

MOLECULAR EVIDENCE FOR EVOLUTION OF PISCIVORY IN *NOCTILIO* (CHIROPTERA: NOCTILIONIDAE)

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Intragenetic relationships within *Noctilio* were examined using the entire 1,140 base pairs of the mitochondrial cytochrome-*b* gene and 1,398 base pairs of the nuclear recombination activating gene-2. Sequences were analyzed to establish the relative time frame during which piscivory, the feeding strategy for hunting and eating fish, evolved in this genus. Piscivory represents the derived morphologic state for the genus *Noctilio*, and *N. leporinus* evolved from a *N. albiventris*-like ancestor. Distance values for specimens of *N. leporinus* from the Antilles, Central America, and South America suggest that *N. leporinus* speciated recently, 0.28–0.7 million years ago. Within *N. albiventris*, evidence exists that specimens from Peru diverged 1.16–2.9 million years ago. Analysis of these data suggests the possibility that 2 biological species are present within *N. albiventris*.

Key words: Chiroptera, cytochrome-*b* gene, molecular phylogeny, *Noctilio*, piscivory, recombination activating gene-2

The superfamily Noctilionoidea has a diversity of species primarily occurring in the New World tropical and subtropical regions. This superfamily includes 4 families (Mormoopidae, Mystacinadae, Noctilionidae, Phyllostomidae), 56 genera, and 165 species (Kirsch et al. 1998; Van Den Bussche and Hooper 2000; Wilson and Reeder 1993). Feeding strategies used by these bats include insectivory, nectivory–pollenivory, carnivory, frugivory, sanguivory, omnivory, and piscivory. Only 1 species within this complex of bats, *Noctilio leporinus*, is piscivorous, having the ability to feed on fish. Most shifts in feeding strategy have been accompanied by extensive morphologic and physiologic evolution (e.g., sanguivory—Baker et al. 1988; Forman et al. 1968; Honeycutt and Sarich 1987; Hood and Smith 1982), and therefore, they have been accorded taxonomic distinction at the subfamily or tribe level

(e.g., pollen and nectar-feeding tribe Glossophagini—Baker et al. 1989; Miller 1907; Solmsen 1998; Webster 1993). A major exception to this pattern is the genus *Noctilio*, where *N. albiventris* primarily is insectivorous, whereas its congener, *N. leporinus*, has evolved to glean fish and other prey from the water's surface and to forage for insects.

The 2 species of *Noctilio* occur sympatrically in the Neotropics and exhibit several similarities with regard to morphology, echolocation, and types of hunting maneuvers. The primary distinguishing characteristics are that *N. leporinus* is the larger of the 2 species, has longer, more robust hind legs, larger feet, and enlarged, laterally compressed claws (Brooke 1994). Echolocation calls of these bats are similar in structure and pattern and are unique to bats known to forage over the surface of the water (Kalko et al. 1998). Differences in echolocation between species of *Noctilio* are attributed to variation in body mass, a rela-

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tionship well documented for a variety of bats (Barclay and Brigham 1991). *N. leporinus* feeds extensively on flying insects, fish, and aquatic invertebrates (Brooke 1994; Goodwin 1928; Kalko et al. 1998; Novick and Dale 1971). *N. leporinus* not only employs aerial captures but gaffing maneuvers, raking its claws through the surface of the water (Altenbach 1989; Bloedel 1955), whereas *N. albiventris* predominantly is insectivorous. Although *N. albiventris* has been trained to take floating fish from the water under laboratory settings (Suthers and Fattu 1973) and fish scales have been reported in its guano (Howell and Burch 1974), *N. albiventris* has not been observed to catch or eat fish in the wild. *N. albiventris* also exhibits behavioral plasticity when foraging for insects. Aerial capture is the primary hunting strategy; however, if an insect falls onto the surface of the water, *N. albiventris* will respond by scooping up the insect in a directed pointed dip or by raking its claws, for a short distance, through the surf (Hooper and Brown 1968; Kalko et al. 1998; Novick and Dale 1971; Suthers and Fattu 1973). Flexibility in hunting strategy allows *Noctilio* to adjust to changes in availability of prey, increasing the breadth of the usable prey base. A change in feeding strategy typically is viewed as a major evolutionary event; however, for the genus *Noctilio*, ability to use both adaptive zones, insectivory and piscivory, has been accomplished without sufficient behavioral or physical modifications to justify generic distinction for these 2 species (Miller 1907).

We explored the intrageneric relationships in *Noctilio* using the mitochondrial cytochrome-*b* gene and the nuclear recombination activating gene-2 (RAG-2) to provide a relative estimate for the origin of piscivory within this complex of bats. Cytochrome-*b* has been used widely to estimate phylogenetic relationships among species (Avice and Walker 1999; Baker et al. 1994; da Silva and Patton 1993; Rosel et al. 1995; Smith and Patton 1993; Talbot and Shields

1996; Van Den Bussche et al. 1998). Much is known about the structure-function relationships of cytochrome-*b*, which enhances its utility as an evolutionary tool (Irwin et al. 1991). Cytochrome-*b* also has been used successfully to establish phylogenetic hypotheses for species within and among the phyllostomid genera *Artibeus*, *Carollia*, *Chiroderma*, *Dermanura*, *Koopmania*, *Phyllostomus*, and *Rhinophylla* (Baker et al. 1994; Van Den Bussche and Baker 1993; Van Den Bussche et al. 1993; Wright et al. 1999). Empirical studies have shown that an accurately resolved gene tree, based on a single gene, may not be congruent with the species trees (Moore 1995; Nei 1987; Pamilo and Nei 1988; Wu 1991). To address this potential problem, we included RAG-2, a nuclear locus, to increase the probability that molecular data depicted the evolution of the taxon under study. RAG-2 is 1 of 2 genes whose protein products are involved in V(D)J recombination, a process involved in assembling functional lymphocytes (Melek et al. 1998; Oettinger et al. 1990). These genes are highly conserved among organisms that undergo V(D)J recombination.

MATERIALS AND METHODS

Mitochondrial DNA was isolated from frozen heart and liver tissue samples using the Wizard Plus Miniprep Kit (Promega, Madison, Wisconsin). Total DNA was isolated from liver tissue stored in ethanol by sodium dodecyl sulfate-proteinase K-NaCl extraction and alcohol-precipitation protocol (Miller et al. 1988). The entire (1,140-base-pair [bp]) cytochrome-*b* gene was amplified by polymerase chain reaction. Conditions for amplifying cytochrome-*b* were as follows: denaturation for 2 min at 94°C followed by 35 cycles of 94°C (60 s) denaturation, 50°C (45 s) annealing, 72°C (90 s) extension, and a final extension period at 72°C (4 min). Amplification reactions were performed in 50- μ l volumes using the following reagents: 10 mM Tris-Cl pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.5 μ M of each primer, 1.6 μ M deoxynucleoside triphosphate, and 0.05 U of *Taq* polymerase (Promega). Two polymerase chain reactions were

TABLE 1.—Primers used for amplification and sequencing of cytochrome-*b* gene and recombination activating gene-2 (RAG-2) for all species within *Noctilio*, *Pteronotus*, and *Mormoops*. The letters H and R refer to reverse direction; L and F refer to forward direction; N = *Noctilio*, P = *Pteronotus*, M = *Mormoops*.

Primer	Sequence of primer	Source
Cytochrome- <i>b</i> primers		
H15915	5'-AAC TGC AGT CAT CTC CGG TTT ACA AGA C-3'	Kocher et al. (1989)
L14724	5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3'	Kocher et al. (1989)
L15513	5'-CTA GGA GAC CCT GAC AAC TA-3'	Irwin et al. (1991)
H15149	5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3'	Kocher et al. (1989)
L15162	5'-GCA AGC TTC TAC GAG GAC AAA TAT C-3'	Kocher et al. (1989)
752R	5'-GAG ACC CCG ATA ATT ACA CTC CTG C-3'	Tiemann-Boege et al. (2000)
P175R	5'-TRT GBG TTA CDG AGT TRA ATG CDG TTG CDG T-3'	This study
P295F	5'-GGC CGA GGV MTR TAC GGA TCV TAY ATR TAC-3'	This study
P644F	5'-CAG AYA TAR TYC CAT TCC ACC C-3'	This study
P653R	5'-GTG GTA AGG GTG GAA TGG GA-3'	This study
P772F	5'-CCA GCH AAC CCA CTR AAY ACY CCA CCA CA-3'	This study
P772R	5'-TGT GGT GGR GTR TTY AGT GGG TTD GCT GG-3'	This study
N360F	5'-ATT CGC CGT CAT AGC CAC AG-3'	This study
N655F	5'-CCA TTC CAC CCC TAC CAC AC-3'	This study
N655R	5'-GTG TGG TAG GGG TGG AAT GG-3'	This study
N956R	5'-RAG GAT TCA RAA YAG GCA TTG GCT TAG GG-3'	This study
M370F	5'-ATA GCA ACA GCA TTC ATA GG-3'	This study
M634F	5'-CAT CGG ACC CAG ACA TAA TC-3'	This study
M658F	5'-TTC CAC CCY TAC TAC ACA WTC-3'	This study
M658R	5'-GAS TGT GTA GTA RGG GTG GAA-3'	This study
M1081R	5'-GGG TGT TCR AGC GGT TGK CC-3'	This study
RAG-2 primers		
RAG-2F1	5'-GGC TGG CCC AAR AGA TCC TG-3'	Baker et al. (2000)
RAG-2F1-Int	5'-GTA CAG TCG AGG GAA RAG CAT GG-3'	Baker et al. (2000)
RAG-2F1B	5'-ATC CTG CCC CAC TGG AAG TTT TC-3'	Baker et al. (2000)
RAG-2F1-Int	5'-GGA YTC CAC TCC CTT TGA AGA-3'	Baker et al. (2000)
RAG-2R1	5'-AAC YTG YTT ATT GTC TCC TGG TAT GC-3'	Baker et al. (2000)
RAG-2R1-Int	5'-GGG GCA GGC AST CAG CTA C-3'	Baker et al. (2000)
RAG-2R2-Int	5'-GCA GCA WGT AAT CCA GTA GC-3'	Baker et al. (2000)
RAG-2R2	5'-GRA AGG ATT TCT TGG CAG GAG T-3'	Baker et al. (2000)

performed to amplify the entire gene for all species. The primer pairs used were N956R–H15915 and L14724–N360F to amplify *Noctilio*, 400F–H15915 and L14725–P772R to amplify *Pteronotus*, and M370F–H15915 and L14724–M1081R to amplify *Mormoops* (Table 1). Partial sequence (1,476 bp) of RAG-2 was amplified, using primers RAG-2F1 and RAG-2R2, following the same thermal profile as for cytochrome-*b* with the exception of raising the annealing temperature to 60°C. Amplicons were purified using QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, California) and sequenced in both directions using ABI Prism dRhodamine Terminator Cycle Sequencing Re-

action Kit (Perkin-Elmer Corporation, Applied Biosystems, Foster City, California) following manufacturer's instructions. Amplicons were sequenced using the primers listed in Table 1. Sequencing products were precipitated with ethanol and analyzed with an ABI Prism[™] 310 Genetic Analyzer (Perkin-Elmer Corporation). Sequences were edited and aligned using Sequencher (version 3.01, Gene Code Corporation, Ann Arbor, MI). Sequences reported in this study have been deposited in GenBank under accession numbers AF330794–AF330808 for cytochrome-*b* and AF330809–AF330818 for RAG-2.

Maximum-parsimony, maximum-likelihood,

and neighbor-joining distance analyses were used to estimate phylogenetic relationships within *Noctilio*. All phylogenetic analyses were performed using PAUP (Swofford 1999). The level of phylogenetic signal within the sequence data was measured with the g_1 -statistic based on 100,000 randomly generated phylogenetic trees for each gene (Hillis and Huelsenbeck 1992). Parsimony analyses initially were conducted with all characters equally weighted as well as applying weighting schemes to reflect the calculated frequency of transition and transversions substitutions at each codon position. Transition-to-transversion ratios and number of changes at each codon position for both cytochrome-*b* gene and RAG-2 were calculated from the most-parsimonious tree. Frequency of occurrence for each of the 6 nucleotide-substitution classes (A↔T, A↔G, A↔C, C↔G, C↔T, G↔T) was identified using the 6-parameter weighting method of Cunningham (1997).

We conducted heuristic searches with 25 random additions of input taxa and tree-bisection-reconnection branch swapping (Swofford and Olsen 1990). Confidence for each node was evaluated by using 10,000 bootstrap iterations with 25 random additions of input taxa and tree-bisection-reconnection branch swapping (Swofford and Olsen 1990). Bremer support (decay) indices were calculated with Autodecay Analysis software (Eriksson 1997). Heuristic searches were conducted under 2 models of maximum-likelihood, F81 and HKY85 (Felsenstein 1985; Hasegawa et al. 1985). Sequence divergence was calculated using the Kimura 2-parameter (Kimura 1980) and the Tamura-Nei (Tamura and Nei 1993) models of evolution. *Mormoops megalophylla* and *Pteronotus parnellii* (Mormoopidae) were used as outgroups to establish polarity of character-state changes.

Specimens examined.—Nineteen specimens were examined. TK numbers identify DNA samples from the Vital Tissue Collection at the Natural Science Research Laboratory, the Museum of Texas Tech University, Lubbock, and CN numbers identify the voucher specimen from which DNA samples were taken from the Center for Biodiversity and Conservation Biology, Royal Ontario Museum, Toronto, Canada. Specimen number and sex of each individual are followed by collecting locality. *Noctilio albiventris*—TK86633, female, Guyana, Berbice District, Dubulay Ranch, 5°40.9'N, 57°51.52'W, 41 m ele-

vation; TK22849, female, Peru, Huanuco Department, Leoncio Prado Province, 1 km S Tingo Maria; TK46004, male, Peru, Coreto, Quebrada, Agas Negras, Cocha Oraidá; TK17528, female, Suriname, Suriname, Paramaribo; TK17633, female, Suriname, Marowijne, Perica; TK19032, male, Venezuela, Bolívar, 18 mi NE El Manteco. *Noctilio leporinus*—TK18513, female, Grenada, St. George, 0.5 km E Confer, Chemin; TK18700, male, Grenadines, Carriacou Island, Craigston Estate; TK86669, male, Guyana, Berbice District, Dubulay Ranch, 5°40.91'N, 57°51.52'W, 41 m elevation; TK86639, male, Guyana, Berbice District, Dubulay Ranch, 5°40.91'N, 57°51.52'W, 41 m elevation; TK86638, female, Guyana, Berbice District, Dubulay Ranch, 5°40.91'N, 57°51.52'W, 41 m elevation; CN97682, male, Mexico, Chiapas, Puerto Arista; TK15708, male, Montserrat, St. Anthony Parish, mouth of Baham River; CN104207, female, Panama, Canal Zone, Gamboa, Gamboa; TK22848, female, Peru, Huanuco Department, Leoncio Prado Province, 1 km S Tingo Maria; TK10224, male, Suriname, Saramacca, Raleigh Falls; TK19126, male, Venezuela, Bolívar, 8 km S, 5.0 km E El Manteco. *Mormoops megalophylla*—TK27640, male, Mexico, Jalisco, Chamela. *Pteronotus parnellii*—TK17953, female, Suriname, Marowijne, Oelemarie.

RESULTS

The DNA sequences were generated from 10 *N. leporinus* and 4 *N. albiventris* for cytochrome-*b* and 5 *N. leporinus* and 3 *N. albiventris* for RAG-2. For all taxa examined, the cytochrome-*b* gene was 1,140 bp. Alignment of cytochrome-*b* sequence data of *Noctilio* with outgroups *Mormoops* and *Pteronotus* resulted in 770 bp (67%) shared among all taxa. Of the remainder, 203 were autapomorphic, leaving 167 potentially phylogenetically informative sites. Among those parsimony-informative characters, 36 occurred at the 1st codon position, 8 at the 2nd codon position, and 123 at the 3rd codon position.

Maximum-parsimony analysis, with all characters weighted equally and uninformative characters excluded, resulted in 4 most-parsimonious trees of 938 steps, con-

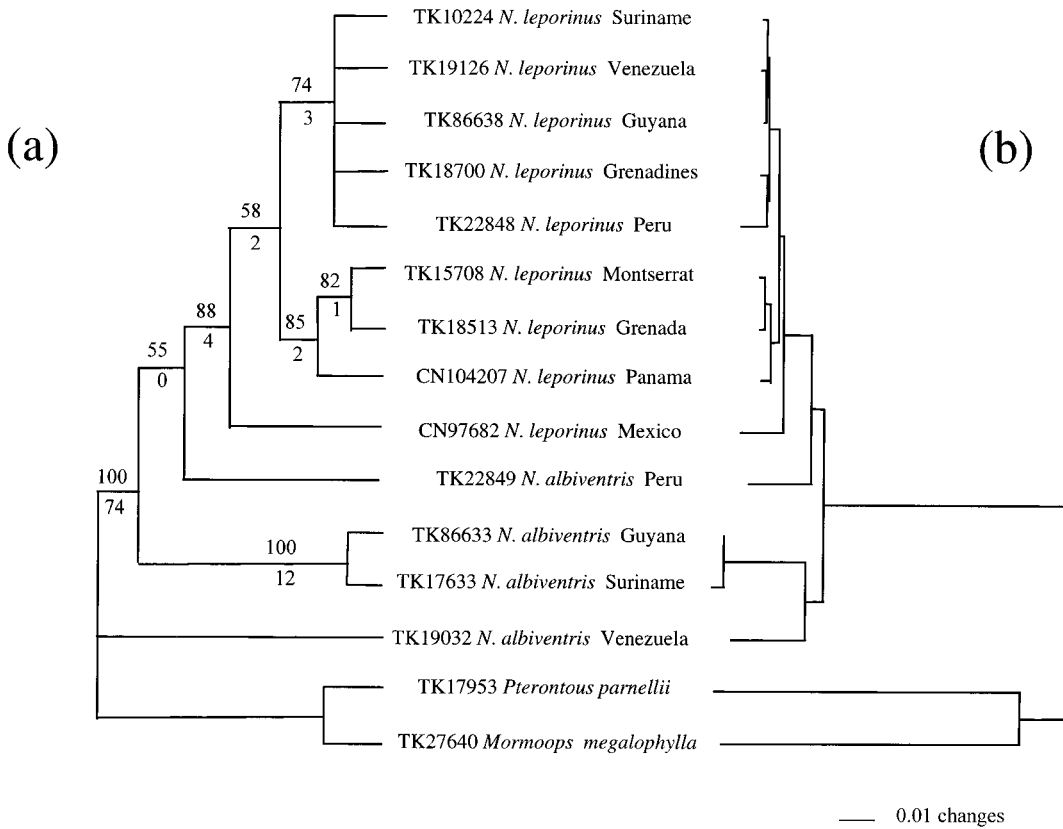


FIG. 1.—Phylogenetic trees representing relationships among specimens of *Noctilio* based on the cytochrome-*b* gene. a) Topology of the strict consensus of the 4 most-parsimonious trees from a heuristic search and 10,000 bootstrap iterations with 25 random additions of input taxa and tree-bisection-reconnection branch swapping; the tree was constructed by applying equal weight to all characters and was 938 steps in length, consistency index (CI) = 0.546, retention index (RI) = 0.712, and $g_1 = -0.652$ ($P < 0.01$); number above each lineage indicates the bootstrap value and number below indicates results from the Bremer decay analysis. b) Neighbor-joining tree generated using the Tamura-Nei (Tamura and Nei 1993) model of evolution.

sistency index (CI) = 0.546, retention index (RI) = 0.712, and a highly significant g_1 -statistic of -0.652 ($P < 0.01$ —Hillis and Huelsenbeck 1992). Variation among the 4 trees was due to positional changes of 3 individuals of *N. leporinus* (TK10224 Suriname, TK22848 Peru, TK18700 Grenadines). Bootstrap and Bremer support values were low for all nodes except those uniting *N. albiventris* from Guyana (TK86633) and Suriname (TK17633) and *N. leporinus* from Montserrat (TK15708), Grenada (TK18513), and Panama (CN104207). *N. albiventris* was depicted as paraphyletic in the most-parsi-

monious trees; however, nodal support for paraphyly was relatively weak (Fig. 1a). Under maximum-parsimony, *N. albiventris* was constrained to be monophyletic. The log-likelihood values obtained for the unconstrained and constrained tree were not significantly different. Maximum-likelihood analyses setting parameter values to the F81 and HKY85 models of nucleotide substitution resulted in a tree identical to the maximum-parsimony consensus tree.

The transition-to-transversion ratio (13.7:1) and number of mutations at each codon position (100:30:332) used in the weighted

maximum-parsimony analyses were calculated from the data. Applying the empirical weight for transversions (13.7) and differentially weighted codon positions (3:11:1) resulted in tree topologies identical to or less resolved than those obtained from the unweighted heuristic analysis.

The 6-parameter weighting scheme resulted in 2 most-parsimonious trees of 1,338 steps with CI = 0.596, RI = 0.769, and $g_1 = -0.58$ ($P < 0.01$ —Hillis and Huelsenbeck 1992). Similar bootstrap values were obtained for those nodes supporting relationships within *N. leporinus* as those obtained for the unweighted analysis. Phylogenetic relationships among *N. albiventris* were better resolved, uniting all representatives in a clade, with the exception of the unresolved placement of TK22849 from Peru.

Neighbor-joining trees constructed under the genetic distance framework of Kimura 2-parameter (Kimura 1980) and Tamura–Nei (Tamura and Nei 1993) models of evolution resulted in identical trees (Fig. 1b). Variation in length of branches among models was minimal. Estimates of mean percentage of sequence divergence for pairwise comparisons within *N. leporinus* and *N. albiventris* were 1.4% and 4.9%, respectively, whereas mean percentage difference between species was 5.5%. Average percentage of sequence divergence between *Noctilio* and outgroup taxa was 24%, and average divergence among outgroup taxa was 17.5% (Table 2).

To eliminate ambiguous sites of RAG-2 in some individuals, sequences were truncated at each end. Therefore, final analysis of the sequence for RAG-2 was limited to 1,398 contiguous bp. Of the 1,398 bp, 1,286 (92%) characters were identical among all taxa, 59 were autapomorphous, and 53 were phylogenetically informative. Of the 53 phylogenetically informative characters 10 were at the 1st codon position, 9 at the 2nd codon position, and 34 at the 3rd codon position. Maximum-parsimony analysis with all characters weighted equally under the

heuristic-search option resulted in 2 trees of 55 steps, CI = 0.964, RI = 0.967, $g_1 = -3.36$ ($P < 0.01$ —Hillis and Huelsenbeck 1992; Fig. 2a). *N. leporinus* was monophyletic and divided into 2 weakly supported clades. *N. albiventris* seemed to be paraphyletic and unresolved with a representative from Peru (TK46004) being the most basal member of the clade. Maximum-likelihood analyses setting parameter values to the F81 and HKY85 models of nucleotide substitution resulted in a tree identical to the maximum-parsimony consensus tree (Fig. 2a).

Again, weighted values used in the heuristic analysis were calculated directly from the data. Downweighting of transitions by a factor of 2 and differentially weighting codon positions (4:15:1) did not influence the tree topology.

The 6-parameter weighting scheme resulted in 2 most-parsimonious trees of 163 steps with CI = 0.988, RI = 0.978, and $g_1 = -3.42$ ($P < 0.01$ —Hillis and Huelsenbeck 1992). The differences in nodal support between the unweighted matrix and the 6-parameter model were minor. *N. leporinus* formed a monophyletic assemblage with 2 moderately supported clades, and *N. albiventris* was again unresolved.

Pairwise comparisons for both Kimura 2-parameter (Kimura 1980) and Tamura–Nei (Tamura and Nei 1993) models of evolution resulted in identical trees and similar percentage of sequence-divergence values (Fig. 2b). Average sequence divergence was 0.32% within *N. leporinus*, 0.33% within *N. albiventris*, 0.5% between species, 6.8% between *Noctilio* and outgroup taxa, and 3.1% among outgroup taxa (Table 3).

DISCUSSION

In systematics, an assumption is made that within a family, genera are of similar genetic geological age (Hennig 1966:160; Sibley and Ahlquist 1990; Vaughan 1986:27). Do analyses of the molecular data suggest that *Noctilio* is about the same age as

TABLE 2.—Percent sequence divergence of the cytochrome-*b* gene for all pairwise comparisons among *Noctilio*, *Mormoops*, and *Pteronotus*, corrected for multiple substitutions using the Tamura–Nei (Tamura and Nei 1993) model of evolution.

Taxon no.	Specimen no.	Species	Taxon no.															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1	TK10224	<i>N. leporinus</i>																
2	TK15708	<i>N. leporinus</i>	1.25															
3	TK18513	<i>N. leporinus</i>	1.25	0.35														
4	TK18700	<i>N. leporinus</i>	0.53	1.42	1.42													
5	TK19126	<i>N. leporinus</i>	0.35	1.25	1.25	0.53												
6	TK22848	<i>N. leporinus</i>	1.16	2.25	2.24	1.15	1.33											
7	CN104207	<i>N. leporinus</i>	1.24	0.71	0.71	1.24	1.24	2.15										
8	CN97682	<i>N. leporinus</i>	2.25	2.44	2.44	2.42	2.25	3.27	2.34									
9	TK86638	<i>N. leporinus</i>	0.26	1.16	1.16	0.44	0.26	1.25	1.15	2.15								
10	TK86633	<i>N. albiventris</i>	5.88	5.69	5.69	6.05	5.87	6.36	5.88	6.18	5.78							
11	TK17633	<i>N. albiventris</i>	6.27	6.08	6.08	6.44	6.26	6.75	6.27	6.57	6.17	0.35						
12	TK19032	<i>N. albiventris</i>	5.61	5.01	5.21	5.78	5.40	5.88	5.39	6.29	5.51	5.48	5.87					
13	TK22849	<i>N. albiventris</i>	4.32	3.94	3.94	4.40	4.32	3.83	4.03	4.61	4.23	5.78	6.07	5.70				
14	TK17953	<i>Pteronotus parnellii</i>	23.79	23.80	24.07	23.78	23.80	23.42	23.80	24.70	23.92	24.46	24.33	24.98	24.59			
15	TK27640	<i>Mormoops megalophylla</i>	22.92	23.86	23.59	23.06	23.20	23.44	23.44	24.46	23.32	24.76	24.77	25.03	23.98	21.31		

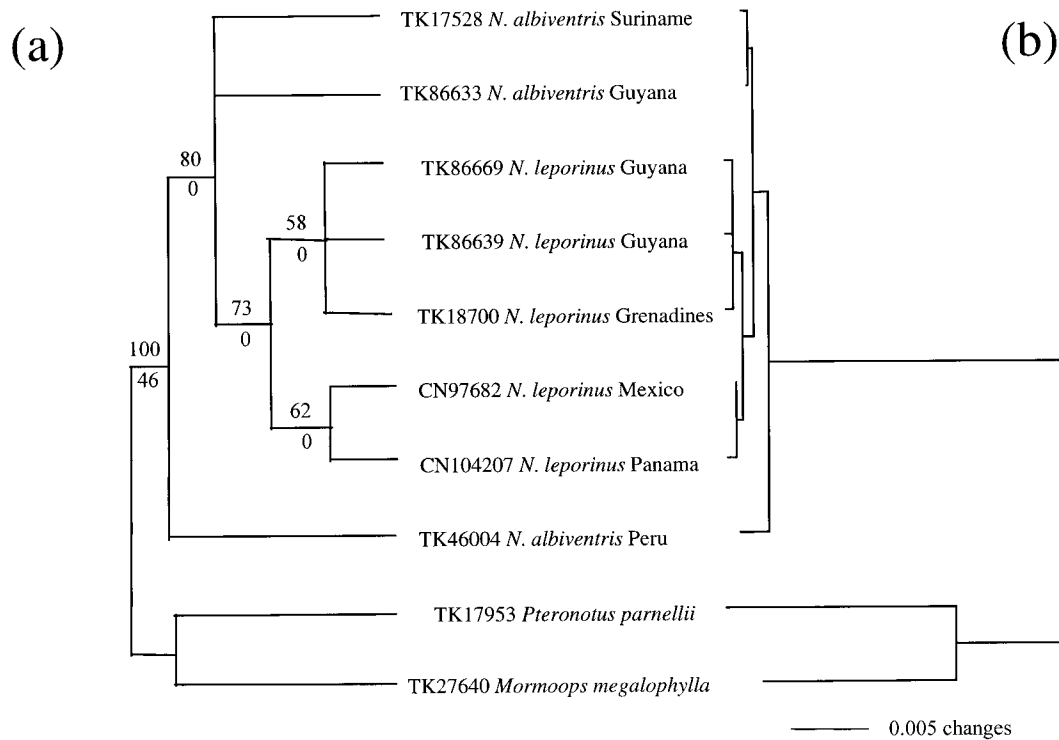


FIG. 2.—Phylogenetic trees representing relationships among specimens of *Noctilio* for the recombination activating gene-2 (RAG-2). a) Topology of the strict consensus of the 2 most-parsimonious trees from a heuristic search and 10,000 bootstrap iterations with 25 random additions of input taxa and tree-bisection-reconnection branch swapping; the tree was constructed by applying equal weight to all characters and was 55 steps in length, consistency index (CI) = 0.964, retention index (RI) = 0.967, and $g_1 = -3.36$ ($P < 0.01$); number above each lineage indicates the bootstrap value and number below indicates results from the Bremer decay analysis. b) Neighbor-joining tree, generated using the Tamura–Nei (Tamura and Nei 1993) model of evolution.

other noctilionoid genera? To test this, we assume that the greatest genetic distance among pairwise comparisons of species within a given genus will be an estimate of the oldest speciation event among extant taxa. Previous studies have demonstrated that rate of heterogeneity among lineages of bats for cytochrome-*b* is not significant at $P = 0.05$ (Van Den Bussche et al. 1998; Wright et al. 1999). Thus, evidence exists that within phyllostomid bats, the cytochrome-*b* gene is evolving in a time-dependent fashion. The average distance value based on cytochrome-*b*, which separates *N. leporinus* from *N. albiventris*, is 5.5% (3.8–6.6%). Cytochrome-*b* sequence data for the entire gene have been published for the rec-

ognized species within 5 other genera of phyllostomid bats (*Artibeus*, *Carollia*, *Chiroderma*, *Dermanura*, and *Rhinophylla*), and we have data from 2 mormoopid genera (*Mormoops* and *Pteronotus*—Baker et al. 1994; Lewis-Oritt 2000; Van Den Bussche et al. 1993; Wright et al. 1999). The greatest distance values within each genus are 11.1%, 14.2%, 13.3%, 13.3%, 19.8%, 19.2%, and 14.5%, respectively. Distance values for *Noctilio* (5.5%, 3.8–6.6%) suggest that time of divergence between *N. leporinus* and *N. albiventris* is more recent than typically found between species in other genera of bats, if cytochrome-*b* is evolving in a clocklike fashion. The distance tree for individuals of *N. leporinus* for cy-

TABLE 3.—Percent sequence divergence of recombination activating gene-2 for all pairwise comparisons among *Noctilio*, *Mormoops*, and *Pteronotus*, corrected for multiple substitutions using the Tamura-Nei (Tamura and Nei 1993) model of evolution.

Taxon no.	Specimen no.	Species	Taxon no.																	
			1	2	3	4	5	6	7	8	9	10								
1	TK17528	<i>N. albiventris</i>																		
2	TK86633	<i>N. albiventris</i>	0.07																	
3	TK46004	<i>N. albiventris</i>	0.43	0.49																
4	TK86669	<i>N. leporinus</i>	0.36	0.28	0.83															
5	TK86639	<i>N. leporinus</i>	0.71	0.28	1.25	0.62														
6	CN97682	<i>N. leporinus</i>	0.29	0.22	0.58	0.22	0.22													
7	TK18700	<i>N. leporinus</i>	0.36	0.28	0.63	0.28	0.63	0.07												
8	CN104207	<i>N. leporinus</i>	0.36	0.28	0.98	0.14	0.50	0.22	0.28											
9	TK17953	<i>Pteronotus parnellii</i>	6.14	6.18	6.15	6.29	6.29	6.15	6.16	6.34										
10	TK27640	<i>Mormoops megalophylla</i>	5.64	5.80	5.78	6.01	6.25	6.06	5.84	6.06	5.92	3.87								

tochrome-*b* (Fig. 1b) has shallow nodes and little resolution, which Avise and Walker (1999) suggested would be characteristic of the most recent species within a group.

Caution should be exercised when calibrating the molecular clock. Vertebrate mitochondrial DNA has been estimated to accumulate mutations at a rate of 2–5%/million years (Arbogast and Slowinski 1998; Brown et al. 1979; Shields and Wilson 1987). If we use this range as an estimate, then the time since divergence of *N. leporinus* from *N. albiventris* would be 1.1–2.75 million years ago, the range of divergence values of the last common ancestor for all *N. leporinus* examined would be 0.28–0.7 million years ago, and the range for all *N. albiventris* examined would be 0.98–2.45 million years ago. The lower cytochrome-*b* distance values for *Noctilio* species compared with those of other genera of bats and the low level of variation among individuals of *N. leporinus* are compatible with the conclusion that *N. leporinus* is the younger of the 2 species (Table 2).

Several examples exist of bats granted taxonomic distinction based on their feeding habits; however, species that have developed these novel feeding strategies also have experienced considerable morphologic and physiologic evolution (Baker et al. 1988, 1989; Forman et al. 1968; Honeycutt and Sarich 1987; Hood and Smith 1982; Miller 1907; Solmsen 1998; Webster 1993). Among genera of bats known to exploit different feeding strategies, *Noctilio* is exceptional. Morphologically, these species are strikingly similar, with the distinction that *N. leporinus* is the larger of the 2 species and has longer, more robust legs and enlarged, laterally compressed claws that enable it to successfully exploit the piscivorous feeding niche. *N. albiventris* has evolved hunting modes to capture insects in midair and pluck insects off the surface of water, whereas *N. leporinus* retains its ability to hunt for insects and forage for fish and other aquatic invertebrates. Although each species is hunting under a different set

of constraints (aerial versus aquatic prey), echolocation frequencies and patterns used are notably similar (Kalko et al. 1998). Not only are these bats using similar calls to respond to different echolocation glint patterns, their kind or genre of echolocation is unique among bats known to forage from the surface of water (Kalko et al. 1998). This evidence lends support to our data and strengthens the hypothesis that the evolution of piscivory in noctilionoid bats is recent and has evolved to allow these species to use different feeding zones without effectuating extensively divergent morphologic or physiologic evolution. Thus, we conclude that the ancestor of *N. leporinus* hunted for insects much as *N. albiventris* does today and that the evolution of piscivory in noctilionoid bats is as recent as the last 3 millions.

Because of the possibility of introgressive hybridization, lineage sorting, and retention of ancestral polymorphisms of mitochondrial DNA, the nuclear RAG-2 was incorporated into the sequence analysis. Although the RAG-2 data cannot be interpreted in a molecular clock framework, these data are valuable for a relative estimate of relationships. Data obtained from RAG-2 sequences generated trees mirroring the same relationships as obtained from cytochrome-*b* (Figs. 1 and 2).

Parsimony analyses demonstrated some evidence for divergence within *N. albiventris*, which may suggest a major subspecific or specific dichotomy. In both cytochrome-*b* and RAG-2 data sets, the specimens from Peru (TK22849 and TK46004) were distinct from the remainder of *N. albiventris* sampled. Based on cytochrome-*b* data, that specimen diverged from the remainder of *N. albiventris* specimens examined about 1.16–2.9 million years ago. The implications of this finding will require examination of additional specimens collected from a broader geographic range. Additional sequence analyses of more rapidly evolving nuclear loci and studies of skins and skulls would be appropriate in resolving the sig-

nificance of the deeper nodes within this species.

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