and islands of the Tethys seaway to explain faunal and floral exchange between Europe and Asia prior to the Oligocene (e.g., Tiffney 1985). The discovery of a recent euprimate skull in China belonging to the same genus as euprimate skulls found in Belgium dating back to the early Eocene (55 MYA) (Ni et al. 2004) indicates that there was exchange between Europe and Asia during this period. Therefore, once bats had reached Eurasia, dispersal to North America would have been likely. The vicariance events that terminated gene flow between the ancestor of the nycterids-emballonurids-noctilionoids in Africa and South America and similarly the ancestor of the molossids, vespertilionids, miniopterids, and natalids in Africa and the Americas (fig. 2) could therefore either have been the disappearance of stepping-stone islands across the South Atlantic in scenario 1 or disappearance for some reason (e.g., elevated sea level) of the connection between Africa and Europe across the Tethys sea and/or Beringia or the transatlantic land connections in scenario 2. We consider both scenarios presented here equally likely given the poor fossil record of bats and their long-distance dispersal capabilities over both land and water bodies (e.g., Webb and Tidemann 1996; Salgueiro et al. 2004). Further fossil discoveries are therefore essential for differentiating between these two scenarios.

In Africa, diversification from the ancestral emballonurid-nycterid-noctilionoid stock appears to have happened rapidly after vicariance (2 MYA interval), whereas there is about an 8 MYA interval between the time of isolation of the emballonurid-nycterid-noctilionoid ancestor in either North or South America and the diversification of the superfamily Noctilionoidea in the late middle Eocene. The similarity in timing between the dispersal/vicariant events in these two groups indicates that they may have made use

of the same dispersal route and were subject to the same vicariant event terminating gene flow.

The presence of extant Mystacinidae in New Zealand, the only Old World member of the otherwise exclusively New World superfamily Noctilionoidea, requires dispersal of the noctilionoid ancestor to Australia between 44 and 42 MYA, followed by cessation of gene flow between South America or North America and Australia at around 42 MYA. Pleisiomorphic mystacinid fossils have recently been recovered in Australia (Hand et al. 1998), and it is therefore thought that the ancestor of the mystacinids first arrived in Australia and then later reached New Zealand via wind-assisted dispersal (Daniel and Williams 1984). South America and Australia remained connected until the Eocene via Antarctica. South America and Antarctica remained in contact until the Oligocene (30 MYA), after which the opening of the Drake Passage separated the two continents. Antarctica and Australia remained in contact until the late Eocene (35 MYA) even though separation had begun in the Late Cretaceous (90 MYA). However, some authors have proposed that dispersal between Australia and Antarctica would have been unlikely after 50 MYA except by a narrow filter or sweepstakes dispersal route (Woodburne and Case 1996). Thus, a likely dispersal route for the ancestral mystacinid from North America (after dispersal into South America) or South America would have been via Antarctica to Australia between 44 and 42 MYA at which time these three land masses were still connected via a narrow filter or sweepstakes dispersal route.

The long-distance dispersal capability of bats over water is illustrated by the presence of the Hoary bat, *Lasiurus cinereus*, in Hawaii. The distance between Hawaii and the closest continental point, namely southwest San Francisco, is roughly 3,800 km, indicating that *L. cinereus* individuals must have traveled at least this distance to colonize Hawaii. Therefore, distances and barriers that are prohibitive for terrestrial mammal dispersal may be easily surmountable for bats, which perhaps explains the high proportion of dispersal to vicariance events explaining the distribution of the extant bat families.

It should be noted that although many bat families appear to have had their origins in Africa, this does not preclude dispersal and diversification elsewhere. Evaluating the likelihood of these dispersal/vicariant events is dependent on further fossil discoveries, especially in putatively key biogeographical areas such as Africa. The recent discovery of *Tanzanycteris*, a 46-MYA fossil bat from Tanzania, indicates that Africa's bat fossil record may extend further into the past than was previously believed (Gunnell et al. 2003). It is also important to note that the Diva analysis reconstructed the possible biogeographic origins of extant families; fossil bat lineages were not taken into account as their phylogenetic affinities to extant chiropterans are uncertain. Furthermore, the poor fossil record for bats, especially in the Southern Hemisphere, precludes a firm understanding of where bats may have originally originated. Archaic bat lineages dating back to the early Eocene have been found in North America, Europe, Africa, and Australia and appear to have overlapped with modern-day bat clades in both space and time (Hand et al. 1998).

Thus, the origin of the Chiroptera may go further back in time than the late Paleocene as suggested by molecular data and perhaps even into the Late Cretaceous (Pettigrew et al. 1989). The Cretaceous was the era during which angiosperms diversified and became dominant (Crane and Lidgard 1989), resulting in radiation of pollinating insects, including the Lepidoptera and Diptera, both major prey items of bats (Grimaldi 1999). The discovery of a 75-MYA noctuid egg (Gall and Tiffney 1983) provides circumstantial evidence for an older age for the bats. Noctuids are one of the families of moths characterized by hearing organs that supposedly evolved in direct response to bat predation (Yack, Scudder, and Fullard 1999). The existence of a 75-MYA noctuid fossil egg indicates that flying, echolocating, insectivorous bats may have been present in the Late Cretaceous. One of the most common sources of error in the fossil record results from its incompleteness, which necessarily results in a consistent underestimation of any given lineage's age, even when multiple calibration points are used (Near and Sanderson 2004; Reisz and Muller 2004). Given the poor fossil record of chiropterans, an underestimation of the age of this clade using dating dependent on fossil calibration points is not wholly unexpected. The hypothesis of a Cretaceous origin of Chiroptera can only be resolved by discoveries of pre-Paleocene fossil bats.

Supplementary Material

Supplementary figure 1 and supplementary tables 1, 2, and 3 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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