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MOLECULAR PHYLOGENETICS AND TAXONOMIC REVISION OF THE GENUS *TONATIA* (CHIROPTERA: PHYLLOSTOMIDAE)

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We conducted a phylogenetic analysis of DNA sequences from 3 adjacent genes (12S rRNA, $tRNA^{Val}$, and 16S rRNA) in the mitochondrial genome from representatives of 14 phyllostomid genera including 7 species of *Tonatia*. Previous studies of the systematic relationships within *Tonatia* document that some members of this group are extremely divergent from others. Data on mitochondrial DNA sequence depict a paraphyletic relationship of a clade of *T. bidens* and *T. saurophila* with respect to other species of *Tonatia* and other members of the Phyllostominae. *Tonatia schulzi* is included as a member of a clade containing all species of *Tonatia* (with the exception of *T. bidens* and *T. saurophila*), congruent with immunologic studies. Results of this study, coupled with those of allozymic, immunologic, and karyotypic data, warrant taxonomic revision of *Tonatia*. We recommend changing the generic name *Tonatia* to *Lophostoma* for all members except *T. bidens* and *T. saurophila*.

Key words: *Lophostoma*, mitochondrial DNA, molecular phylogenetics, Phyllostominae, *Tonatia*

Round-eared bats of the genus *Tonatia* (family Phyllostomidae) are a widespread, Neotropical group of 7 species (*T. bidens*, *T. brasiliense*, *T. carrikeri*, *T. evotis*, *T. saurophila*, *T. schulzi*, and *T. silvicola*). Relationships among these species are not well understood, partly as a result of a number of recent taxonomic changes within the genus. For example, the smaller forms of *Tonatia* that most previous studies recognized as valid species (*brasiliense*, *minuta*, *nicaraguae*, *venezuealae*) typically are now regarded as subspecies of *T. brasiliense* (Gardner 1976; Genoways and Williams 1984). Williams et al. (1995) also, recently elevated individuals of *T. bidens* (occurring from southern Mexico, south to Peru, and east to northeastern Brazil) to the species *T. saurophila*. Therefore, many studies prior to 1995 that reported data on *T. bidens* may have examined *T. saurophila*. Monophyly of *Tonatia* was also questioned by Honeycutt and Sarich (1987) because albumin immunologic distances between *T. bidens* and other species of *Tonatia* were equally as great as between *T. bidens* and *Phyllostomus*. Finally, the sister-taxon of *Tonatia* are yet to be resolved unambiquously (Baker et al. 1989, 2000; Van Den Bussche 1991, 1992; Wetterer et al. 2000).

These problems exist because few studies have evaluated phylogenetic relationships among all species of *Tonatia*. Those that have, unfortunately, did not include representatives of other phyllostomine taxa to test for monophyly of *Tonatia*. Furthermore, previous studies used characters that provided ambiguous resolution of intergeneric relationships (Arnold et al. 1983; Honeycutt and Sarich 1987; Patton and Baker 1978). It therefore seems warranted to examine phylogenetic relationships among currently recognized species of *Tonatia*.

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Resolution of these questions has implications beyond phyllostomid classification. A better understanding of intergeneric relationships would help develop hypotheses concerning primitive character states of ancestral stocks for lineages that evolved into new feeding niches (Dumont 1999; Ferrarezi and Gimenez 1996; Freeman 2000) and might provide insight into karyotypic evolution and mechanism(s) of speciation in *Tonatia*. For example, *T. bidens*, *T. saurophila*, and *T. schulzi* have undergone radical reorganization of G-banded karyotypes (Baker and Bickham 1980; Patton and Baker 1978), yet this chromosomal reorganization is not correlated with increased changes at allozyme loci (Arnold et al. 1983).

In this study, we analyzed DNA sequences from 3 adjacent genes (12S rRNA, $tRNA^{Val}$, and 16S rRNA) in the mitochondrial genome (about 2.7 kilobase pairs of contiguous sequence) from 7 species of *Tonatia* (*bidens*, *brasiliense*, *carrikeri*, *evotis*, *saurophila*, *schulzi*, *silvicola*) and representatives of all other phyllostomine genera (*Chrotopterus*, *Macrophyllum*, *Mimon*, *Phylloderma*, *Phyllostomus*, *Trachops*, and *Vampyrum*). We chose several outgroups for our phylogenetic analysis, including representatives of Desmodontinae (*Desmodus*, *Diaemus*, *Diphylla*), Macrotinae (*Macrotus*), and Micronycterinae (*Lampronycteris* and *Micronycteris*). We interpreted our results in the light of previous allozymic, immunologic, and karyotypic data.

Materials and Methods

Genomic DNA was extracted (Longmire et al. 1997) from skeletal muscle or liver tissue of *Tonatia carrikeri*, *T. evotis*, *T. saurophila*, and *T. schulzi*. For each specimen, we amplified 3 adjacent mitochondrial genes (12S rRNA, $tRNA^{Val}$, 16S rRNA) by performing 2 polymerase chain reactions (PCRs), 1 for 12S rRNA and partial $tRNA^{Val}$ and another for 16S rRNA and the remaining $tRNA^{Val}$. Conditions for PCR amplification followed Van Den Bussche and Hoofer (2000). Double-stranded amplicons were purified using the Wizard PCR Prep DNA Purification System (Promega Corporation, Madison, Wisconsin) and sequenced in both directions using Big-Dye chain terminators and a 377 automated DNA sequencer (Applied Biosystems, Inc., Foster City, California). Amplicons were sequenced entirely in both directions using a combination of flanking and internal primers (Van Den Bussche and Hoofer 2000). The computer program AssemblyLIGN-1.0.9 (Oxford Molecular Group PLC 1998) was used to piece together overlapping fragments of contiguous genes for each taxon. Additionally, we included complete sequences retrieved from GenBank for 15 additional taxa (GenBank accession numbers follow the taxon name in parentheses): *Chrotopterus auritus* (AF411538), *Desmodus rotundus* (AF263228), *Diaemus youngi* (AF411534), *Diphylla ecaudata* (AF411533), *Lampronycteris brachyotis* (AF411536), *Macrotus waterhousii* (AF263229), *Macrophyllum macrophyllum* (AF 411540), *Micronycteris schmidtorum* (AF411535), *Mimon crenulatum* (AF411543), *Phylloderma stenops* (AF411542), *Phyllostomus hastatus* (AF 411541), *T. bidens* (AF179288), *T. brasiliense* (AF411544), *T. silvicola* (AF263230), *Trachops cirrhosus* (AF411539), and *Vampyrum spectrum* (AF411537).

The computer program CLUSTAL W (Thompson et al. 1994) was used to obtain a multiple sequence alignment, and the resulting alignment was refined based on published secondary structural models (Anderson et al. 1982; De Rijk et al. 1994; Springer and Douzery 1996). As positional homology was equivocal in some regions of the multiple alignments, we recoded these potentially ambiguous regions using the computer programs INAASE 2.3b and CompAlgin 1.0b (Lutzoni et al. 2000). For this analysis, all invariant characters were removed and potentially ambiguously aligned regions were unequivocally coded as new characters employing equal weights for transitions, transversions, and gaps. The resulting coded characters were appended to the end of the sequence data, replacing their respective ambiguous region. Both data sets (the original multiple alignment and the alignment that recodes ambiguously aligned regions) were included in parsimony analysis.

Phylogenetic analyses were performed using PAUP* 4.0b8 (Swofford 2001). Nucleotides were coded as unordered, discrete characters (G, A, T, C) and gaps as missing data. For the align-

ment that was modified using the approach of Lutzoni et al. (2000), each of the coded characters in ambiguously aligned regions were subjected to a specific step matrix to account for the differential number of changes needed to transform 1 sequence to another. Following the suggestion of Lutzoni et al. (2000), we excluded ambiguously aligned regions that required >15 character states. Parsimony analyses were conducted with equal weights for all characters and substitutions. The most-parsimonious trees employed a heuristic search with 50 random additions and tree-bisection–reconnection (TBR) branch-swapping. To test for monophyly of *Tonatia*, we included representatives of *Chrotopterus*, *Macrophyllum*, *Mimon*, *Phylloderma*, *Phyllostomus*, and *Trachops* as ingroup taxa and used multiple putative outgroups (*Desmodus*, *Diaemus*, *Diphylla*, *Lampronycteris*, *Macrotus*, and *Micronycteris*); *Macrotus* was used to root the resulting phylogenetic tree(s). Stability or accuracy of inferred topologies was assessed via bootstrap analysis (Felsenstein 1985) of 500 branch and bound iterations with 25 random additions of input taxa and TBR branch-swapping for each iteration using PAUP. Decay analysis used AutoDecay 3.0.3 (Eriksson 1997). Percentage sequence differences were calculated between all pairwise comparisons of ingroup taxa using the Tamura and Nei (1993) model of DNA sequence evolution.

All tissues used in this study are represented by voucher specimens deposited in the Natural Sciences Research Laboratory of the Museum of Texas Tech University (TK) or the Royal Ontario Museum (ROM). The following specimens were used: *T. carrikeri* (ROM107391) Guyana: Potaro-Siparuni: Buro Buro R, 25 km WNW Kurupukari; *T. evotis* (ROM95626) Mexico: Campeche: 44 km S Constitucion; *T. saurophila* (ROM104559) Ecuador: Napo: Parque Nacional Yasuni, 37 km S Pompeya, (ROM103401) Guyana: Upper Demerara-Berbice: Tropenbos, 20 km SSE Mabura Hill; *T. schulzi* (ROM101128) Guyana: Barima-Waini: Baramita; *T. silvicola* (TK17946) Suriname: Marowijne: Oelemarie.

RESULTS

Mitochondrial 12S rRNA, $tRNA^{Val}$, and 16S rRNA genes were sequenced from 5 representatives of *Tonatia* representing 4 species, the sequences have been deposited in GenBank (accession numbers: AF411528–AF411532). Length of the amplified products, not including primer sequences, ranged from 2,596 to 2,625 base pairs (bp). Alignment of 21 phyllostomid sequences resulted in 2,758 aligned sites, of which 821 sites (29.77%) were phylogenetically informative; 301 (36.67%) in the 12S rRNA, 18 (2.19%) in the $tRNA^{Val}$, and 502 (61.14%) in the 16S rRNA.

A single most-parsimonious tree of 3,261 steps (consistency index [CI] = 0.2968, retention index [RI] = 0.4333) resulted with equal weights applied to all characters and substitutions and with *Desmodus*, *Diaemus*, *Diphylla*, *Lampronycteris*, *Macrotus*, and *Micronycteris* as outgroups. Most clades were supported strongly based on bootstrap and decay analyses (Fig. 1). The most surprising result was the paraphyly of *Tonatia*. *Tonatia brasiliense*, *T. carrikeri*, *T. evotis*, *T. schulzi*, and *T. silvicola* formed a strongly supported clade (bootstrap support [bs] = 100%, decay support [ds] = 31) that was sister (bs = 79%, ds = 8) to another strongly supported clade of *Phyllostomus*, *Phylloderma*, and *Mimon* (bs = 92%, ds = 11). *Tonatia bidens* and *T. saurophila* formed a strongly supported clade (bs = 100%, ds = 93) that was sister to another strongly supported clade of *Trachops* and *Macrophyllum* (bs = 87%, ds = 12). Finally, the sister-group arrangements for *T. evotis* and *T. silvicola* (bs = 100%, ds = 27) and for *Vampyrum* and *Chrotopterus* (bs = 99%, ds = 23) were strongly supported. *Vampyrum* and *Chrotopterus* formed the most basal phyllostomine clade (Fig. 1).

A CI of 0.3968 for unweighted parsimony analysis indicated a relatively high degree of homoplasy. Relatively low CI-values are typical for mitochondrial ribosomal genes and may be because of length differences among taxa resulting in ambiguously aligned regions (Allard and Honeycutt 1992; Hoofer and Van Den Bussche 2001; Springer and Douzery 1996; Van Den Bussche and Hoofer 2000, 2001). Forty-six regions of the alignment con-

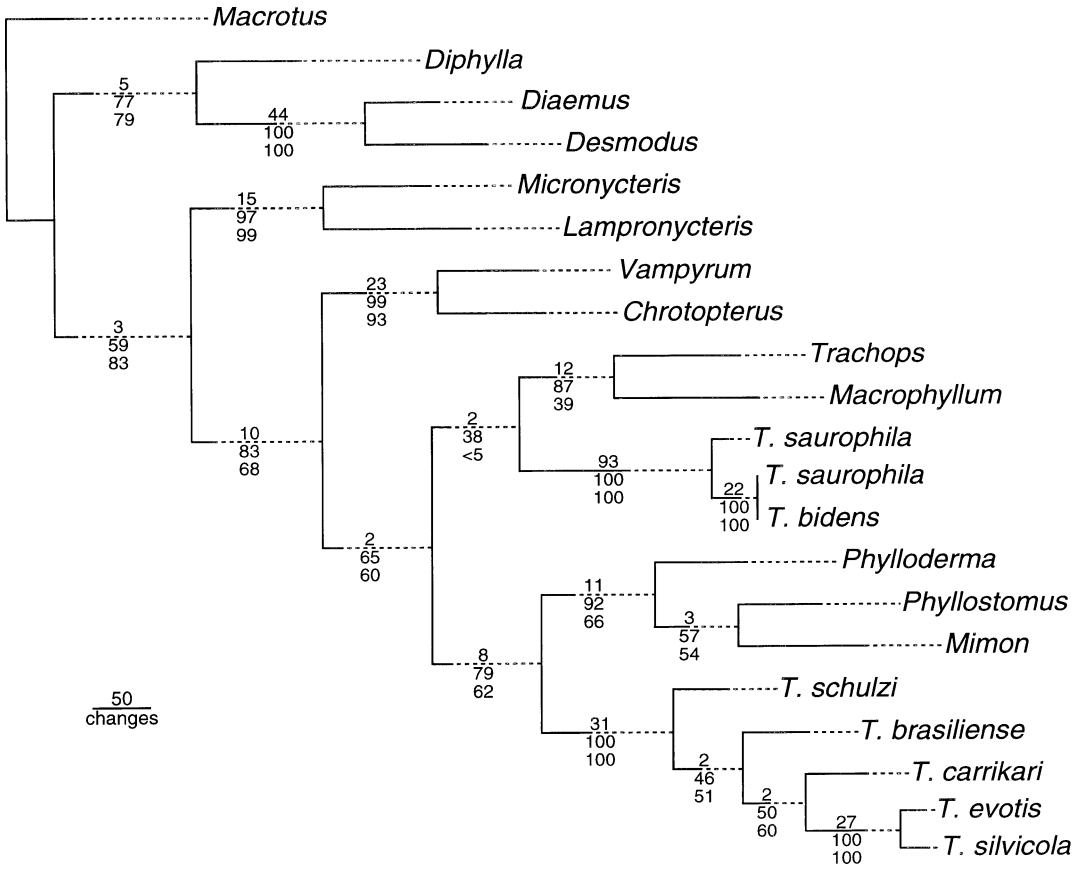

FIG. 1.—Topology of the single most-parsimonious tree based on equally weighted parsimony analysis. Solid portion of each branch represents minimal branch length; dashed portion represents maximal branch length. Numbers above branches indicate number of additional steps required to collapse each clade (decay support). The 2 numbers below each branch are bootstrap values (percentage of 500 iterations that each clade was detected) for equally weighted analysis of the entire data set and the modified data set (Lutzoni et al. 2000), respectively.

tained insertion or deletions events (indels) that potentially violate the assumption of positional homology. These 46 regions ranged in size from 2 to 34 bp (mean = 11 bp). Twenty-six of these ambiguously aligned regions were eliminated in subsequent parsimony analysis because they required >15 character states (Lutzoni et al. 2000). These 26 regions are positions 84–105, 128–137, 320–339, 758–766,773–789, 808–815, 909–931, 945–962, 1,106–1,119, 1,122–1,155, 1,172–1,183, 1,254–1,258, 1,519–1,526, 1,612–1,619, 1,679–1,697, 1,705–1,719, 1,855–1,879, 1,891–1,904, 2,200–2,206, 2,252–2,274, 2,284–2,299, 2,352–2,360, 2,383–2,398, 2,666–2,672, 2,682–2,693, and 2,729–2,740 of the aligned sequences, respectively.

Parsimony analysis of this truncated data set, applying equal weights to all transition and transversion substitutions and region specific step matrices for each of the remaining 20 regions that possessed unique character codings resulted in 4 most-parsimonious trees of 2,058 steps (CI = 0.4490, RI = 0.4623). Although none of these 4 most-parsimonious trees was identical to the most-parsimonious tree identified by the previous analysis, differences among these 5 trees were because of the movement of

Trachops and *Macrophyllum*. The ambiguity associated with the relationships of *Trachops* and *Macrophyllum* (to each other and to other phyllostomine taxa) is reflected by the low-bootstrap support for the sister association between them (bs = 39%) and for their relationship to other phyllostomine taxa (bs < 5%—Fig. 1). Nevertheless, paraphyly of *Tonatia* as described earlier was detected in all 4 trees. Bootstrap support for all clades also was similar to that described earlier (Fig. 1).

Percentage sequence divergence based on the Tamura and Nei (1993) model of evolution ranged from 0.3% between *T. bidens* and *T. saurophila* to 17.1% between *Chrotopterus* and *Mimon*, with a mean of 13.2% among ingroup taxa (Table 1). Average percentage sequence difference among *T. brasiliense*, *T. carrikeri*, *T. schulzi*, *T. evotis*, and *T. silvicola* was 7.3%, whereas it was 15.1% between the *T. bidens–T. saurophila* clade and the remaining 5 species of *Tonatia*. This value of 13.4% falls within the range (11.6–17.1%) for comparisons of recognized phyllostomine genera (Table 1).

Discussion

Although members of the *T. bidens–T. saurophila* species group are similar morphologically to *T. silvicola* and distinguished only by width of the lower incisors, analyses of other characters indicate that they are highly divergent from other species of *Tonatia*. Karyotypic studies of G-banded chromosomes reveal numerous rearrangements in *T. bidens* and *T. saurophila* that are highly differentiated from other species of *Tonatia* and from the primitive karyotype of Phyllostomidae (Baker and Bickham 1980; Patton and Baker 1978). For example, the G-banded karyotype of *T. brasiliense* (and most other species of *Tonatia*) is highly conserved with almost all chromosomal arms referable to homologous arms in *M. waterhousii*, which has the most primitive karyotype for the Phyllostomidae (Baker et al. 1982). However, the magnitude of rearrangements in *T. bidens* and *T. saurophila* prevent definite conclusions regarding chromosomal homologies with *M. waterhousii* (Patton and Baker 1978).

Phylogenetic analyses of allozymic and albumin immunologic data also document a high degree of divergence between the *T. bidens–T. saurophila* species group and all other species of *Tonatia*. They were distinguished from all other species of *Tonatia* by 11 fixed allozymic differences (Arnold et al. 1983); no other species of *Tonatia* were so divergent. Based on albumin immunology, *T. bidens* and *T. saurophila* were as divergent from other species of *Tonatia* as they were from *Phyllostomus* (Honeycutt and Sarich 1987). Although Honeycutt and Sarich (1987) indicated that these immunologic data document a magnitude of divergence inconsistent with a monophyletic grouping of *Tonatia*, they felt their data were not conclusive enough to justify taxonomic revision of *Tonatia*.

We add about 2.7 kb of mitochondrial DNA sequence data to this list of characters indicating that *Tonatia* is not monophyletic. Parsimony analysis of all currently recognized genera of Phyllostominae and 7 species of *Tonatia* depicted *Tonatia* paraphyly (Fig. 1). *T. bidens* and *T. saurophila* formed a strongly supported clade (bs = 100, ds = 93) separate from the other 5 species of *Tonatia*. The least amount of support for any of the relationships (Fig. 1) concerns the relationships of the *Trachops–Macrophyllum* and *T. bidens–T. saurophila* clades to other phyllostomine lineages. In fact, collapsing clades with ≤60% bootstrap support (Hillis and Bull 1993) results in an unresolved polytomy of these 2 lineages with a lineage that contains *Phylloderma*, *Phyllostomus*, *Mimon*, and the other 5 species of *Tonatia* (Fig. 1). Even though our data provide little support for the relationships among these 3 lineages, our data clearly are inconsistent with *Tonatia* monophyly.

Percentage sequence divergence within *Tonatia* and other phyllostomines (Table 1) further supports paraphyly of *Tonatia*. Al-

TABLE 1.—Percentage sequence differences for all pairwise comparisons of ingroup taxa under study (Tamura and Nei 1993). *T.* = *Tonatia*.

	Vampyrum	*Chrotopterus*	*Trachops*	*Macrophyllum*	*Phyllostomus*	*Phylloderma*	*Mimon*
Vampyrum	—						
Chrotopterus	12.9	—					
Trachops	15.6	15.6	—				
Macrophyllum	16.2	16.7	15.1	—			
Phyllostomus	13.9	14.4	13.3	15.5	—		
Phylloderma	14.8	16.3	15.1	16.1	11.6	—	
Mimon	16.4	17.1	14.9	16.0	12.3	14.4	—
T. saurophila	15.5	15.7	13.9	15.7	13.6	14.2	14.7
T. saurophila	15.3	16.0	14.4	16.0	14.0	14.6	14.5
T. bidens	15.5	16.2	14.7	16.2	14.2	14.7	14.9
T. brasiliense	14.8	14.8	13.2	14.6	12.0	13.2	14.0
T. carrikeri	13.7	15.4	13.6	14.8	11.9	13.4	13.9
T. schulzi	14.0	15.4	13.8	14.7	12.7	14.6	14.2
T. evotis	14.1	15.0	13.6	15.0	12.0	13.2	13.3
T. silvicola	13.7	14.2	13.0	14.4	12.0	11.8	13.4

though percentage sequence divergence alone should not be used to recognize taxa, when our data are viewed together with allozymic, immunologic, and karyotypic data, *Tonatia* does not appear monophyletic. Mean percentage sequence divergence among all species of *Tonatia* (including *bidens* and *saurophila*) is 11.0%, with most variation caused by the divergence between the *T. bidens*–*T. saurophila* species group and the other 5 species of *Tonatia* (13.4%). This value of 13.4% falls within the range (11.6–17.1%) for comparisons between other phyllostomid genera (Table 1).

Regarding the remaining species of *Tonatia*, mitochondrial DNA sequences provide a genealogy consistent with previous studies. Davis and Carter (1978) elevated the smallest members of the *T. silvicola* complex to *T. evotis*. Our data support a close relationship between *T. evotis* and *T. silvicola* (Fig. 1), with percentage sequence divergence between these taxa (2.4%; Table 1) similar to that found between 2 specimens of *T. saurophila* (Table 1). Furthermore, this clade was sister to *T. carrikeri*, supporting the close association between *T. carrikeri* and *T. silvicola* found by Arnold et al. (1983).

Tonatia schulzi is the most basal lineage within the clade of 5 species of *Tonatia*, which agrees with allozyme studies (Arnold et al. 1983). Furthermore, like *T. bidens*, the karyotype of *T. schulzi* is highly divergent from other species of *Tonatia* and may have undergone karyotypic megaevolution (Baker and Bickham 1980; Genoways and Williams 1984). Baker et al. (1982) found the G-banded karyotype of *T. schulzi* so derived that none of the arms proposed as primitive for the family could be identified. In contrast, immunologic data do not show *T. schulzi* to be as divergent; however, the relationship of *T. schulzi* to *T. carrikeri* or *T. silvicola* is unclear (Honeycutt and Sarich 1987).

The presumed radiation of species in the Phyllostominae has caused problems with the elucidation of phylogenetic relationships and assignment of taxonomic rank. The possible explanation for the confusion in the phylogenetic analysis is that the Phyllostominae have undergone a rapid adaptive radiation that results in the phylogenetic grouping of species with primitive character states rather than of species with a shared common ancestry. Allozymic, immunologic, karyotypic, and mtDNA data reveal a blueprint with implications for the interpretation of evolution within the Phyllostomi-

TABLE 1.—Extended.

T. saurophila	*T. saurophila*	*T. bidens*	*T. brasiliense*	*T. carrikeri*	*T. schulzi*	*T. evotis*	*T. silvicola*
—							
2.4	—						
2.6	0.3	—					
13.5	14.1	14.4	—				
13.2	13.4	13.7	7.8	—			
13.2	13.7	13.9	7.4	8.1	—		
12.9	13.2	13.4	8.3	8.0	7.0	—	
12.6	13.2	13.4	8.3	8.3	7.1	2.4	—

nae. Though not always congruent, these other data sets lend support to the assertion that diversification within Phyllostomidae proceeded rapidly, producing an assemblage of bats within which some species retain primitive morphotypes, whereas others have highly derived character states.

TAXONOMIC REVISION

Results of this study, coupled with those of allozymic (Arnold et al. 1983), immunologic (Honeycutt and Sarich 1987), and karyotypic (Baker and Bickham 1980; Patton and Baker 1978) studies warrant taxonomic revision of *Tonatia*. Specifically, we recommend changing the genus *Tonatia* to *Lophostoma* for *brasiliense*, *carrikeri*, *evotis*, *schulzi*, and *silvicola*. *T. bidens* and *T. saurophila* retain their generic name because *bidens* is the type species of *Tonatia* (Spix 1823). The appropriate available name for the remaining members of *Tonatia* is *Lophostoma* D'Orbigny (1836). Spix (1823) described *Vampyrus bidens* from Rio San Francisco, Bahia, Brazil. Gray (1827) recognized the generic status of *Tonatia* (Medellín and Arita 1989). D'Orbigny (1836:11) described *Lophostoma sylvicolum* from ''des grandes foréts qui bordent le pied oriental de la Cordill ére bolivienne, au pays des sauvages Yuracarés'' (or, the large forest bordering the east of the Bolivian mountain range, in the country of the Yuracaré Indians). This new genus conforms to the distribution and morphologic attributes offered under the formal description of the genus *Tonatia*.

Tonatia Gray, 1827

Vampyrus Spix, 1823:65. Type species *Vampyrus bidens* Spix.

Tonatia Gray 1827:71. Type species *Vampyrus bidens* Spix.

Lophostoma D'Orbigny, 1836:11. Type species *Lophostoma silvicolum* D'Orbigny.

Phyllostoma Gray, 1838:488. Type species *Phyllostoma childreni* (=*Vampyrus bidens* Spix).

Tylostoma Gervais, 1855:49. Type species *Tylostoma bidens* Gervais.

Chrotopterus Allen, 1910:147. Type species *Chrotopterus carrikeri*.

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