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Phylogenies, fossils and functional genes: the evolution of echolocation in bats

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1.1 Introduction

Bats are one of the most successful orders of mammals on this planet. They account for over 20% of living mammalian diversity (~1200 species), and are distributed throughout the globe, absent only from the extreme latitudes (Simmons, 2005). Bats are the only living mammals that are capable of true self-powered flight, and likewise they are the only mammals capable of sophisticated laryngeal echolocation (Macdonald, 2006). Their global success is largely attributed to these novel adaptations (Jones and Teeling, 2006). Echolocation occurs when a bat emits a brief laryngeal-generated sound that can vary in duration (0.3–300 ms) and in frequency (8–210 kHz) and interprets the returning echoes to perceive its environment (Fenton and Bell, 1981; Thomas *et al.*, 2004). Calls and echoes can be separated either in time or in frequency (Jones, 2005). Some bats (e.g., horseshoe bats, leaf-nosed bats and mustached bats) emit long constant-frequency calls with Doppler shift compensation (CF/DSC) by taking the velocity of their flight into account and adjusting the frequency of their outgoing calls to ensure that the incoming echoes return at a specific frequency (Thomas *et al.*, 2004; Jones, 2005). Most other bats emit low-duty-cycle frequency-modulated calls, and separate outgoing calls and incoming echoes temporally (Thomas *et al.*, 2004; Jones, 2005).

Echolocation calls show a great diversity in shape, duration and amplitude, and are correlated with the parameters of a bat's environment (Jones and Teeling, 2006; Jones and Holderied, 2007). The auditory capabilities of bats are extraordinary. Bats produce and interpret some of the "loudest" naturally produced airborne sounds ever recorded (130 dB; Jones, 2005), and are also capable of hearing some of the "quietest" sounds of any mammal (~-20 dB; Neuweiler, 1990). Despite the magnitude and functionality of this spectacular form of sensory perception, the evolutionary history of echolocation is still controversial.

Evolutionary History of Bats: Fossils, Molecules and Morphology, ed. G. F. Gunnell and N. B. Simmons. Published by Cambridge University Press.

This has stemmed from inconsistent and unresolved phylogenies (Simmons and Geisler, 1998; Van Den Bussche and Hoofer, 2004; Eick *et al.*, 2005; Teeling *et al.*, 2005), and an incomplete (Teeling *et al.*, 2005; Eiting and Gunnell, 2009) and differentially interpreted fossil record (Simmons *et al.*, 2008; Veselka *et al.*, 2010) that allows for alternate interpretations of gain and loss of auditory function, and lack of molecular echolocation signatures (Teeling, 2009).

1.2 Phylogenetic controversies

Traditionally bats were divided into two subordinal groups, Megachiroptera and Microchiroptera (Koopman, 1994; Simmons, 1998; Simmons and Geisler, 1998). Megachiroptera includes the Old World family Pteropodidae, and Microchiroptera contains the remaining 17 bat families (Simmons and Geisler, 1998). This division was based mainly on morphological and paleontological data, but it also highlighted the difference in the dominant mode of sensory perception used by megabats (vision) and microbats (ultrasound). Given that all microbats are capable of sophisticated laryngeal echolocation, whereas megabats are not (Jones, 2005), it was believed that laryngeal echolocation had a single origin in the common ancestor of microbats (Teeling *et al.*, 2000). The 17 families of microbats were subsequently divided into two infraorders Yinochiroptera (rhinolophids, hipposiderids, megadermatids, craseonycterids, rhinopomatids, emballonurids, nycterids) and Yangochiroptera (vespertilionids, molossids, natalids, phyllostomids, noctilionids, furipterids, thyropterids, mormoopids, mystacinids, myzopodids), based on whether their premaxillaries were moveable/absent or fused relative to their maxillaries (Koopman, 1994; Simmons and Geisler, 1998; Hutcheon and Kirsch, 2006). This arrangement was largely supported by morphological data sets (Gunnell and Simmons, 2005) and supertree consensus studies (Jones *et al.*, 2002). However, superfamilial groupings ranged in content and number between studies (Koopman, 1994; Simmons and Geisler, 1998; Jones *et al.*, 2002; Gunnell and Simmons, 2005).

From the advent of modern molecular techniques during the 1980s and 1990s, it became apparent that molecular data did not support the monophyly of Microchiroptera and consequently, did not support a single origin of laryngeal echolocation. Rather, molecular data supported a basal division between Yinpterochiroptera (rhinolophoid microbats and pteropodids) and Yangochiroptera (all other bats; Teeling *et al.*, 2000, 2005; Hutcheon and Kirsch, 2004; Van Den Bussche and Hoofer, 2004; Eick *et al.*, 2005; Miller-Butterworth *et al.*, 2007). This topology suggested that laryngeal echolocation either originated in the ancestor of all bats and was subsequently lost in the common ancestor of megabats, or originated on more than one occasion in

the microbats (Teeling *et al.*, 2000). Initially immunological distance data (Pierson, 1986), single gene data sets (Stanhope *et al.*, 1992; Porter *et al.*, 1996), single-copy DNA–DNA hybridization (Hutcheon *et al.*, 1998), studies of repetitive genomic elements (Baker *et al.*, 1997) and taxonomically limited consensus studies (Liu and Miyamoto, 1999) all supported microbat paraphyly to different degrees (Jones and Teeling, 2006). However, strong support and congruence for the association of the rhinolophoid microbats with the pteropodids was only derived from large concatenated nuclear data sets with representatives from nearly all putative bat families (Eick *et al.*, 2005 – 4 kb, four nuclear introns; Teeling *et al.*, 2005 – 13.7 kb, 18 nuclear exons and UTRs; Miller-Butterworth *et al.*, 2007 – 11 kb, 16 nuclear exons and UTRs) and rare cytogenetic signature events (Ao *et al.*, 2007).

Molecular data in the form of large nuclear and mitochondrial concatenations (Teeling *et al.*, 2000, 2005; Van Den Bussche and Hoofer, 2004; Eick *et al.*, 2005; Miller-Butterworth *et al.*, 2007) provided strong support for the monophyly of four different lineages of echolocating microbat lineages:

(1) Rhinolophoidea (rhinolophids, hipposiderids, rhinopomatids, craseonycterids, megadermatids)
(2) Emballonuroidea (nycterids and emballonurids)
(3) Vespertilionoidea (vespertilionids, molossids, natalids, miniopterids)
(4) Noctilionoidea (noctilionids, phyllostomids, mormoopids, furipterids, thyropterids, mystacinids, myzopodids).

Myzopodidae was recovered with robust support as the sister taxon to other Noctilionoidea (Teeling *et al.*, 2005; Miller-Butterworth *et al.*, 2007), or as the sister taxon to Vespertilionoidea (Eick *et al.*, 2005), albeit with weak support. Two other differences between the exon + UTR tree (Teeling *et al.*, 2005; Miller-Butterworth *et al.*, 2007; Figure 1.1) and the intron tree (Eick *et al.*, 2005; Figure 1.2) are as follows (Figure 1.3):

(1) *Thyroptera* was either the sister group to *Mystacina* (Eick *et al.*, 2005) or grouped in a clade with *Noctilio* and *Furipterus* (Teeling *et al.*, 2005; Miller-Butterworth *et al.*, 2007).
(2) Emballonuroidea and Noctilionoidea were sister taxa (Eick *et al.*, 2005) or Emballonuroidea and Vespertilionoidea were sister taxa (Teeling *et al.*, 2005; Miller-Butterworth *et al.*, 2007).

Finally, the phylogenetic position of Craseonycteridae within Rhinolophoidea was robust based on exons + UTRs (Teeling *et al.*, 2005; Miller-Butterworth *et al.*, 2007), but Craseonycteridae was not included in the Eick *et al.* (2005) data set.

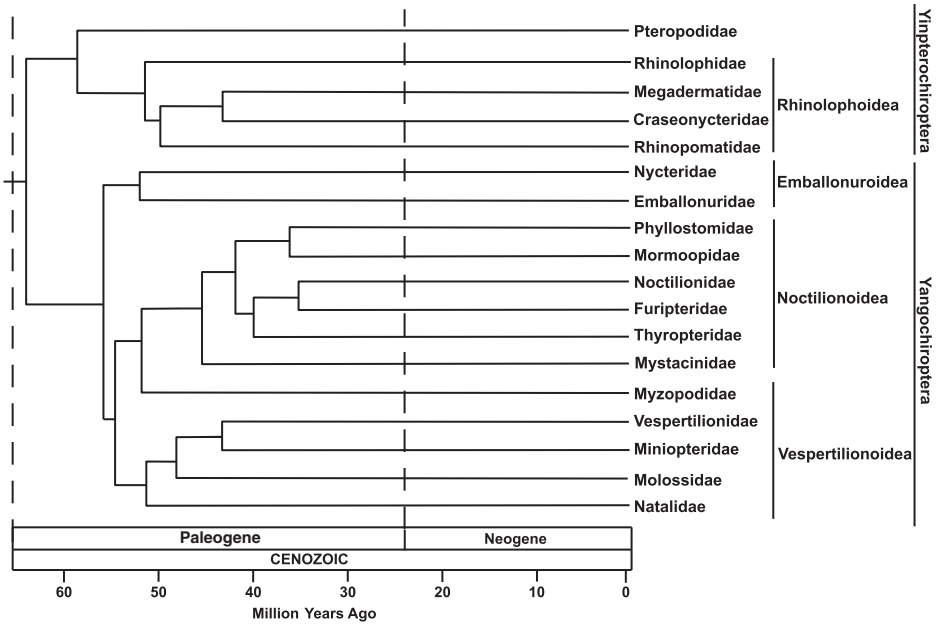

Figure 1.1 Miller-Butterworth *et al.*, 2007 bat phylogeny.

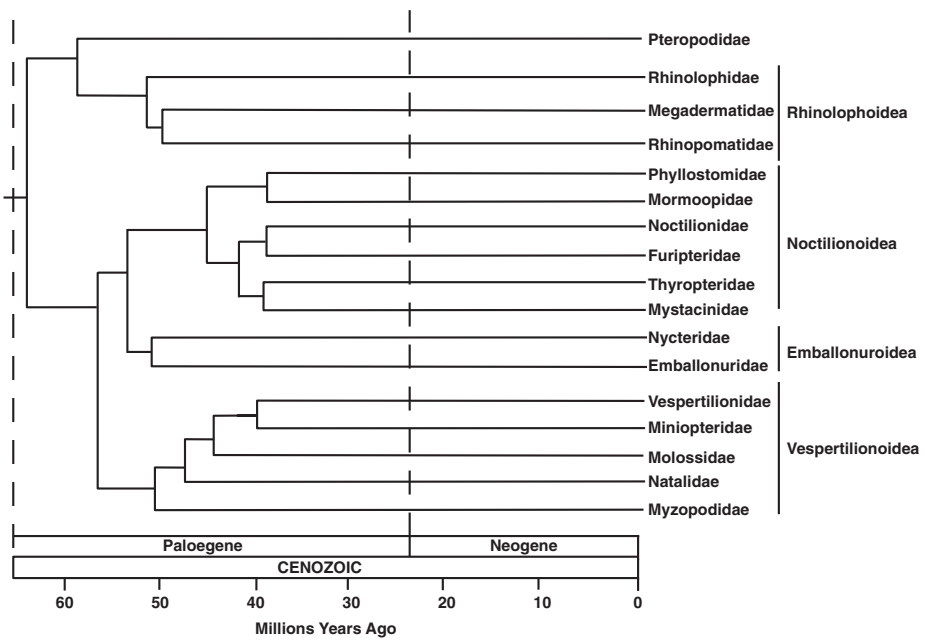

Figure 1.2 Eick *et al.*, 2005 bat phylogeny.

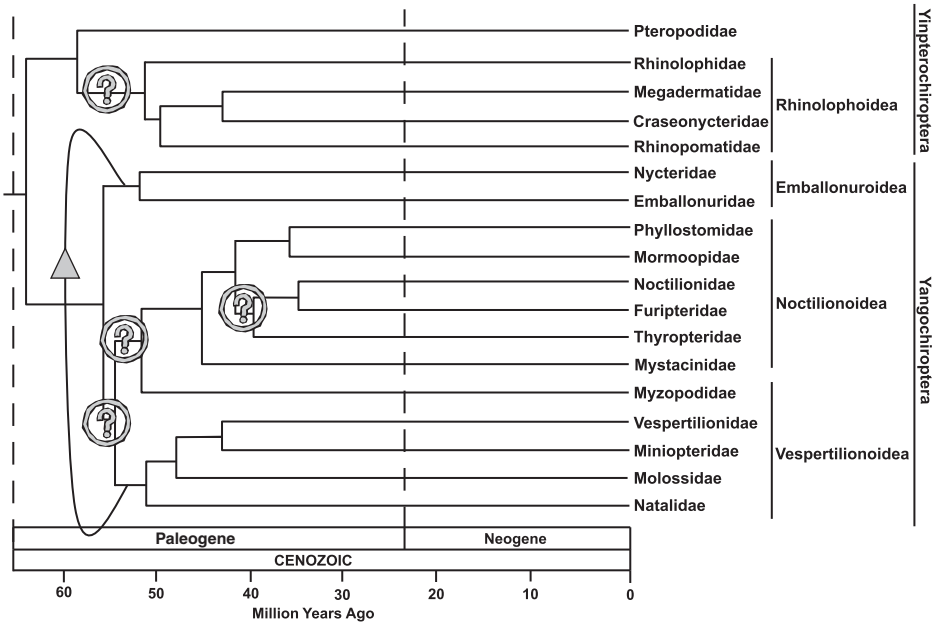

Figure 1.3 Tree depicting the questionable nodes that differ in support and familial representation between Miller-Butterworth *et al.*, 2007 and Eick *et al.*, 2005.

Here, we combine the largest published nuclear data sets for bats (Eick *et al.*, 2005; Teeling *et al.*, 2005; Miller-Butterworth *et al.*, 2007) and reconstruct phylogenetic relationships based on this concatenation. Next, we examine the evolutionary history and molecular basis of echolocation in the context of our phylogenetic results, and discuss recent fossil evidence in light of these findings. We also discuss ongoing molecular investigations into candidate genes that underlie echolocation, and describe how these studies inform the echolocation gain vs. loss debate.

1.3 Phylogenetic analysis

1.3.1 Molecular data sets

We combined nuclear gene sequences from Teeling *et al.* (2005), Miller-Butterworth *et al.* (2007) and Eick *et al.* (2005). Teeling *et al.*'s (2005) data set comprised ~13 kb of nuclear sequence (exons and UTRs) from 18 genes, and included representatives of all bat families except Miniopteridae. Miller-Butterworth *et al.*'s (2007) data set expanded on Teeling *et al.*'s (2005) data set by including two species of *Miniopterus* and an additional vespertilionid, but omitted sequences for the *ADRA2B* and *VWF* genes (these are omitted from subsequent analyses). Eick *et al.*'s (2005) data set consisted of ~4 kb from four

Table 1.1 Chimeric relationships formed between species (based on Eick *et al.*, 2005; Miller-Butterworth *et al.*, 2007).

Miller-Butterworth *et al.*, 2007	*Eick et al.*, 2005
Pteropus rayneri	No suitable taxon found
Cynopterus brachyotis	*Cynopterus sphinx*
Rousettus lanosus	*Rousettus aegytiacus*
Nyctimene albiventer	No suitable taxon found
Rhinolophus creaghi	*Rhinolophus capensis*
Hipposideros commersoni	*Hipposideros commersoni*
Megaderma lyra	*Megaderma lyra*
Macroderma gigas	*Cardioderma cor*
Nycteris grandis	*Nycteris grandis*
Rhinopoma hardwicki	*Rhinopoma hardwicki*
Emballonura atrata	No suitable taxon found
Taphozous nudiventris	*Taphozous mauritianus*
Rhynchonycteris naso	*Peropteryx kappleri*
Tonatia saurophila	No suitable taxon found
Artibeus jamaicensis	*Artibeus jamaicensis*
Desmodus rotundus	*Desmodus rotundus*
Anoura geoffroyi	*Glossophaga soricina*
Noctilio albiventris	*Noctilio albiventris*
Antrozous pallidus	No suitable taxon found
Rhogeesa tumida	*Scotophilus dinganii*
Myotis daubentoni	*Myotis tricolor*
Myzopoda aurita	*Myzopoda aurita*
Pteronotus parnellii	*Pteronotus parnellii*
Thyroptera tricolor	*Thyroptera tricolor*
Mystacina tuberculata	*Mystacina tuberculata*
Furipterus horrens	*Furipterus horrens*
Natalus stramineus	*Natalus micropus*
Tadarida brasiliensis	*Tadarida aegyptiaca*
Eumops auripendulus	*Otomops martiensseni*
Craseonycteris thonglongyai	*Craseonycteris thonglongyai*[1]
Miniopterus schreibersii	*Miniopterus natalensis*
Miniopterus fraterculus	*Miniopterus fraterculus*
Eptesicus fuscus	*Eptesicus hottentotus*

[1] Data generated in this study.

nuclear introns for 17 of the 18 bat families (missing Craseonycteridae). Our concatenation of data from Miller-Butterworth *et al.* (2007) and Eick *et al.* (2005) included several chimeric taxa (Table 1.1). When possible we concatenated the data set at the species level. When the same species was not present in

both data set we concatenated the taxa at the generic level, using published phylogenies to assess intergeneric relationships (Hollar and Springer, 1997; Jones *et al.*, 2002; Baker *et al.*, 2003; Hoofer and Van den Bussche, 2003; Table 1.1). In addition to sequences from the aforementioned studies, we amplified and sequenced the missing intronic fragments for *Craseonycteri*s using primers and PCR amplification conditions described in Eick *et al.* (2005) (GenBank Accession Numbers HQ231220- HQ231221). Our final data set comprised ~14 kb and consisted of exonic sequences from 12 genes (*ADORA2, ADRB2, ATP7A, BDNF, BRCA1, EDG1, PNOC, RAG1, RAG2, TITIN, TYR, ZFX*), UTR sequences from four genes (*APP, BMI1, CREM, PLCB4*) and intronic sequences from four genes (*SPTBN, PRKC1, THY, STAT5A*) for 35 taxa, of which 33 are bats and two are outgroup sequences from the laurasiatherian orders Perissodactyla and Carnivora.

1.3.2 Phylogenetic methods

The concatenated data set was aligned with the program Clustal W (Higgins and Sharp, 1988) and optimized using Se-Al (Rambaut, 1996). Insertion-deletion events were observed among taxa, and gaps were introduced (by Se-Al) to maintain the alignment. All alignment gaps were treated as missing characters in subsequent phylogenetic analyses. Alignment-ambiguous regions were identified by eye and were excluded from phylogenetic analyses. PAUP*4.0 (Swofford, 2002) was used to perform maximum parsimony (MP), maximum likelihood (ML) and minimum evolution (ME) analyses. Modeltest v3.06 (Posada and Crandall, 1998) was used to select the nucleotide substitution model that best fit the data. This was a general time reversible (GTR) model of sequence evolution with a proportion of invariant sites (I) and an allowance for a gamma (Γ) distribution of rates (GTR + I + Γ). Parameter estimates for ML and ME analyses were as follows: Base = (0.2649 0.2456 0.2379), Nst = 6, Rmat = (1.1770 3.9466 0.5501 1.2454 4.3633), Rates = gamma, Shape = 0.7992, Pinvar = 0.2626. MP analyses employed stepwise addition with ten randomized input orders. ME and ML analyses started with neighbor-joining (NJ) trees. All heuristic searches employed tree-bisection and reconnection (TBR) branch-swapping, except for ML bootstrap analyses, which employed nearest- neighbor interchange (NNI) branch-swapping. MP and ME bootstrap analyses were performed with 500 pseudoreplicate data sets; ML bootstraps were carried out with 100 pseudoreplicate data sets.

1.3.3 Phylogenetic results

Figure 1.4 shows the maximum likelihood tree with ML bootstrap percentages for the concatenated data set. The results for all bootstrap analyses are depicted in Table 1.2. The overall topology of the Miller-Butterworth *et al.*

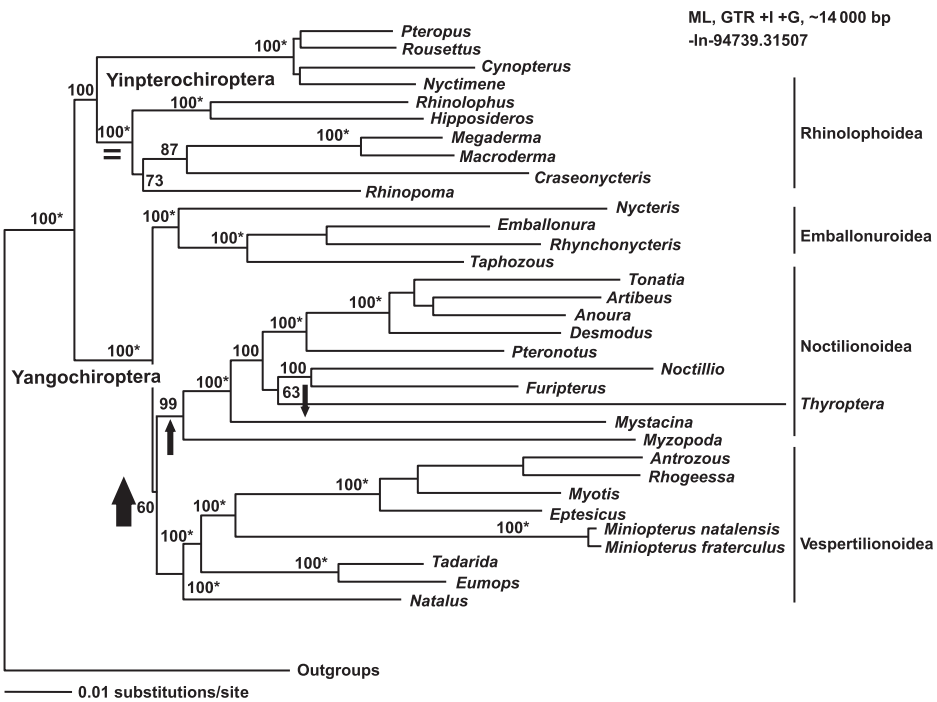

Figure 1.4 Maximum likelihood phylogram with ML bootstrap support on the nodes. Arrows indicate where bootstrap support is increased or decreased on the questionable nodes depicted in Figure 1.3. *Indicates when ML, MP and ME bootstrap analyses agree.

(2007) phylogeny is still supported and the majority of nodes received robust bootstrap support. All of the uncertain relationships depicted in Figure 1.3 were resolved in favor of the Miller-Butterworth *et al.* (2007) tree. *Myzopoda* is the sister group to other noctilionoid families, and Vespertilionoidea and Noctilionoidea are sister taxa, although the branch that groups these two superfamilies together only received moderate bootstrap support (Figure 1.4). The branch uniting the Neotropical families Noctilionidae, Furipteridae and Thyropteridae was also recovered, albeit with lower ML bootstrap support (60% vs. 91%) than in Miller-Butterworth *et al.* (2007). Analyses of the nuclear intronic data set alone do not support this node, nor do phylogenetic analyses of mitochondrial data (Van Den Bussche and Hoofer, 2004). Further phylogenetic investigations will be required to assess the validity of this clade. *Craseonycteris* still groups within Rhinolophoidea and is the sister taxon to Megadermatidae.

Table 1.2 Bootstrap values for the various clades depicted in Figure 1.4. Abbreviations are as follows: ML = maximum likelihood, MP = maximum parsimony, ME = minimum evolution.

	ML	MP	ME
Pteropodidae	100	100	100
Rhinolophidae	100	100	100
Megadermatidae	100	100	100
Megadermatidae + Craseonycteridae	87	100	100
Megadermatidae + Craseonycteridae + Rhinopomatidae	73	83	<50
Rhinolophoidea	100	100	100
Yinpterochiroptera	100	100	<50
Yangochiroptera	100	100	100
Emballonuridae	100	100	100
Nycteridae + Emballonuridae	100	56	100
Phyllostomidae	100	100	100
Phyllostomidae + Mormoopidae	100	100	100
Noctilionidae + Furipteridae	100	70	99
Noctilionidae + Furipteridae + Thyropteridae	63	<50	<50
Noctilionoidea	99	57	54
Noctilionoidea + Emballonuroidea	60	50	79
Vespertilionidae	100	100	100
Vespertilionidae + Miniopteridae	100	100	100
Miniopteridae	100	100	100
Molossidae	100	100	100
Molossidae + Miniopteridae + Vespertilionidae	100	100	100
Vespertilionoidea	100	100	100

1.4 Implications for echolocation

The suborder Yinpterochiroptera, which unites the non-echolocating Pteropodidae and the echolocating superfamily Rhinolophoidea, is still supported. This association was previously supported by independent nuclear data sets based on exon + UTRs (Teeling *et al.*, 2005; Miller-Butterworth *et al.*, 2007) and introns (Eick *et al.*, 2005), and received additional support when analyses were performed on a concatenation that included both data sets (Figure 1.4). This has direct implications for the evolution of echolocation. Two scenarios are depicted in Figure 1.5:

(A) Laryngeal echolocation was gained in the ancestor of all living bats and subsequently lost in the Pteropodidae.
(B) Laryngeal echolocation was gained independently in at least two echolocating lineages (Jones and Teeling, 2006; Teeling, 2009).

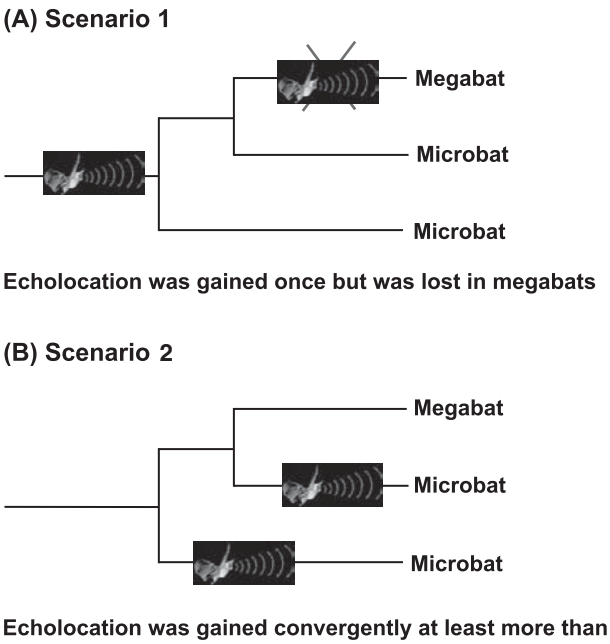

Figure 1.5 Two scenarios depicting the evolution of echolocation given the molecular phylogeny supported.

Both scenarios are equally parsimonious if we only consider the topology for living bats, although echolocation loss in Pteropodidae is more parsimonious if we consider living and fossil bats (Springer *et al.*, 2001; Teeling *et al.*, 2005). Attempts to reconstruct an ancestral type of echolocation call of extant bats given this molecular phylogeny that could indicate a single origin of echolocation failed to draw any conclusive evidence, suggesting that echolocation variation reflected environmental niche adaptation rather than shared ancestry (Jones and Teeling, 2006).

The chronogram of Teeling (2009) suggests that the four major lineages of echolocating bats originated ~58 mya, whereas basal cladogenesis within each group is in the range of 52–50 mya (Teeling *et al.*, 2005; Figure 1.1). The origin of the major lineages is coincident with the Paleocene/Eocene thermal maximum (PETM), where global temperatures increased by ~5°C. The PETM commenced at 55.8 mya and lasted ~170 ky (Woodburne *et al.*, 2009). This global warming event was associated with a significant increase in plant diversity and the peak of Tertiary insect diversity (Teeling *et al.*, 2005). The basal diversification of the echolocating lineages appears to coincide with the late Early Eocene climatic optimum (EECO, 53–50 mya; Woodburne *et al.*, 2009). This climatic episode is associated with a marked global temperature increase,