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Nuclear gene sequences confirm an ancient link between New Zealand's short-tailed bat and South American noctilionoid bats

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Abstract

Molecular and morphological hypotheses disagree on the phylogenetic position of New Zealand's short-tailed bat *Mystacina tuberculata*. Most morphological analyses place *Mystacina* in the superfamily Vespertilionoidea, whereas molecular studies unite *Mystacina* with the Neotropical noctilionoids and imply a shared Gondwanan history. To date, competing hypotheses for the placement of *Mystacina* have not been addressed with a large concatenation of nuclear protein sequences. We investigated this problem using 7.1 kb of nuclear sequence data that included segments from five nuclear protein-coding genes for representatives of 14 bat families and six laurasiatherian outgroups. We employed the Thorne/Kishino method of molecular dating, allowing for simultaneous constraints from the fossil record and varying rates of molecular evolution on different branches on the phylogenetic tree, to estimate basal divergence times within key chiropteran clades. Maximum likelihood, minimum evolution, maximum parsimony, and Bayesian posterior probabilities all provide robust support for the association of *Mystacina* with the South American noctilionoids. The basal divergence within Chiroptera was estimated at 67 mya and the mystacinid/noctilionoid split was calculated at 47 mya. Although the mystacinid lineage is too young to have originated in New Zealand before it split from the other Gondwanan landmasses (80 mya), the exact geographic origin of these lineages is still uncertain and will not be answered until more fossils are found. It is most probable that *Mystacina* dispersed from Australia to New Zealand while other noctilionoid bats either remained in or dispersed to South America.
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1. Introduction

Mystacina tuberculata (Gray, 1843), otherwise known as the New Zealand short-tailed bat, is unique among chiropterans in having a partially terrestrial mode of life. *Mystacina* has enigmatic phylogenetic affinities and has been associated with three of the four recognized bat superfamilies [*sensu* Koopman, 1994): Noctilionoidea (Kennedy et al., 1999; Kirsch et al., 1998; Pierson et al., 1986; Van Den Bussche and Hoofer, 2000); Emballonuroidea (Gray, 1843); and Vespertilionoidea (Koopman, 1994; Smith, 1976; Van Valen, 1979). At the heart of this confusion are spectacular autapomorphies and a limited fossil record (Kirsch et al., 1998).

Found only in New Zealand, *M. tuberculata* is the sole living representative of the family Mystacinidae. The other member of the family, *Mystacina robusta*, is listed as extinct (Hilton-Taylor, 2002). *M. tuberculata* is one of three species of non-marine mammals endemic to New Zealand and exhibits many unique morphological adaptations for arboreal and terrestrial locomotion that are suggestive of protracted in situ evolution: a large claw with a talon on the thumb; the ability to roll, fold and constrict the wing membrane into a wing pocket allowing use of the forelimb in climbing and walking;

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and robust hind legs (Daniel, 1979; Pierson et al., 1986). These peculiar adaptations, along with the capability for flight, allow *M. tuberculata* to exploit a diverse range of ecological niches: catching insects on the wing; gleaning prey from surfaces (insects, small vertebrates, and carrion); feeding on fruit and plants; and burrowing/roosting in fallen trees (Altringham, 1996; Daniel, 1979; Kirsch et al., 1998; Pierson et al., 1986).

Morphological [these datasets also included 12S rDNA restriction site and mtDNA d-loop R-1 tandem repeat characters (Simmons, 2000; Simmons and Geisler, 1998)] and molecular data (Kennedy et al., 1999; Kirsch et al., 1998; Pierson et al., 1986; Van Den Bussche and Hoofer, 2000; Van Den Bussche and Hoofer, 2001) disagree on the phylogenetic position of *Mystacina.* Although all agree that *Mystacina* is a yangochiropteran microbat (Koopman, 1994), the agreement stops here. Analyses of morphological data place *Mystacina* either in its own superfamily as the sister-group to a clade containing Molossoidea, Nataloidea and Vespertilionoidea (Simmons and Geisler, 1998; Table 1); within the superfamily Vespertilionoidea (Koopman, 1994; Van Valen, 1979); or as sister-group to the Molossidae within the superfamily Vespertilionoidea (Novacek, 1991; Smith, 1976).

Molecular data have suggested entirely different hypotheses. All molecular data, including transferrin immunological distances (Pierson, 1986), single copy DNA–DNA hybridization distances (Kirsch et al., 1998), cytochrome *b* sequences (Kennedy et al., 1999), and mitochondrial RNA gene sequences (Van Den Bussche and Hoofer, 2000; Van Den Bussche and Hoofer, 2001), have concluded that *Mystacina* is a member of the Neotropical superfamily Noctilionoidea and is not associated with molossoids, nataloids, or vespertilionoids. Although previous molecular studies agree that *Mystacina* is a member of the superfamily Noctilionoidea, which also includes Noctilionidae, Mormoopidae, and Phyllostomidae, they do not agree on the branching pattern within this superfamily. Pierson et al. (1986) supported a sister-group relationship between Noctilionidae and Mystacindae; Kirsch et al. (1998) suggested a basal position for Mystacindae within the Noctilionoidea; Kennedy et al. (1999) associated *Mystacina*, both with *Noctillio* and *Pteronotus* (Mormoopidae) and with *Mormoops* (Mormoopidae) depending on analyses; Van Den Bussche and Hoofer (2000, 2001) and Van Den Bussche et al. (2002) reported a basal position for Noctilionidae within the Noctilionoidea (including *Mystacina*; Fig. 1). The supertree

Table 1
Bat classifications incorporated in this paper

Simmons and Geisler (1998)	New classification expanded from Teeling et al. (2002)
Order Chiroptera	Order Chiroptera
Suborder Megachiroptera	Suborder Yinpterochiroptera
Family Pteropodidae (*Pteropus*, *Cynopterus*, *Nyctimene*, and *Rousettus*)	Superfamily Pteropodoidea
Suborder Microchiroptera	Family Pteropodidae (*Pteropus*, *Cynopterus*, *Nyctimene*, and *Rousettus*)
Superfamily Emballonuridae	Superfamily Rhinolophoidea
Family Emballonuridae (*Emballonura*, *Taphozous*)	Family Rhinopomatidae (*Rhinopoma*)
Infraorder Yinochiroptera	Family Megadermatidae (*Megaderma*)
Superfamily Rhinopomatoidea	Family Rhinolophidae
Family Rhinopomatidae (*Rhinopoma*)	Subfamily Rhinolophinae (*Rhinolophus*)
Superfamily Rhinolophoidea	Subfamily Hipposiderinae (*Hipposideros*)
Family Nycteridae (*Nycteris*)	Suborder Yangochiroptera
Family Megadermatidae (*Megaderma*)	Family Nycteridae (*Nycteris*) *incertae sedis*
Family Rhinolophidae	Superfamily Emballonuroidea
Subfamily Hipposiderinae (*Hipposideros*)	Family Emballonuridae (*Emballonura*, *Taphozous*)
Subfamily Rhinolophinae (*Rhinolophus*)	Superfamily Noctilionoidea
Infraorder Yangochiroptera	Family Mystacinidae (*Mystacina*)
Family Mystacinidae (*Mystacina*)	Family Mormoopidae (*Pteronotus*)
Superfamily Noctilionoidea	Family Noctilionidae (*Noctilio*)
Family Noctilionidae (*Noctilio*)	Family Phyllostomidae (*Tonatia*, *Desmodus*)
Family Phyllostomidae (*Tonatia*, *Desmodus*)	Superfamily Vespertilionoidea
Family Mormoopidae (*Pteronotus*)	Family Natalidae (*Natalus*)
Superfamily Nataloidea	Family Vespertilionidae (*Antrozous*, *Rhogeessa*, and *Myotis*)
Family Natalidae (*Natalus*)	Family Molossidae (*Tadarida*)
Superfamily Molossoidea	
Family Antrozoidae (*Antrozous*)	
Family Molossidae (*Tadarida*)	
Superfamily Vespertilionoidea	
Family Vespertilionidae (*Rhogeessa*, *Myotis*)	

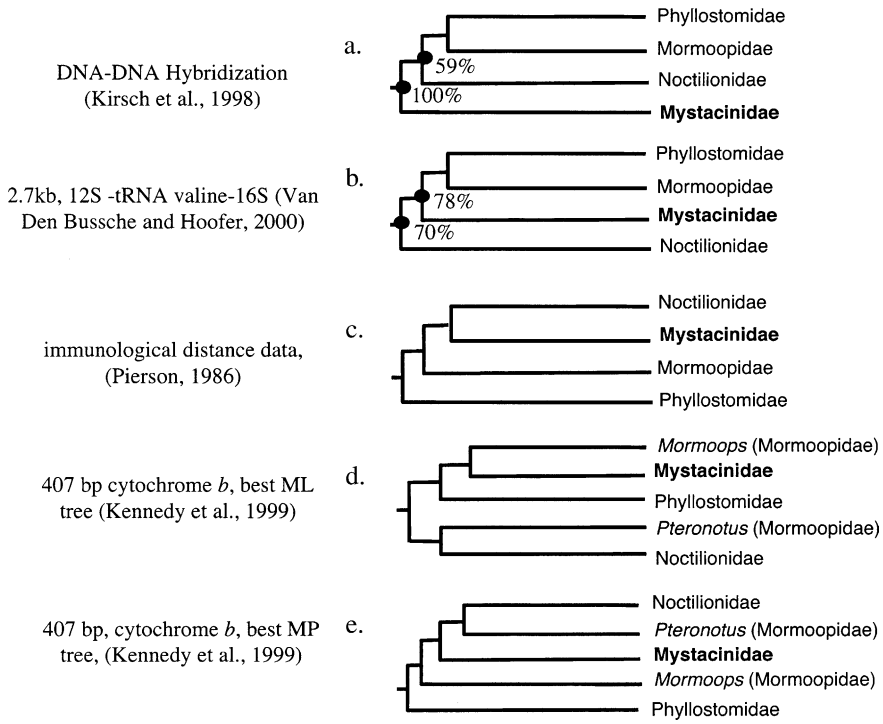

Fig. 1. Five molecular topologies that differ in their placement of Mystacinidae within the superfamily Noctilionoidea (using the families represented in this paper): (a) DNA–DNA hybridization data based on FITCH analyses, showing % bootstrap support (Kirsch et al., 1998); (b) 2.7 kb, 12S rRNA + tRNA-valine + 16S rRNA mitochondrial sequence data, MP analyses, showing % bootstrap support (Van Den Bussche and Hoofer, 2000); (c) transferrin albumin immunological distance data, pg. 199 (Pierson, 1986); (d) 407 bp, cytochrome *b* mitochondrial sequence data, ML tree of Kennedy et al. (1999); (e) 407 bp cytochrome *b* mitochondrial sequence data, MP tree, codon weightings 5:61:1 of Kennedy et al. (1999).

analyses of Jones et al. (2002), based on 105 topologies of bat phylogenetic relationships published since 1970 (both molecular and morphological), support a basal position for *Mystacina* within the superfamily Noctilionoidea.

The association between Old World mystacinids and New World noctilionoids raises important questions pertaining to the role of vicariance and dispersal in the historical biogeography of this group (Kirsch et al., 1998). Was the common ancestor of the mystacinid/noctilionoid clade present in New Zealand prior to its vicariant sundering from other Gondwanan landmasses at approximately 80 million years ago (mya)? If not, was the common ancestor of the mystacinid/noctilionoid clade present in South America, Antarctica, and Australia before the final separation of Australia from Antarctica? Veevers (1991) date the final separation of Australia from Antarctica at 35 mya, which coincides with the opening of the Drake Passage, establishment of circumpolar oceanic circulation, and Pierson's (1986) 35 mya estimate for the separation of mystacinids and noctilionoids. However, Woodburne and Case (1996) provide a more synthetic overview that incorporates both geophysical and paleoclimatic data and argue that overland dispersal between Antarctica and Australia had become improbable by 64 mya, with decreasing opportunities for dispersal after this time. Kirsch et al. (1998) estimated the noctilionoid/mystacinid split between 44 and 66 mya, favoring a separation date of 54 mya. This is well before the formation of the Drake passage, but after Woodburne and Case's (1996) estimate for the vicariant separation of Australia from Antarctica.

Given controversies pertaining to the phylogenetic placement and biogeographic history of *Mystacina*, we sequenced portions of five nuclear genes for *M. tuberculata* and *Pternontus parnelli* (Mormoopidae). These sequences were combined with those of Teeling et al. (2002), yielding a dataset comprising 7.1 kb for 22 bats and six laurasiatherian outgroups. This dataset is the first based on a large concatenation of nuclear gene sequences to examine the phylogenetic affinities of *Mystacina*. Our results agree with previous molecular studies that support an association of *M. tuberculata* with the noctilionoid families Noctilionidae, Mormoopidae, and Phyllostomidae. Further, we estimate that *Mystacina* separated from other noctilionoids approximately 47 mya, suggesting that stem mystacinids dispersed to New Zealand after this island had separated from other Gondwanan landmasses.

2. Materials and methods

2.1. Taxa and sequences

New protein-coding sequences were obtained for fragments of *ADRA2B* (α-2B adrenergic receptor gene, 1.3 kb), *BRCA1* (breast cancer susceptibility gene, 2.8 kb of exon 11), *RAG1* (recombination activating gene 1, 1.1 kb), *RAG2* (recombination activating gene 2, 0.8 kb), and *VWF* (von Willebrand factor gene, 1.2 kb of exon 28) for *M. tuberculata* and *P. parnelli* as previously described (Madsen et al., 2001; Teeling et al., 2000). Additional sequences are from Teeling et al. (2002). Our dataset included four pteropodids (*Cynopterus*, *Nyctimene*, *Pteropus*, and *Rousettus*), one rhinopomatid (*Rhinopoma*), two rhinolophids (*Rhinolopus*, *Hipposideros*), one megadermatid (*Megaderma*), two nycterids (*Nycteris*), one molossid (*Tadarida*), one natalid (*Natalus*), two vespertilionids (*Myotis*, *Rhogeessa*), one antrozoid (*Antrozous*), two phyllostomids (*Desmodus*, *Tonatia*), one mystacinid (*Mystacina*), and one mormoopid (*Pteronotus*) (families sensu Simmons and Geisler, 1998, Table 1). Outgroup taxa included six laurasiatherians (Waddell et al., 1999). The nuclear genes were aligned with CLUSTAL W (Thompson et al., 1994) and the EYEBALL sequence editor (Cabot and Beckenbach, 1989). Due to alignment ambiguities, repeat regions in *BRCA1* and *ADRA2B*, were removed prior to phylogenetic analysis. GenBank accession numbers for all taxa in this dataset are given in Table 2.

2.2. Phylogenetic analyses

Using de Queiroz's (1993) method for evaluating dataset incongruence with a bootstrap support/conflict criterion of 90%, we deemed it appropriate to concatenate all gene fragments as there were no conflicting nodes. Phylogenetic analyses were performed on the concatenated dataset (~7.1 kb). Maximum likelihood (ML), minimum evolution (ME), and maximum parsimony (MP) analyses were performed with PAUP 4.0b (Swofford, 1998). Bayesian analyses were carried out using MrBayes 2.01 (Hulsenbeck and Ronquist, 2001). ML analyses were performed using the GTR (general time reversible) + Γ (gamma distribution of rates) + I (proportion of invariant sites) model of sequence evolution, as was suggested by Modeltest (Posada and Crandall, 1998), with the following parameters settings: R-Matrix = (1.0531, 4.1015, 0.6338, 1.1118, 4.7496, 1.0000); base frequencies = (0.2806, 0.2460, 0.2533, 0.2199); proportion of invariant sites = 0.129; and shape parameter of Γ distribution = 0.9408. In all ML analyses, starting trees were obtained by neighbor-joining. Due to computational demands, the following taxonomic constraint was used in ML bootstrap analyses using tree-bisection and reconnection branch swapping: ((*Tonatia*, *Desmodus*), (*Myotis*, *Antrozous*, *Rhogeessa*), (*Hipposideros*, *Rhinolophus*), (*Nycteris thebaica*, *Nycteris grandis*), (*Cynopterus*, *Pteropus*, *Nyctimene*, *Rousettus*), (*Taphozous*, *Emballonura*), *Tadarida*, *Megaderma*, *Rhinopoma*, *Natalus*, *Noctilio*, *Mystacina*, *Pteronotus*). ML bootstrap analyses were also performed without this molecular constraint using nearest-neighbor interchange branch swapping.

All constrained clades received 100% support from MP and ME analyses and are well supported by other studies (Simmons, 2000; Teeling et al., 2002). ME analyses were performed with: (i) ML distances corrected according to the GTR + Γ + I model of evolution and (ii) logdet distances (Lockhart et al., 1994). Starting trees were obtained using neighbor-joining. In MP analyses, we used stepwise addition with 10 randomized input orders. Nucleotide positions were unweighted and gaps were coded as missing data. Bootstrap analyses included 100 replicates with ML and 500 replicates with ME and MP. We used heuristic searches with tree-bisection and reconnection branch swapping (TBR) in all analyses except unconstrained ML bootstrap replicates.

Bayesian posterior probabilities were calculated using a Metropolis-coupled, Markov chain, Monte Carlo (MCMCMC) sampling approach implemented in MrBayes 2.01 (Hulsenbeck and Ronquist, 2001). Bayesian analyses used the GTR + Γ + I model of sequence evolution. Starting trees were random, phylogenetic constraints were not used, four simultaneous Markov chains were run for 300,000 generations, burn-in values were set at 40,000 generations (based on empirical values of stabilizing likelihoods), and trees were sampled every 20 generations.

2.3. Statistical testing

We used the Kishino and Hasegawa (1989) test, with RELL optimization and 1000 bootstrap replicates, to examine the statistical significance of five a priori hypotheses regarding the phylogenetic position of *Mystacina*: (1) *Mystacina* is included in the superfamily Vespertilionoidea (Van Valen, 1979); (2) *Mystacina* is a sister-group to the Molossidae (Novacek, 1991); (3) Mystacinidae + Mormoopidae + Phyllostomidae form a monophyletic clade, rendering Noctilionidae basal within the superfamily Noctilionoidea (Van Den Bussche and Hoofer, 2000); (4) *Mystacina* is basal within Noctilionoidea (Kirsch et al., 1998); and (5) *Noctilio* and *Mystacina* are sister-taxa (Pierson, 1986).

2.4. Molecular dating

The programs *estbranches* and *divtime5b* were used to estimate divergence times following the Bayesian methods of Thorne et al. (1998) and Kishino et al. (2001).

Table 2
GenBank accession numbers for sequences included in this study

	ADRA2B	*BRCA1*	*RAG1*	*RAG2*	*VWF*
Order Chiroptera					
Cynopterus sphinx	AJ251181	AF203750	AF203758	AF203768	U31605
Pteropus rayneri	AF337539	AF203751	AF203759	AF203769	
Pteropus hypomelanus					AF203777
Rousettus amplexicaudatus		AF447500	AF447512	AF447529	AF447547
Nyctimene albiventer	AF447549	AF447502	AF447514	AF447530	AF447549
Rhinolophus creaghi	AJ419806	AF447499	AF447511	AF447528	AF447546
Hipposideros commersoni	AF337538	AF203752	AF203760	AF203770	AF203778
Megaderma lyra	AF337537	AF203749	AF203757	AF203767	U31616
Nycteris grandis	AJ419807	AF447494	AF447506	AF447523	AF447541
Nycteris thebaica	AJ419808	AF447501	AF447513	AF447530	AF447548
Rhinopoma hardwickei	AJ419809	AF447504	AF447518	AF447535	AF447551
Emballonura atrata	AJ419810	AF447505	AF447519	AF447536	AF203776
Taphozous nudiventris	AF337543	AF203748	AF203756	AF203766	AF447540
Tonatia bidens	AF337541	AF203745	AF203753	AF203763	U31622
Desmodus rotundus	AJ419811	AF447503	AF447517	AF447534	AF447550
Noctilio albiventris	AJ419812	AF447497	AF447509	AF447526	AF447544
Antrozous pallidus	AJ419813	AF447495	AF447507	AF447524	AF447542
Rhogeessa tumida	AJ419814	AF447496	AF447508	AF447525	AF447543
Myotis daubentoni	AF337540	AF203746	AF203754	AF203764	
Myotis velifer					AF203775
Natalus stramineus	AJ419815	AF447498	AF447510	AF447527	AF447545
Tadarida brasiliensis	AF337542	AF203747	AF203755	AF203765	AF061061
Mystacina tuberculata	AY245423	AY245829	AY245419	AY245417	AY245421
Pteronotus parnelli	AY245422	AY245828	AY245418	AY245416	AY245420
Order Carnivora					
Felis catus	AJ251174	AF284018	AF203761	AF203771	U31613
Order Cetartiodactyla					
Bos taurus	Y15944	AF284013	AF447520	AF447537	AF004285
Megaptera novaeangliae		AF284017	AF447522	AF447539	AF226849
Balaenoptera physalus	AJ251175				
Order Insectivora					
Talpa europaea	Y12520		AF447515	AF447532	
Scalopus aquaticus		AF284007			AF076479
Order Perissodactyla					
Equus asinus					U31610
Equus caballus	Y15945	AF284010	AF447516	AF447533	
Order Pholidota					
Manis sp.	AJ251185	AF284009	AF447521	AF447538	U97535

This approach relaxes the molecular clock assumption, permits the incorporation of multiple constraints from the fossil record, and provides estimates of posterior probabilities of divergence times. Branch lengths were estimated with the *estbranches* program of Thorne et al. (1998) for the complete dataset in conjunction with the ML topology shown in Fig. 2. Thorne et al.'s (1998) program permits a single outgroup. We used mole as the outgroup based on previous studies that suggest a sister-group relationship between eulipotyphlan insectivores and other laurasiatherians (Murphy et al., 2001; Waddell et al., 1999). We used the F84 (Felsenstein, 1984) model of sequence evolution with an allowance for a gamma (Γ) distribution of rates with four discrete rate categories. The transition/transversion parameter and estimates of the rate categories of the Γ distribution were estimated with PAUP 4.0b (Swofford, 1998). Divergence times were estimated using the program *divtime5b* (Kishino et al., 2001; Thorne et al., 1998). Markov chain Monte Carlo analyses were run for 1 million generations after a burn-in of 100,000 generations to allow Markov chains to approach stationarity before states were sampled; chains were sampled every 100 generations. Application of the *divtime5b* program requires a value for the mean of the prior distribution for the time separating the ingroup root from the present (rttm). We used a conservative estimate of 65 mya following a strict interpretation of the Explosive model of placental diversification, which places the placental root at or near the K/T boundary (Archibald and

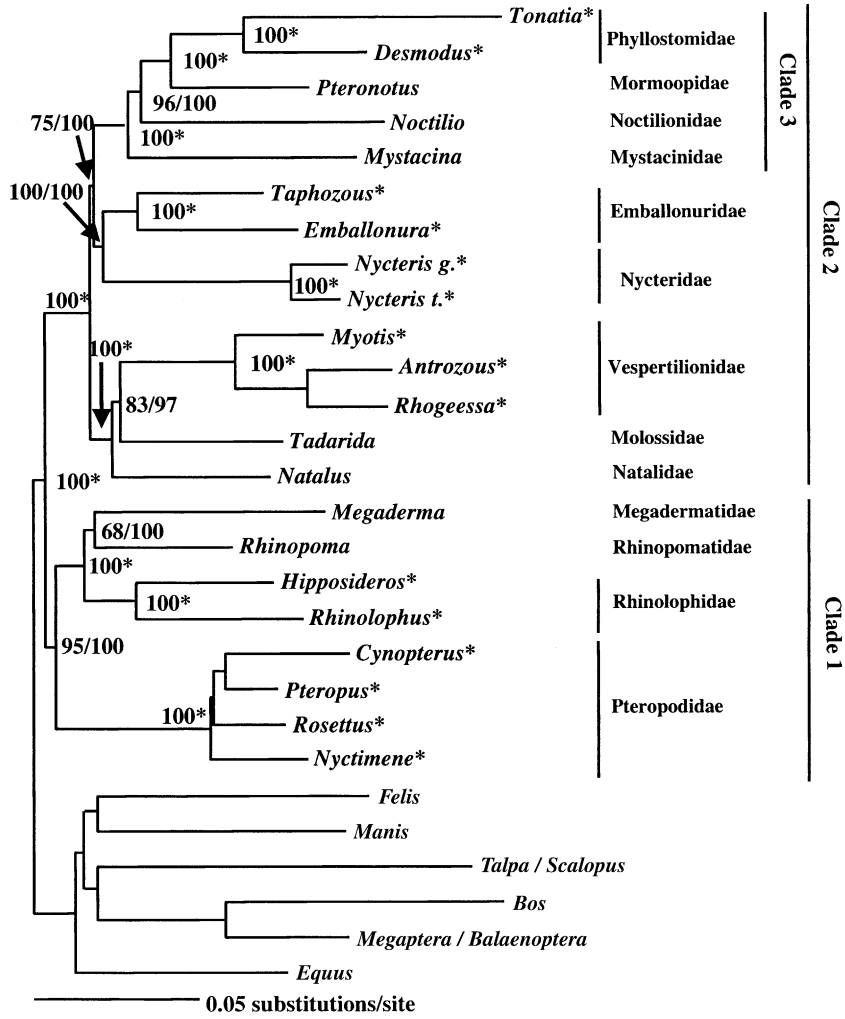

Fig. 2. The maximum likelihood tree (−ln likelihood = 57211.96) for the concatenated dataset under the GTR + Γ + I model of sequence evolution. Asterisks indicate taxa that were constrained in ML bootstrap analyses which received 100% bootstrap support in unconstrained MP and ME analyses. 100* = clades that received 100% bootstrap support with all analyses and had a posterior probability of 1.000. Numbers at the nodes are the ML bootstrap values (left of the slash); posterior probabilities are shown as percentages (right of the slash).

Deutschman, 2001). Other settings for running *divtime5b* were as follows: starttype = 0; standard deviation of rttm (rttmsd) = 0.5 × rttm; rtrate = *X*/rttm, where rtrate is the mean of prior distribution for the rate at the root node and *X* is the median amount of evolution from the ingroup root to the ingroup tips; rtratesd = 0.5 × rtrate, where rtratesd is the standard deviation of rtrate; rttm × brownmean = 1, where brownmean is the mean of the prior distribution for the autocorrelation parameter (v); and brownsd = brownmean, where brownsd is the standard deviation of the prior distribution for v.

The *divtime5b* program allows for both minimum and maximum fossil constraints. Whereas minima are often based on earliest occurrences in the fossil record, maxima are intrinsically more difficult to estimate and incorporate allowances for unobserved segments of taxon ranges (Strauss and Sadler, 1989). Nevertheless, incorporating at least one of each of these kinds of constraints is important for obtaining reasonably narrow posterior distributions for divergence times (Kishino et al., 2001; Thorne and Kishino, 2002). We used the following seven constraints (four minima and three maxima):

(a) A minimum of 34 mya and a maximum of 55 mya for the split between *Megaderma* and *Rhinopoma*. The minimum is based on megadermatid fossils from the late Eocene (McKenna and Bell, 1997). Given that *Megaderma* and *Rhinopoma* are sister-taxa in our analyses, and that there are no fossils belonging to either family from the middle or early Eocene, we constrained the maximum age for this split at the Paleocene/Eocene boundary (i.e., ~55 mya).

(b) A minimum of 37 mya and a maximum of 55 mya for the separation of hipposiderids and rhinolophids, both of which have been reported from the middle Eocene of Europe (McKenna and Bell, 1997; Simmons and Geisler, 1998). Given that *Hipposideros* and *Rhinolophus*

are sister-taxa in our analyses, and that there are no early Eocene hipposiderid or rhinolophid fossils, we constrained the maximum age for this split at the Paleocene/Eocene boundary (i.e., ~55 mya).

(c) A minimum of 63 mya for the carnivore-pangolin split based on the occurrence of carnivores from the early Paleocene (McKenna and Bell, 1997).

(d) A minimum of 53 mya and a maximum of 65 mya for the bovine-cetacean split. The minimum is based on the earliest cetacean fossil (*Himalayacetus*; Bajpai and Gingerich, 1998); the maximum allows that this split may have occurred as early as the K/T boundary (e.g., see Gatesy and O'Leary, 2001).

3. Results

3.1. Phylogenetic analyses

Similar to the topology reported in Teeling et al. (2002), the concatenated dataset provided robust support for a basal split between two groups of bats: Yinpterochiroptera (Clade 1, Fig. 2; Table 3; 67–98% bootstrap support; posterior probability of 1.000); and Yangochiroptera (Clade 2, Fig. 2; Table 3; 100% bootstrap support with all methods; posterior probability of 1.000).

Both *Mystacina* and *Pteronotus* grouped within Yangochiroptera (Clade 2, Fig. 2). The concatenated dataset provided strong support for the association of *Mystacina* and *Pteronotus* within the Noctilionoidea (Clade 3, Fig. 2; Table 3; 100% bootstrap support with all methods; posterior probability of 1.000). *Pteronotus* grouped with the phyllostomids with 100% bootstrap support in all analyses (Fig. 2, Table 3; posterior probability of 1.000). With ML, ME, and Bayesian analyses, Noctilionidae + Mormoopidae + Phyllostomidae formed a monophyletic group rendering *Mystacina* basal within Noctilionoidea (Fig. 2; 96–98% bootstrap support; posterior probability of 1.000). However, this branching pattern received no bootstrap support with MP. Rather, MP analyses produced weak support for a sister-group relationship between *Noctilio* and *Mystacina* (64% bootstrap support; Table 3). This sister-group relationship between *Noctilio* and *Mystacina* received little to no support with ML and ME analyses (Table 3; >3% bootstrap support; posterior probability of 0.000). All analyses rejected the monophyly of Mystacinidae + Mormoopidae + Phyllostomidae and therefore rejected a basal position for Noctilionidae (Table 3; 0% bootstrap support with all methods; posterior probability of 0.000). *Mystacina* did not group with the superfamily Vespertilionoidea, nor with the Molossidae (Table 3; 0% bootstrap support with all methods; posterior probability of 0.000).

The association of the emballonurids with the nycterids received 69–100% bootstrap support and had a posterior probability of 1.000 (Fig. 2, Table 3). A sister-group relationship between the emballonurid/nycterid clade and the superfamily Noctilionoidea + *Mystacina* was supported by 58–75% bootstrap values and a posterior probability of 1.000 (Fig. 2, Table 3). Vespertilionids, molossids, and natalids formed a monophyletic clade in all analyses (Fig. 2; Table 3; 100% bootstrapsupport with all methods; posterior probability 1.000).

Table 3
Bootstrap support and posterior probabilities for selected phylogenetic hypotheses

	Bootstrap support percentages				Bayesian posterior probabillities
	MP	ME-ML (GTR + Γ + I)	ME-ML (logdet)	ML (GTR + Γ + I) with constraints/ without constraints	
Yinpterochiroptera (Clade 1, Fig. 2)	70	67	68	95/98	1.000
Yangochiroptera (Clade 2, Fig. 2)	100	100	100	100/100	1.000
Mystacina + Noctilionoidea, (Clade 3, Fig. 2)	100	100	100	100/100	1.000
Basal position for *Mystacina* within the Noctilionoidea	0	98	97	96/96	1.000
Mystacina + *Noctilio*	64	2	3	2/2	0.000
Basal position for *Noctilio* within the Noctilionoidea	0	0	0	0/0	0.000
Mystacina + Vespertilionoidea	0	0	0	0/0	0.000
Mystacina + Molossidae	0	0	0	0/0	0.000
Emballonuridae + Nycteridae	74	72	76	100/100	1.000
Emballonuridae + Nycteridae + *Mystacina* + Noctilionoidea	58	89	91	75/69	1.000
Vespertilionids + molossids + natalids	98	100	100	100/100	1.000

ME-ML, minimum evolution with ML-corrected distances; GTR, general time reversible model; Γ, gamma distribution of rates; I, allowance for invariant sites. All analyses were performed on the concatenated dataset that included fragments of *ADRA2B*, *BRCA1*, *RAG1*, *RAG2*, and *VWF* for all taxa.

3.2. Statistical tests

Kishino–Hasegawa (ML) tests were used to make pairwise comparisons between competing phylogenetic hypotheses for the placement of *Mystacina*. A basal position for Mystacinidae within the superfamily Noctilionoidea was significantly better than inclusion of *Mystacina* in the Vespertilionoidea ($P < 0.005$; Table 4), a sister-group relationship between the Mystacinidae and the Molossidae ($P < 0.0001$, Table 4), and the monophyly of Mystacinidae + Mormoopidae + Phyllostomidae, rendering Noctilionidae basal within the superfamily Noctilionoidae ($P = 0.049$, Table 4). Kishino–Hasegawa tests did not discriminate between a basal placement for *Mystacina* (within Noctilionoidea) versus *Mystacina* + *Noctilio* ($P = 0.067$, Table 4).

Table 4
Results of the Kishino–Hasegawa (ML) tests comparing various a priori phylogenetic hypotheses

Phylogenetic hypotheses	Log likelihood score	*P* Values				
		Versus Kirsch	Versus Van Valen	Versus Novacek	Versus Van Den Bussche	Versus Pierson
Basal position for Mystacainidae within the Noctilionoidea (best tree, Kirsch et al., 1998)	57211.96	—	<0.001*	<0.001*	0.049*	0.067
Mystacina + Vespertilionoidea (Van Valen, 1979)	57317.82		—	0.055	<0.001*	<0.001*
Mystacina + *Tadarida* (molossid) (Novacek, 1991)	57350.34			—	<0.001*	<0.001*
Basal position for *Noctilio* within Noctilionoidea (Van Den Bussche and Hoofer, 2000)	57224.43				—	7.04
Sistergroup relationship between *Noctilio* + *Mystacina* (Pierson et al., 1986)	57223.68					—

* Asterisks indicate hypotheses that were rejected at $P = 0.05$ in pairwise comparisions.

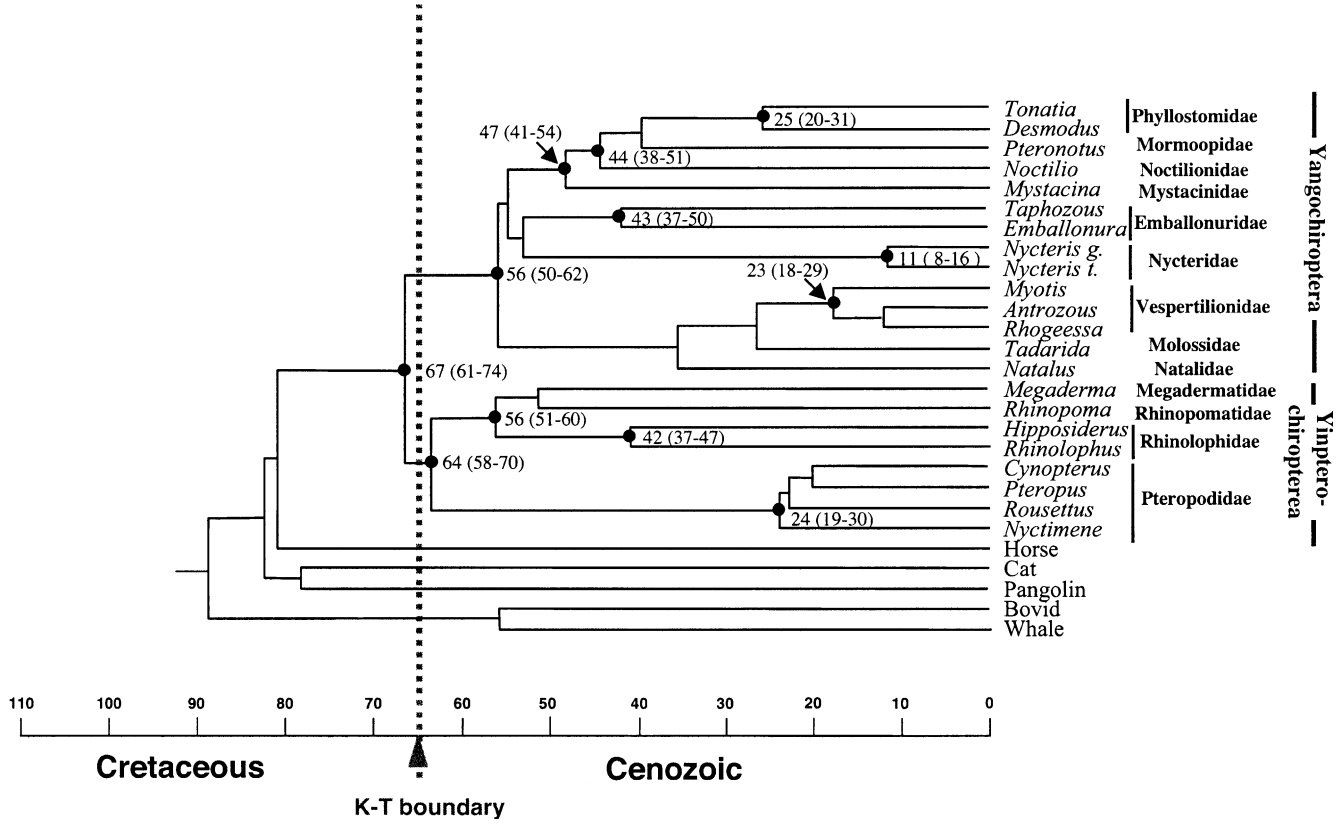

Fig. 3. Molecular timescale for the order Chiroptera based on the 7.1 kb dataset and ML tree shown in Fig. 2, with mole as the outgroup, seven fossil constraints (Methods), and a mean prior of 65 mya for the placental root. Values at nodes are in millions of years; values in brackets are 95% confidence intervals.

3.3. Molecular dating

Molecular divergence estimates obtained with the Thorne et al. (1998)/Kishino et al. (2001) approach estimate the split of Chiroptera from other laurasiatherian orders at 81 mya. Within Chiroptera, the deepest split is between Yinpterochiroptera (Fig. 3) and Yangochiroptera (Fig. 3) at 67 mya. Within Yinpterochiroptera, the lineages leading to the Pteropodidae and Rhinolophoidea diverged at 64 mya (Fig. 3). Extant crown group pteropodids originated at 24 mya while basal splits within the superfamily Rhinolophoidea are estimated at 56 mya (Fig. 3). Interfamilial splits within Yangochiroptera range from 39 to 56 mya. Noctilionoids, inclusive of *Mystacina*, diverged from other yangochiropterans at 47 mya (Fig. 3). *Mystacina* diverged from the South American noctilionoids (Noctilionidae, Mormoopidae, and Phyllostomidae) at 44 mya. Extant crown group emballonurids, phyllostomids, nycterids, vespertilionids originated 43, 26, 11, 23 mya, respectively (Fig. 3).

4. Discussion

4.1. Phylogenetic affinities

Pierson et al. (1986) were the first to address the phylogenetic position of *Mystacina* using molecular data (transferrin immunological distance data) and reported a sister-group association between the New Zealand mystacinids and the New World noctilionids (fisherman bats; Pierson, 1986; Pierson et al., 1986). This result contradicted previous morphological analyses that associated *Mystacina* with the superfamily Vespertilionoidea (Koopman, 1994; Smith, 1976; Van Valen, 1979) and in the process implied an ancient biogeographic link between the bats of New Zealand and those of South America.

Kirsch et al. (1998) re-visited the *Noctilio*/*Mystacina* association using single copy DNA–DNA hybridization. They compared 15 different bat families and also found strong support (99% bootstrap support) for the inclusion of *Mystacina* within the superfamily Noctilionoidea (Noctilionidae, Phyllostomidae, and Moormopidae), as opposed to the Vespertilionoidea. Kirsch et al. (1998) rejected a sister-group relationship between *Noctilio* and *Mystacina* (which received only 13% bootstrap support), and tentatively suggested a basal position for the Mystacinidae within the Noctilionoidea (Kirsch et al., 1998).

Kennedy et al. (1999) analyzed 402 bp (178 phylogenetically informative sites) of the mitochondrial cytochrome *b* gene for 34 chiropteran species (4 pteropodids, 1 mystacinid, 11 phyllostomids, 3 mormoopids, 2 noctilionids, 7 molossids, and 6 vespertilionids) to test the mystacinid/noctilionoid association. All tree reconstruction methods resulted in strong bootstrap support for the inclusion of *Mystacina* in the Noctilionoidea and again rejected any affiliation with the Vespertilionoidea. Parsimony (Mormoopidae + Noctilionidae + *Mystacina*), likelihood (*Mormoops* + Phyllostomidae + *Mystacinia*), and spectral analyses (*Mystacina* + Phyllostomidae) disagreed on the placement of *Mystacina* within the Noctilionoidea, perhaps due to the long terminal branch leading to *Mystacina* (Kennedy et al., 1999). However, they did not find a sister-group relationship between *Noctilio* and *Mystacina*, as was suggested from immunological distance data (Pierson et al., 1986), and only found secondary support from spectral analyses for a basal position for Mystacinidae within the Noctilionoidea, previously suggested by DNA–DNA hybridization data (Kirsch et al., 1998).

In an attempt to resolve the branching pattern within the superfamily Noctilionoidea (including *Mystacina*), Van Den Bussche and Hoofer (2000) analyzed the phylogenetic position of *Mystacina* based on sequence data from three mitochondrial genes (12S rRNA, tRNA-valine, 16S rRNA, 2.7 kb, 1099 phylogenetically informative sites) for nine microchiropteran families. Van Den Bussche and Hoofer (2001) also examined the same mitochondrial genes but increased their taxonomic sampling to include four additional families (Furipteridae, Thyropteridae, Natalidae, and Myzopodidae). Both analyzes resulted in high bootstrap support for the inclusion of *Mystacina* within the Noctilionoidea and concluded that the family Noctilionidae was basal to a clade containing Mystacinidae + Mormoopidae + Phyllostomidae. With the increased taxonomic sampling Noctilionidae and Furipteridae were sister-taxa (Van Den Bussche and Hoofer, 2001). However, these results were only based on maximum parsimony analyses and were perhaps influenced by long-branch attraction (see Kennedy et al., 1999).

Even with the addition of two new families, Mystacinidae and Mormoopidae, our results mirror those of Teeling et al. (2002). Concurrent with previous molecular phylogenetic analyses, the Neotropical families Phyllostomidae and Mormoopidae are found in our analyses to be sister-taxa (Kirsch et al., 1998; Van Den Bussche and Hoofer, 2000; Van Den Bussche and Hoofer, 2001). Our nuclear protein sequence data unequivocally support the association of *Mystacina* with the Neotropical superfamily Noctilionoidea and refute any mystacinid/vespertilionoid affiliations. Similar to the findings reported from DNA hybridization data (Kirsch et al., 1998), our results provide robust support for a basal position for *Mystacina* within the Noctilionoidea. However, Kishino–Hasegawa tests were unable to reject a sister-group relationship between *Noctilio* and *Mystacina*. Currently, four microchiropteran families (Furipteridae, Thyropteridae, Myzopodidae, and Craseonycteridae) are missing from this dataset. Van Den Bussche and Hoofer

(2001) identified a well-supported clade that contained Furipteridae + Thyropteridae + Mystacinidae + Noctilionidae + Mormoopidae + Phyllostomidae based on mitochondrial sequence data (12S rRNA, tRNA valine, and 16S rRNA). Hoofer et al. (in press) reported a high bayesian posterior probability for a basal position for Mystacinidae within the Noctilionoidea (Phyllostomidae, Mormoopidae, Thyropteridae, Furitpteridae, Noctilionidae, and Mystacinidae) using a 4 kb concatenation of RAG2 (1.4 kb) and mtDNA (2.6 kb, 12S-tRNA-16S).

Before any firm conclusions regarding the phylogenetic position of *Mystacina* within the superfamily Noctilionoidea are drawn, the above families should be added to this large nuclear DNA concatenation.

4.2. Dating and estimation of divergence times

Molecular divergence estimates using the Thorne–Kishino approach indicate that extant chiropterans originated 67 mya, roughly corresponding to the K/T boundary given the 95% confidence intervals for this estimate. This date is compatible with the results of Springer et al. (2003), that estimated basal splits within Chiroptera at 62–68 mya.

Yinpterochiroptera represents one of the oldest bat lineages. Fig. 3 places the basal split within this clade near the K/T boundary (64 mya) followed by diversification within the superfamily Rhinolphoidea at 56 mya. The latter date coincides with the late Paleocene, whereas the earliest rhinolophoid fossils are from middle Eocene deposits (Simmons and Geisler, 1998). In contrast, an extremely long branch leads to extant megabats, which have a most recent common ancestry at 24 mya. This is approximately 40 million years after the initial split within Yinpterochiroptera and is corroborated by DNA–DNA hybridization data, which place basal divergences within Pteropodidae at 25 mya (Kirsch et al., 1998). The oldest putative crown group megabat fossil is *Archaeopteropus transiens* from the late Oligocene (Meschinelli, 1903; single specimen destroyed in World War II) and a late Eocene representative from Thailand (Ducrocq et al., 1993). The Eocene representative consists of a single tooth believed to be from an epomorphorine pteropodid (Ducrocq et al., 1993). We argue that this "Thailand tooth" is not from an epomorphorine megabat as independent molecular datasets (Hollar and Springer, 1997; Kirsch et al., 1995) estimate that this subfamily is Miocene in origin.

Yangochiropteran microbats began to split from their most recent common ancestor at approximately 56 mya (Fig. 3). Within Yangochiroptera, our dating results place the noctilionoid/mystacinid divergence at 47 mya. This result is older than the 35 million year date estimated by Pierson et al. (1986) and younger than the 68 million year date estimated by Kennedy et al. (1999). Instead our findings are more congruent with those of Van Den Bussche and Hoofer (2000), who estimated the divergence time of *Noctilio* from the mystacinid/mormoopid/phyllostomid clade at 39–50 mya, and Kirsch et al. (1998), who estimated the mystacinid/noctilionoid split at 44–66 mya using DNA–DNA hybridization data.

4.3. Historical biogeography of Mystacina

We estimate that mystacinids and noctilionoids last shared a common ancestor 47 mya in the middle Eocene. Several paleogeographic events have relevance for understanding the biogeographic context of this divergence: (1) New Zealand was vicariantly separated from the other Gondwanan landmass at 80 mya (Fig. 4). (2) Australia began rifting from East Antarctica at 80 mya. The South Tasman Rise, which previously connected the two landmasses, became submerged by 64 mya. This resulted in a shallow sea that separated Australia from East Antarctica and effectively terminated overland dispersal at this time, approximately 17 million years prior to the mystacinid/noctilionoid split. However, sweepstakes dispersal via 'island hopping' may have been possible up until 52 mya (Fig. 4; Woodburne and Case, 1996). (3) Antarctica was joined to terminal South America up until 36 mya, 11 million years after the mystacinid/noctilionoid split. At this time the Drake Passage seaway opened between South America and Antarctica, separating the two landmasses and terminating overland dispersal between both regions (Fig. 4; Woodburne and Case, 1996). (4) Glacial ice covered

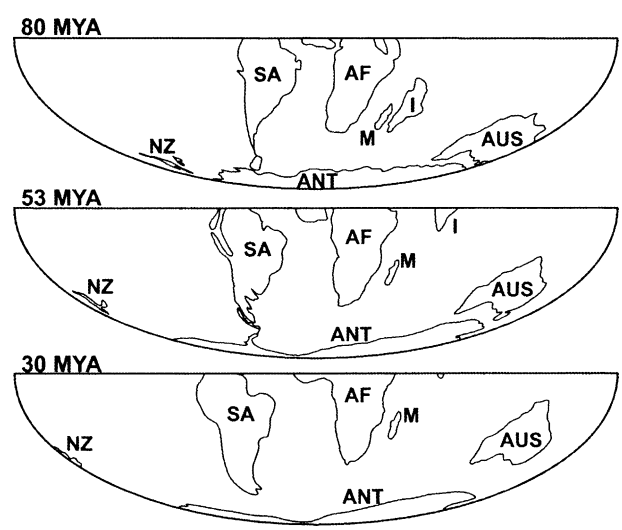

Fig. 4. Position of Gondwanan landmasses at 80, 53, and 30 mya, respectively. Based on Smith et al., 1994. SA, South America; AF, Africa; ANT, Antarctica; AUS, Australia; I, India, M, Madagascar; NZ, New Zealand.

Antarctica 1000 m above sea level at 46 mya, with temperatures dropping until the continent froze entirely at 36 mya (Ehrmann and Mackensen, 1992; Woodburne and Case, 1996).

The ancestral mystacinid and noctilionoid lineages had been separated from each other for approximately 11 million years at the time of the South American/Antarctic separation. Therefore, it is possible that they co-mingled together within the South American/Antarctic Gondwanan landmass, as suggested by Kirsch et al. (1998). As the stem noctilionoid/mystacinid clade is only 55 million years old, we can rule out its presence in New Zealand prior to the vicariant separation of this island (approx. 80 mya) from other Gondwanan landmasses. However, the question arises as to whether the mystacinid/noctilionoid lineage arose in Australia, Antarctica, or the New World prior to dispersal of a mystacinid ancestor to New Zealand.

The noctilionoid/mystacinid lineage could have arisen in Australia with subsequent dispersal of the noctilionoid linegae to South America and the mystacinid lineage to New Zealand. Fossils from Queensland (Hand et al., 1998) indicate that mystacinids were present in Australia in the early Miocene. As discussed by Kirsch et al. (1998), there are at least two possibilities for dispersing from Australia to New Zealand. First, a direct crossing over the Tasman Sea through wind aided flight. Second, crossing an archipelagic arc that existed between northeastern Australia and New Zealand's North Island 45–40 mya (Kirsch et al., 1998; Sibley and Ahlquist, 1981).

Another possibility, and perhaps one that is more likely given a richer New World fossil record, is that the mystacinid/noctilionoid ancestor originated, either in North or South America, and later dispersed to Antarctica, Australia, and finally to New Zealand. The oldest putative noctilionoid fossils are mormoopids and phyllostomids from peninsular Florida, including a 30–31 mya mormoopid that is intermediate between *Mormoops* and *Pteronotus* (Morgan, 2002). This suggests that noctilionoids had a more northern distribution then demonstrated today. In contrast, *Mystacina* is the only Old World representative of this otherwise exclusively New World superfamily.

The arrival time of the mystacinid lineage in New Zealand is open for debate. Their peculiar terrestrial adaptations, plus the presence the indigenous parasitic plant *Dactylanthus taylorii* or "wood rose" that is exclusively pollinated by *Mystacina*, suggests a lengthy period of in situ evolution and indicates an ancient arrival of mystacinid bats in New Zealand (Kirsch et al., 1998). Unfortunately, these questions can only be answered by finding key fossils at crucial geological times that will identify the dispersal path taken by mystacinid/noctilionoid bats.

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