

Loren K. Ammerman
Department of Biology
Angelo State University
Box 10890
San Angelo, TX 76909
Ph#: (915) 942-2189 ext. 243
Fax#: (915) 942-2184
E-mail: loren.ammerman@angelo.edu

Running title: Phylogenetics of small-footed *Myotis*

MOLECULAR PHYLOGENETICS OF THE *MYOTIS CALIFORNICUS*—*MYOTIS*
CILIOLABRUM COMPLEX: MORPHOLOGICAL SEPARATION IS NOT
CORROBORATED BY MITOCHONDRIAL DNA DIVERGENCE

Rogelio M. Rodriguez* and Loren K. Ammerman

Department of Biology, Angelo State University, San Angelo, TX 76909

ABSTRACT

The California myotis, *Myotis californicus*, and the western small-footed myotis, *Myotis ciliolabrum*, are two morphologically similar bats that have had a complex taxonomic history due to intraspecific geographic variation across their sympatric distribution in western North America. Despite several published differences, field identification remains problematic in the southwestern United States. Mitochondrial DNA, cytochrome-*b* and control region, sequences were amplified from tissue samples ($n = 20$) from Texas, Oklahoma, New Mexico, Utah, and California, for detection of genetic markers and to examine species boundaries. A combined total of 1184 bp from cytochrome-*b* and the control region were utilized in parsimony and maximum-likelihood analyses. To corroborate species identification, cranial measurements from all specimens included in molecular analysis along with additional specimens were analyzed statistically using principal components analysis (PCA). Individuals were separated into a group identified either as *M. californicus* or *M.*

*Correspondent: r_rod77@hotmail.com

ciliolabrum. Molecular analysis resulted in *M. leibii* being nested within clades containing both *M. californicus* and *M. ciliolabrum*. Results of intraspecific and interspecific levels of sequence divergence and phylogenetic analysis indicate that there are no distinct lineages and possible species intergradation between *M. californicus* and *M. ciliolabrum*. Phylogenetic analysis via network estimation of cytochrome-*b* haplotypes supports the results of the parsimony and maximum-likelihood trees in demonstrating possible gene flow between the two species. Phylogeographic structure is minimally represented between haplotypes with a western and eastern separation in the southwestern United States.

Key words: control region, cytochrome-*b*, *Myotis*, phylogenetic, phylogeography

INTRODUCTION

Myotis species of the *leibii* group have had a complex taxonomic history due to intraspecific geographic variation in morphology (Bogan 1974, 1975; Glass and Baker 1965, 1968; Miller and Allen 1928; van Zyll de Jong 1984). This intricate problem has been particularly evident in *M. californicus* and *M. ciliolabrum* which have overlapping ranges in western North America, while *M. leibii* inhabits a distinct region from the central United States to southeastern Canada (Fig. 1). In 1918, the distinction between *M. subulatus* and *M. californicus* was recognized (Miller and Allen 1928). *Myotis subulatus* was later reclassified as *M. leibii* with *M. ciliolabrum* as a subspecies of *M. leibii* (Glass and Baker 1965, 1968). Upon reassessment of craniometric data, *M. ciliolabrum* was elevated to specific status (van Zyll de Jong 1984). Additionally, Herd (1987) provided protein electrophoretic data that supported the separation of *M. leibii* and *M. ciliolabrum* as distinct species.

Among the various published characters (e.g., pelage color, face mask, naked snout length, and presence of free-tail) used to distinguish between *M. californicus* and *M. ciliolabrum*, there has been either overlap or inconsistency (Bogan 1974; Constantine 1998; Gannon et al. 2001; Holloway and Barclay 2001; Schmidly 1991; Simpson 1993; van Zyll de Jong 1984, 1985). Pelage color, which varies in several degrees of brown for both species, was described as tri-colored in *M. californicus* and bi-colored in *M. ciliolabrum* (Bogan 1978; Chaney 1993). Conversely, *M. ciliolabrum* also has been described as having a tri-colored pelage (Davis and Schmidly 1994). As Bogan (1974, 1975) pointed out, pelage color varies directly with mean annual precipitation and altitude; for instance montane bats appear darker in coloration. The face mask of *M. ciliolabrum* is characterized as being black whereas that of *M. californicus* is said to be brown or less dark compared to *M. ciliolabrum* (Constantine 1998; van Zyll de Jong 1985). The length of naked snout is described for *M. californicus* as equal to the width between the nostrils, while the naked snout length is greater than the width between nostrils in *M. ciliolabrum* (van Zyll de Jong 1985). In California, Constantine (1998) also documented the presence of a “free-tail,” a 2-mm protuberance from the border of the interfemoral membrane, in *M. ciliolabrum* and absence of a free-tail in *M. californicus*. These differences are so subtle and variable that they rarely allow for differentiation between the two species in the field and have been questionable for bats captured in Texas (Higginbotham and Ammerman 2002).

Although cranial morphology seems to be the most useful character in differentiating between the species, it is not always definitive. *Myotis californicus* is characterized as having a globose cranium with an abruptly sloping forehead, while *M. ciliolabrum* has a flattened cranium with a gradually sloping forehead. Bogan (1974) found that rostral breadth

and cranial depth were significantly different between the two species in Hidalgo County, New Mexico. Similarly, van Zyll de Jong (1984) found rostral breadth, cranial depth and the height of the coronoid process were discriminatory between the two species from various localities throughout their distributions. These cranial differences were examined with bivariate plots of large sample sizes; however, this method does not always allow for distinction between a few skulls in hand when there is a lack of comparative material.

Recent studies of bat echolocation calls provide an additional feature for distinction between *M. californicus* and *M. ciliolabrum* (Gannon et al. 2001; O'Farrell and Gannon 1999; O'Farrell et al. 1999). *Myotis californicus* has a characteristic frequency of 50 kHz, while *M. ciliolabrum* has a characteristic frequency of 40 kHz. Gannon et al. (2001) found the pinnae of *M. ciliolabrum* to be slightly larger than those of *M. californicus*. Although this morphological character provides an additional support for further differentiation between the two species, pinna size still demonstrates a trivial deviation similar to those characters previously described.

Mitochondrial genes, such as the control region, cytochrome-*b*, and NADH dehydrogenase 1, have been used in the determination of chiropteran species boundaries (Cooper et al. 2001; Piaggio et al. 2002) and in the detection of cryptic chiropteran species (Barratt et al. 1997; Mayer and Helversen 2001a, 2001b). The cytochrome-*b* gene has been useful on many taxonomic levels and more specifically has been utilized as a source of phylogenetic characters within the genus *Myotis* (Cooper et al. 2001; Ruedi and Mayer 2001) as well as in other bat genera (Hoffman and Baker 2001; Sudman et al. 1994). Analysis of the control region has demonstrated greater resolution of phylogenetic relationships of closely related species (Duvernell and Turner 1998; Macey et al. 1998) than

other genetic markers such as cytochrome-*b* (Lamb et al. 1994) and has been helpful in resolving chiropteran relationships (Cooper et al. 1998; Spitzenberger et al. 2001).

In the investigation of the taxonomic relationships of the *leibii* group unexpected relationships have been found. Through protein electrophoresis, Herd (1987) unexpectedly found that the long-eared myotis, *M. evotis*, was more genetically similar to *M. ciliolabrum* than to *M. leibii*. Additionally, Bickham et al. (1986) reported that *M. leibii* has a metacentric autosome 25 that is shared by long-eared myotis which includes *M. evotis*, *M. auriculus*, *M. thysanodes* and *M. milleri*. Such evidence suggests that *M. evotis* demonstrates a close relationship to the *leibii* group, thus, the investigation of this relationship is under the confines of this study.

This study examines sequences of cytochrome-*b* and control region genes to determine the species boundaries between *M. californicus* and *M. ciliolabrum* in the southwestern United States. Analysis of molecular data was used to compare to traditional analysis of morphological characters. Additionally, this study assesses the phylogenetic relationships within the *leibii* group based upon mitochondrial DNA.

MATERIALS AND METHODS

Morphometric analysis.—Specimens of *M. californicus* ($n = 15$) and *M. ciliolabrum* ($n = 17$) from Texas, Oklahoma, New Mexico, Arizona, California and Utah were examined for morphological characters that have been used in past studies (Appendix I; Bogan 1974; van Zyll de Jong 1984). To avoid problems of misidentification, all specimens examined were identified on the basis of the following criteria. Specimens of *M. californicus* were identified as those having a brown facial mask, brown membranes, and a globose, abruptly

sloping cranium. Specimens of *M. ciliolabrum* were those that had a black facial mask, black membranes, and a flat, gradual sloping cranium. As previous authors (Bogan 1974; van Zyll de Jong 1984) have stated, pelage color was not always reliable in distinguishing between the two species, although for the majority of specimens *M. californicus* appeared tri-colored while *M. ciliolabrum* was bi-colored. Morphological descriptions were recorded along with all pertinent data from the museum label. Sexes were pooled since sexual dimorphism has not been found in these bats (Bogan 1974; Constantine 1998; van Zyll de Jong 1984; Williams and Findley 1979).

Ten cranial measurements adapted from van Zyll de Jong (1984) (Table 1) were taken for each specimen using digital calipers calibrated to the nearest 0.01 mm. Since both species also have been differentiated based on forehead slope, this character was taken as an additional measurement. The slope was recorded by using a protractor to measure the angle of the forehead from the top of the skull from a digital image of the skull profile (Fig. 2). All skulls were mounted in the same way and placed equidistant from the camera to obtain photographs that would produce consistent measurements.

All eleven cranial characters were subjected to principal components analysis (PCA) using SYSTAT version 9 (SPSS Inc., 1998) to demonstrate the ability of traditional methods to discriminate between the two species.

Molecular analysis.—Twenty of the specimens that were examined morphologically were analyzed using molecular techniques (Appendix I). *Myotis leibii* tissue ($n = 1$) was obtained for analysis of phylogenetic structure within the *leibii* group. *Myotis evotis* tissue ($n = 1$) was obtained for examination of its relationship to the *leibii* group. For further outgroup comparison, *M. yumanensis* ($n = 1$; Appendix I), which shows no close relation to the four

other species, was chosen along with published sequences of *M. lucifugus* (Ruedi and Mayer 2001; Wilkinson et al. 1997) to provide structure and resolution to the ingroup.

Total genomic DNA was extracted from frozen or alcoholic-preserved tissues of liver, kidney, or heart using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, California) following the manufacturer's protocol. Mitochondrial DNA sequences were amplified following standard polymerase chain reaction (PCR) methods (Palumbi 1996). A pair of cytochrome-*b* primers known to work generally for vertebrates was used (Kocher et al. 1989). This pair consists of L14841, 5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3' which binds about 80 base pairs within cytochrome-*b* nearest to the glutamate tRNA gene (Kocher et al. 1989; Fig. 3) and H15547, 5'-GGC AAA TAG GAA ATA TCA TTC -3' which binds about 820 base pairs within the cytochrome-*b* gene (Edwards et al. 1991). The following control region primers known to work specifically for bats were used; P, 5'-TCC TAC CAT CAG CAC CCA AAG C-3' that binds to the proline tRNA gene and F, 5'-GTT GCT GGT TTC ACG GAG GTA G-3' that binds to the conserved sequence block F (Wilkinson and Chapman 1991; Wilkinson et al. 1997). Reactions were amplified for an initial cycle of denaturation at 93°C for 3 min, 39 cycles of denaturation at 94°C, annealing at 48°C, extension 72°C each at 1 min, and a final cycle of extension at 72°C for 3 min. PCR products were gel purified for cloning using low-melt agarose (1.0% w/v) containing 0.05% (10 µg/mL) ethidium bromide. PCR products were cloned following the manufacturer's protocol according to the TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, California). Plasmids containing PCR product were extracted and purified following the protocol of the QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, California) and digested with the restriction enzyme *EcoRI* to confirm presence of PCR fragments. Plasmid

DNA was precipitated and sequenced following the manufacturer's protocol for the SequiTherm EXCEL II™ DNA Sequencing Kit – LC (Epicentre, Madison, Wisconsin) using one quarter of the amounts they recommend. Cycle sequencing of both strands was performed with dye-labeled M13 primers on a LI-COR Long Read Dual Laser 4200 automated sequencer (LI-COR, Inc., Lincoln, Nebraska).

Sequences were aligned to a previously sequenced control region sequence (Wilkinson et al. 1997) and a cytochrome-*b* sequence (Ruedi and Mayer 2001) of *M. lucifugus* obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>; accession numbers U95342 and AF376854) using the computer program Sequencher version 4.1 (Gene Codes Corp. 2001). Parsimony analyses was performed with the heuristic and branch-and-bound search methods using the computer program PAUP* version 4.0b10 (Swofford 2001). To assess confidence in branching patterns, bootstrap analyses (Felsenstein 1985) were performed using 1,000 pseudoreplicates. Bremer support indices (Bremer 1994) were calculated using the program TreeRot version 2.0 (Sorenson 1999) for further nodal support. To test for hierarchical structure of the data, the degree of skewness was determined by calculating the g_1 statistic based upon 1,000 random trees in PAUP* version 4.0b10 (Swofford 2001).

The computer program MODELTEST version 3.06 (Posada and Crandall 1998) was used to determine the best fit model of DNA evolution for the observed sequence data. The suggested model was used to calculate pairwise genetic distances between taxa and as a basis for maximum-likelihood trees generated in PAUP* version 4.0b10 (Swofford 2001).

Due to a lack of resolution in phylogenetic trees, sequence data was analyzed using network methods. Recently, the use of network representation in the analysis of intraspecific

phylogenies has been the preferred method for studies of population structure and gene flow (Bandelt and Dress 1992; Excoffier and Smouse 1994; Fitch 1997; Posada and Crandall 2001; Templeton et al. 1992). Phylogenetic reconstruction using networks takes into account processes that act at the species level, for instance coalescent and recombination events that result in homoplasies (Posada and Crandall 2001). Traditional phylogenetic methods aim to limit these events by eliminating homoplasies. The computer program TCS version 1.13 (Clement et al. 2000) was utilized to make a network diagram for *M. californicus* and *M. ciliolabrum* individuals. TCS estimates and constructs a network using a procedure of statistical parsimony (Templeton et al. 1992), which has been shown to have greater statistical power and accuracy when there are few variable sites (Crandall 1994). Treating gaps as a fifth state, all haplotypes were incorporated into the network based upon a 95% parsimony connection limit. Various parsimony limits were set to observe the number of taxa incorporated into the network. Network estimation was used with cytochrome-*b* sequence data due to few variable sites between haplotypes. Because network analysis is based on minimal changes among taxa, it was not surprising that the highly variable control region sequences were incapable of forming a network.

RESULTS

Morphometric analysis.—In general all standard measurements overlapped between *M. californicus* and *M. ciliolabrum* (total length, tail length, hindfoot length, and forearm length) (Table 1). In the principal component analysis of all 11 cranial characters, PC1 accounted for 53.5% of the total variation. The first principal component was dominated by characters associated with cranial length and width, such as greatest skull length (GSL),

rostral breadth (RB), rostral width (RW) and mastoid width (MW) (Table 2). Additionally, the length of the P4M3 series (P4M3L) and height of the coronoid process (HCP) strongly contributed to the overall variation explained by PC1. The variation explained by PC2 (14.4%) was most affected by cranial depth (CD), interorbital width (IOW), and forehead slope. Projection of the first 2 principal components revealed a distinct separation between specimens of *M. californicus* and *M. ciliolabrum* (Fig. 3). Two specimens (ASK 5171 and TTU 45840) lacked a lower mandible, therefore the height of the coronoid process could not be measured. Upon principal components analysis of all characters except HCP these taxa fell out with their suggested groups (not shown). Additional principal components analyses showed this similar pattern (Rodriguez 2002).

Molecular analysis.—Approximately 758 base pairs of sequence were obtained from the cytochrome-*b* gene for each taxon (GenBank Accession No. XXXX-XXXX). From the total cytochrome-*b* sequence, 556 characters were constant, 130 were parsimony uninformative, and 72 were parsimony informative. Parsimony analysis of cytochrome-*b* produced one most parsimonious tree (length = 251 steps; Fig. 4A) with a consistency index (CI) = 0.829 and retention index (RI) = 0.811. A g_1 statistic ($g_1 = -0.864$) was calculated based upon 1000 random trees. Both heuristic and branch-and-bound search methods produced identical topologies in all parsimony analyses. Strong bootstrap support was found for two monophyletic groups containing a mixture of *M. californicus* and *M. ciliolabrum* individuals. Bootstrap analysis, as well as the Bremer support index, did support the exclusion of *M. yumanensis* and *M. lucifugus* from the *leibii* group (Fig. 4A), yet failed to separate *M. evotis* from the *leibii* group.

Utilizing the HKY + Γ (Hasegawa et al. 1995) model of evolution suggested by MODELTEST, genetic distances were calculated in PAUP with transition/transversion ratio = 7.065, Γ = 0.6018 and base frequencies; A = 0.285, C = 0.271, G = 0.139, and T = 0.305. The average divergences of *M. yumanensis* (19.5%) and *M. lucifugus* (18.6%) compared to all the other taxa, were higher than all other species comparisons (Table 3). *Myotis evotis* compared to *M. yumanensis* and *M. lucifugus* displayed divergence values equally as high (18.8% and 19.5%). Yet, when compared to each member of the *leibii* group, average divergences were unexpectedly low ranging from 1.1% to 4.1%. Within the *leibii* group, values were expectedly low overall. Average percent distances for *M. leibii* compared to both *M. californicus* (3.7%) and *M. ciliolabrum* (3.8%) were practically equal and generally greater than distances between *M. californicus* and *M. ciliolabrum* (2.3%). The averages and ranges for interspecific and intraspecific percent divergences for both *M. californicus* and *M. ciliolabrum* were nearly identical (Table 3).

A maximum-likelihood tree ($\ln L = -2312.835$) based on cytochrome-*b* using the HKY + Γ model of evolution produced a tree that was similar in topology to the most parsimonious tree (Fig. 4B). Exceptions were shown by the *M. evotis*-*M. leibii*-*M. ciliolabrum* clade falling deeper within the tree and *M. ciliolabrum* NM (39583) clustering with the larger *M. californicus*-*M. ciliolabrum* clade instead of the reverse situation in the most parsimonious tree. To solely look at the phylogenetic relationships of the *leibii* group, all other taxa were excluded with *M. leibii* defined as the outgroup. One most parsimonious tree ($L = 98$ steps; CI = 0.827; RI = 0.888, $g_1 = -0.518$) was obtained (tree not shown). Although the topology of this tree did not differ much from the tree with all taxa, strong

bootstrap support (93%) was once more found for two monophyletic groups with combined individuals of *M. californicus* and *M. ciliolabrum*.

For the control region, variable sequence lengths were obtained among taxa because of extensive insertions/deletions which made alignment troublesome. Therefore, the remainder of the alignment containing these areas was excluded resulting in a total of 426 base pairs with 262 constant, 90 parsimony uninformative, and 74 parsimony informative characters (GenBank Accession No. XXXXX-XXXXX). MODELTEST suggested the HKY + Γ (Hasegawa et al. 1995) model for the control region data with base frequencies; A = 0.378, C = 0.176, G = 0.107, T = 0.339, transition/transversion ratio = 2.523 and the proportion of variable sites (Γ) = 0.4216. As expected, the control region produced overall higher genetic distances compared to cytochrome-*b*. Comparisons of *M. yumanensis* (29.5%) and *M. lucifugus* (18.0%) to all the other taxa produced exceptionally high average divergences (Table 3). The substantially high divergence values found for *M. yumanensis* are most likely meaningless for phylogenetic inference at this level due to expected saturation and a high degree of homoplasy. In contrast to cytochrome-*b* data, *M. evotis* sequences had higher average percent divergences compared to the *leibii* group (18.0%). Average percent distances for *M. leibii* compared to both *M. californicus* (5.1%) and *M. ciliolabrum* (5.0%) largely overlapped. Yet, these values were noticeably lower than the average distances between *M. californicus* and *M. ciliolabrum* (7.3%). Again, comparison of interspecific percent divergences to average values within *M. californicus* (7.7%) and within *M. ciliolabrum* (7.3%) were very similar (Table 3).

Due to the extensive differences found from pairwise comparisons with outgroup taxa, all outgroup taxa were excluded from phylogenetic analyses and *M. leibii* was defined

as the outgroup for interpretation of relationships within the *leibii* group. A strict consensus tree was calculated from ten most parsimonious trees (Fig. 5). Moderate bootstrap support (69%) was found for two monophyletic groups containing individuals of *M. californicus* and *M. ciliolabrum*. These groups do not correspond to the two monophyletic groups found from cytochrome-*b* data (Fig. 4A). The maximum-likelihood tree ($\ln L = -1465.049$) based on the control region using the HKY + Γ model of evolution resulted in the same topological structure as the strict consensus tree (not shown).

Both cytochrome-*b* and control region data were combined for total mtDNA sequence analysis. A strict consensus tree was produced from six most parsimonious trees each with $L = 605$, $CI = 0.676$, and $RI = 0.598$ (Fig. 6). Combining both gene sequences produced little phylogenetic signal (g_1 statistic = -0.574). Combined mtDNA data failed to resolve relationships beyond those produced from individual analysis of the two genes. Similar relationships were retained, but a few individuals clustered with alternate groups.

Traditional phylogenetic methods produced no distinct structuring that differentiated between *M. californicus* and *M. ciliolabrum*. Because these results were indicative of gene flow or hybridization between the two species, patterns of gene flow were investigated by network estimation. Since interspecific divergence did not differ from intraspecific divergence, all taxa of *M. californicus* and *M. ciliolabrum* were included in the network estimation. Network reconstruction of cytochrome-*b* haplotypes resulted in two groups, a “western” and “eastern” clade (Fig. 7). The two groups appeared to be separated in Arizona with the exception of *M. californicus* (32149) in southcentral NM (Otero Co.) belonging to the “western” clade. These phylogroups were also found as distinct clades within the most parsimonious cytochrome-*b* tree (Fig. 4A). All taxa demonstrated unique haplotypes with

the exception of *M. californicus* NM (3658) and *M. californicus* AZ (5170) exhibiting the same haplotype. The same three individuals (*M. californicus* UT (34800), *M. ciliolabrum* NM (39583), and *M. ciliolabrum* NM (32086)) that fell outside any *M. californicus*-*M. ciliolabrum* clade in the most parsimonious tree did not form any connections to the network (Fig. 7). The resulting network was based on a 95% parsimony connection limit equal to 11 steps. For taxa that did not form a connection to any group, it took one more step (total of 12 steps) for them to connect with any other taxa. It took a total of 16 steps to connect all individuals and groups into one complete network.

DISCUSSION

Based on cranial morphology, *M. californicus* and *M. ciliolabrum* can be distinguished from one another. As other authors (Bogan 1974; Constantine 1998; Miller and Allen 1928; van Zyll de Jong 1984) have found; *M. californicus* generally has a smaller skull with an abrupt slope of the forehead and greater cranial depth, while *M. ciliolabrum* has a larger skull with a gradual slope of the forehead and lesser cranial depth. Among those characters that contributed to separation between the two species in this study, rostral breadth, cranial depth and height of the coronoid process are consistent with characteristics observed by van Zyll de Jong (1984).

Analysis of molecular data failed to support the hypothesis that *M. californicus* and *M. ciliolabrum* are monophyletic species. Interspecific and intraspecific divergences based on cytochrome-*b* for both *M. californicus* and *M. ciliolabrum* were similar ranging from 0.0% to 4.8% (Table 3). In a comparison of several small mammal taxa, Bradley and Baker (2001) found interspecific distances to be typically greater than 11% for cytochrome-*b*, while

intraspecific values were generally less than 2%. They pointed out that among sister species of bats, distances averaged 6.83% and within a single bat species values averaged 3.0%. More specifically for *Myotis* species, Ruedi and Mayer (2001) found that divergences generally averaged greater than 10.0% between species, while intraspecific values were generally lower than 5.0%. Here, the overall average of interspecific (2.3%) and intraspecific (*M. californicus* 2.1% and *M. ciliolabrum* 2.5%) pairwise genetic divergences do not differ greatly and are more consistent with intraspecific values based on the aforementioned studies. The results of this study suggest that *M. californicus* and *M. ciliolabrum* have not diverged to the same degree as other *Myotis* species and should be possibly considered a single species. The same could be suggested of *M. leibii*, but more data from this species is necessary to validate its phylogenetic relationship to the group. Comparisons among outgroups (*M. yumanensis*, *M. lucifugus*, and *M. evotis*) found sufficient support for specific status. Unexpectedly, sequence divergence between *M. evotis* and the *leibii* group was small (2.9%) and within the intraspecific range (Table 3). This close relationship is supported by previous genetic studies (Bickham et al. 1986; Herd 1987). Further sampling of *M. evotis* is necessary to establish the level of divergence between *M. evotis*, as well as other long-eared *Myotis*, and the *leibii* group.

Control region sequence data analysis produced similar results to those of the cytochrome-*b* gene. Overall, genetic distances between and within *M. californicus* and *M. ciliolabrum* overlapped and did not differentiate between the species (Table 3). Cooper et al. (2001) found that control region distances generally averaged greater than 15.0% between species of rhinolophid bats, while intraspecific values were generally lower than 7.0%. Greater divergences were found among species of *Plecotus* (> 20.0%) with closely related

species ranging from 14.0% to 17.5% (Spitzenberger et al. 2001). An average divergence of 3.8% was found among clades of *M. myotis* (Castella et al. 2001). Comparing these results with those found here for *M. californicus* and *M. ciliolabrum*, interspecific and intraspecific values rarely exceeded 14.0% ($n = 10$ pairwise comparisons) and largely ranged within 3.9% to 10.7%. The lack of a geographic pattern in relation to tree topology and variable intraspecific and interspecific divergences can be attributed to the high sequence variability of the control region (Table 3). In fact, the low g_1 statistic (-0.544) suggested that control region data lacked phylogenetic structure. In contrast to cytochrome-*b* distances, control region pairwise distances between *M. leibii* and *M. californicus*/*M. ciliolabrum* were lower than intraspecific and interspecific distances for *M. californicus* and *M. ciliolabrum*. Again, these results could be indicative of synonymy; however, additional samples of *M. leibii* would be necessary to test this hypothesis. Control region sequence appeared diagnostic for separating *M. evotis* from the *leibii* group with overall high divergences (Table 3).

Parsimony and likelihood analyses of cytochrome-*b* sequence did not illustrate any clear pattern of monophyly within either species (Fig. 4). These results were supported by similar analyses of control region data (Fig. 5). In fact, relationships demonstrated from topologies of each gene tree conflicted marginally, that is, taxa shown to be close relatives in one gene tree were distant relatives in the other tree. The combined parsimony analysis of cytochrome-*b* and control region sequence did not resolve relationships among the *leibii* group (Fig. 6). Individuals of *M. californicus* and *M. ciliolabrum* were dispersed throughout the combined gene tree and showed minimal recognizable geographic structure or little congruence with geographic distance. The lack of support for separate lineages of each species suggests that gene flow is occurring between the two species. Phylogenetic analysis

using network construction of cytochrome-*b* sequence illustrated a better geographic representation of gene flow, yet produced minimal phylogeographic structure (Fig. 7). Two explanations could be derived from the overall phylogenetic and phylogeographic patterns observed. If *M. californicus* and *M. ciliolabrum* do represent two distinct species, then some level of introgression is occurring and the southwestern United States serves as a potential hybrid zone for these two species. However, hybridization appears to be rare in bats and has only been described for megachiropterans (Webb and Tidemann 1995) and subspecies of microchiropterans (Baker 1981; Greenbaum 1981). A direct test of hybridization was not performed and would most likely be apparent from comparisons of allelic frequencies at nuclear loci for both species. The alternative explanation could be that *M. californicus* and *M. ciliolabrum* do not represent two distinct species and instead represent a single species with a diversity of phenotypic polymorphisms.

The same pattern between closely related yet phenotypically different organisms has been shown (Orr and Smith 1998) and especially has been found in birds (Ball et al. 1988; Greenberg et al. 1998; Piertney et al. 2001; Seutin et al. 1995; Zink 1996; Zink and Dittman 1993). These studies have found no phylogeographic structure and no genetic distinctiveness in what appears to be morphologically differentiated organisms. This pattern has been referred to as the “ecophenotype phenomenon” in which morphological differences in birds can be attributed to environmental factors and not genetic differences (James 1983; Seutin et al. 1995; Zink and Remsen 1986). The lack of genetic structuring among phenotypically diverse avian species can be interpreted as the result of birds having a mode of dispersal that largely frees them from obstructions to gene flow. Similarities in phylogeographic patterns between bats and birds have been described (Ditchfield and Burns 1998) and can be

explained by their shared mode of unrestricted dispersal. More recently, bats have demonstrated a phylogeographic pattern that is sharply contrasted to other mammals (Ditchfield 2000). Nonvolant small mammals show low levels of sequence divergence within populations and high levels among populations, while bats show low levels of sequence divergence across a continental distribution. In contrast to bats, small mammalian taxa, such as rodents and some marsupials, have populations that are strictly confined due to distance and geographical isolation (Ditchfield 2000). This overall phylogeographic pattern shown in birds and bats appears to be shared among some fish and insect species (see Orr and Smith (1998) for a review).

Application of the ecophenotype phenomenon to *M. californicus* and *M. ciliolabrum* would suggest that differences in skull morphology are attributed to trophic differences. Strong selective ecological pressures could cause rapid morphological divergence in response to resource competition in the presence of neutral mtDNA polymorphisms. This has been the case in describing differences in bill size for birds (James 1983; Piertney et al. 2001; Smith et al. 1997). It is questionable whether *M. californicus* and *M. ciliolabrum* share this phenomenon and overall phylogeographic pattern without additional sampling, especially since several studies of cryptic chiropteran species *have* revealed patterns of genetic differentiation (Arlettaz et al. 1997b; Barratt et al. 1997; Herd and Fenton 1983; Kingston et al. 2001; Mayer and von Helversen 2001a; Piaggio et al. 2002).

Myotis californicus and *M. ciliolabrum* appear to demonstrate a relationship similar to other vespertilionid bats (Arlettaz et al. 1997b; Barratt et al. 1997; Herd and Fenton 1983; Mayer and von Helversen 2001a; Piaggio et al. 2002), with the majority of these cases in the genus *Myotis* (Arlettaz et al. 1997b; Herd and Fenton 1983; Piaggio et al. 2002). As Jones

(1997) stated, bats contain many cryptic species which can be discovered through molecular and acoustic divergence. In addition to molecular characterization, many of these cases have demonstrated ecological differences between cryptic species (Arlettaz 1995; Barlow 1997; Herd and Fenton 1983; Vaughan et al. 1997). For example, Arlettaz (1995, 1996, 1999) with others (Arlettaz et al. 1997a, 1997b) has extensively studied the relationships of the cryptic species *M. myotis* and *M. blythii* throughout Europe and has found these morphologically similar species to be different both ecologically and genetically.

It may be that the specific mtDNA genes examined in this study were unable to differentiate between the species; however, the phylogenetic utility of mtDNA certainly has been substantiated (Avice 2000; Moritz et al. 1987). Likewise it has proven useful for differentiating between cryptic chiropteran species (Barratt et al. 1997; Kingston et al. 2001; Mayer and von Helversen 2001a; Piaggio et al. 2002). It is possible that diagnostic characters might be found in other genes of the mitochondria, for instance cytochrome oxidase II (Piaggio et al. 2002) or NADH dehydrogenase 1 (Mayer and von Helversen 2001a).

Currently, studies of these two species suggest that *M. californicus* and *M. ciliolabrum* demonstrate ecologically divergent species (Black 1974; Constantine 1998; Gannon et al. 2001; Woodsworth 1981). Woodsworth (1981) has observed *M. californicus* foraging mainly over or near water, while *M. ciliolabrum* foraged in areas over or adjacent to rock bluffs. Additionally, Constantine (1998) observed *M. californicus* at lower elevations (below 1220 m) and *M. ciliolabrum* at higher elevations. Black (1974) suggested that one may be a “beetle strategist,” while the other a “moth strategist.” This suggestion was made on analysis of the combined dietary data for both species since Black (1974) was uncertain

about the identification of either species. Evidence, at least in their northern distribution, suggests they do not partition their diets (Whitaker et al. 1981; Woodsworth 1981), but they do spatially partition their available food sources (Woodsworth 1981). Additionally, differences in echolocation suggest that *M. californicus* and *M. ciliolabrum* partition their ‘auditory space’ (Gannon et al. 2001; O’Farrell and Gannon 1999). Since these frequency differences are apparently maintained across their range (Gannon et al. 2001), it may be possible that echolocation call differences could be correlated to dietary and cranial differences (Barlow et al. 1997; Bogdanowicz et al. 1999).

If *M. californicus* and *M. ciliolabrum* demonstrate differences in habitat use, acoustics and morphology, why is there not genetic divergence? For these species the question remains unanswered as to the evolutionary processes involved in the speciation of these bats. A complete ecological investigation (i.e., diet analysis and spatial partitioning) for these two species in the southwestern United States as well as across their distributions is required to assess their ecological relationships. Additionally, a phylogeographic study of their entire range should be performed to assess whether or not interspecific or intraspecific populations are geographically distributed. Sequencing of additional genes, such as nuclear DNA, for diagnostic characters needs to be initiated to assess the degree of genetic similarity and the possibility of hybridization between the two species both in areas of allopatry and sympatry.

ACKNOWLEDGMENTS

We are grateful to A. Baldwin for technical assistance and J. Apodaca for laboratory assistance. We thank M. A. Bogan and R. J. Baker for discussions and advice on data interpretation. We thank R. C. Dowler and J. K. McCoy for comments on earlier versions of this manuscript. We are thankful to E. W. Valdez and M. A. Bogan for their help in identification of specimens. We thank the curators and institutions for their loans of specimens and tissues.

LITERATURE CITED

- Arlettaz, R. 1995. Ecology of the sibling mouse-eared bats (*Myotis myotis* and *Myotis blythii*): zoogeography, niche, competition, and foraging. Ph.D. Thesis, University of Lausanne, Lausanne, Switzerland. Horus Publishers, Martigny, Switzerland. 208 pp.
- Arlettaz, R. 1996. Feeding behaviour and foraging strategy of free-living mouse-eared bats (*Myotis myotis* and *Myotis blythii*). *Animal Behaviour* 51:1-11.
- Arlettaz, R. 1999. Habitat selection as a major resource partitioning mechanism between the two sympatric sibling bat species *Myotis myotis* and *Myotis blythii*. *Journal of Animal Ecology* 68:460-471.
- Arlettaz, R., N. Perrin, and J. Hausser. 1997a. Trophic resource partitioning and competition between the two sibling bat species *Myotis myotis* and *Myotis blythii*. *Journal of Animal Ecology* 66:897-911.
- Arlettaz, R., M. Ruedi, C. Ibanez, J. Palmeirim, and J. Hausser. 1997b. A new perspective on the zoogeography of the sibling mouse-eared bat species *Myotis myotis* and

- Myotis blythii*: morphological, genetical and ecological evidence. *Journal of Zoology (London)* 242:45-62.
- Avise, J. C. 2000. *Phylogeography: the history and formation of species*. Cambridge, MA: Harvard University Press. 447 pp.
- Baker, R. J. 1981. Chromosome flow between chromosomally characterized taxa of a volant mammal, *Uroderma bilobatum* (Chiroptera: Phyllostomatidae). *Evolution* 35:296-305.
- Ball, R. M. Jr., S. Freeman, F. C. James, E. Bermingham, and J. C. Avise. 1988. Phylogeographic population structure of red-winged blackbirds assessed by mitochondrial DNA. *Proceedings of the National Academy of Sciences USA* 85:1558-1562.
- Bandelt, H. J. and A. W. M. Dress. 1992. Split decomposition: a new and useful approach to phylogenetic analysis of distance data. *Molecular Phylogenetics and Evolution* 1:242-252.
- Barlow, K. E. 1997. The diets of two phonic types of *Pipistrellus pipistrellus* (Chiroptera: Vespertilionidae) in Britain. *Journal of Zoology* 243:597-603.
- Barlow, K. E., G. Jones, and E. M. Barratt. 1997. Can skull morphology be used to predict ecological relationships between bat species? A test using two cryptic species of pipistrelle. *Proceedings of the Royal Society of London, Series B* 264:1695-1700.
- Barratt, E. M., R. Deaville, T. M. Burland, M. W. Bruford, G. Jones, P. A. Racey, and R. K. Wayne. 1997. DNA answers the call of pipistrelle bat species. *Nature* 387:138-139.
- Best, T. L. and J. B. Jennings. 1997. *Myotis leibii*. *Mammalian Species* 547:1-6.
- Bickham, J. W., K. McBee, and D. A. Schlitter. 1986. Chromosomal variation among

- seven species of *Myotis* (Chiroptera: Vespertilionidae). *Journal of Mammalogy* 67(4):746-750.
- Black, H. L. 1974. A north temperate bat community: structure and prey populations. *Journal of Mammalogy* 55:138-157.
- Bogan, M. A. 1974. Identification of *Myotis californicus* and *M. leibii* in southwestern North America. *Proceedings of the Biological Society of Washington* 87(7):49-56.
- Bogan, M. A. 1975. Geographic variation in *Myotis californicus* in the southwestern United States and Mexico. Fish and Wildlife Service, Wildlife Research Report 3:iv + 1-31.
- Bogan, M. A. 1978. A new species of *Myotis* from the Islas Tres Marias, Nayarit, Mexico, with comments on variation in *Myotis nigricans*. *Journal of Mammalogy* 59(3):519-530.
- Bogdanowicz, W., M. B. Fenton, and K. Daleszczyk. 1999. The relationships between echolocation calls, morphology and diet in insectivorous bats. *Journal of Zoology (London)* 247:381-393.
- Bradley, R. D. and R. J. Baker. 2001. A test of the genetic species concept: cytochrome *b* sequences and mammals. *Journal of Mammalogy* 82(4):960-973.
- Bremer, K. 1994. Branch support and tree stability. *Cladistics* 10:395-404.
- Castella, V., M. Ruedi, and L. Excoffier. 2001. Contrasted patterns of mitochondrial and nuclear structure among nursery colonies of the bat *Myotis myotis*. *Journal of Evolutionary Biology* 14:708-720.
- Chaney, A. H. 1993. Keys to the vertebrates of Texas (exclusive of birds). Kingsville: Texas A & I University. 101 pp.

- Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9:1657-1659.
- Constantine, D. G. 1998. An overlooked external character to differentiate *Myotis californicus* and *Myotis ciliolabrum* (Vespertilionidae). *Journal of Mammalogy* 79(2):624-630.
- Cooper, S. J. B., P. R. Day, T. B. Reardon, and M. Schulz. 2001. Assessment of species boundaries in Australian *Myotis* (Chiroptera: Vespertilionidae) using mitochondrial DNA. *Journal of Mammalogy* 82(2):328-338.
- Cooper, S. J. B., T. B. Reardon, and J. Skilins. 1998. Molecular systematics of Australian rhinolophid bats (Chiroptera: Rhinolophidae). *Australian Journal of Zoology* 46:203-220.
- Crandall, K. A. 1994. Intraspecific cladogram estimation: accuracy at higher levels of divergence. *Systematic Biology* 43:222-235.
- Davis, W. B. and D. J. Schmidly. 1994. *The mammals of Texas*. Austin: Texas Parks and Wildlife Press. 338 pp.
- Ditchfield, A. D. 2000. The comparative phylogeography of neotropical mammals: patterns of intraspecific mitochondrial DNA variation among bats contrasted to nonvolant small mammals. *Molecular Ecology* 9:1307-1318.
- Ditchfield, A. D. and K. Burns. 1998. DNA sequences reveal phylogeographic similarities of neotropical bats and birds. *Journal of Comparative Biology* 3(2):165-170.
- Duvernell, D. D. and B. J. Turner. 1998. Evolutionary genetics of Death Valley pupfish populations: mitochondrial DNA sequence variation and population structure. *Molecular Ecology* 7:279-288.

- Edwards, S. V., P. Arctander and A. C. Wilson. 1991. Mitochondrial resolution of a deep branch in the genealogical tree for perching birds. *Proceedings of the Royal Society of London, Series B* 243:99-107.
- Excoffier, L. and P. E. Smouse. 1994. Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. *Genetics* 136:343-359.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution* 39:783-791.
- Fitch, W. M. 1997. Networks and viral evolution. *Journal of Molecular Evolution* 44:S65-S75.
- Gannon, W. L., R. E. Sherwin, T. N. deCarvalho, and M. J. O'Farrell. 2001. Pinnae and echolocation call differences between *Myotis californicus* and *Myotis ciliolabrum* (Chiroptera: Vespertilionidae). *Acta Chiropterologica* 3(1):77-91.
- Gene Codes Corporation Inc. 2001. Sequencher: The complete software solution for sequencing DNA. Version 4.1. Gene Codes Corporation Inc, Ann Arbor, Michigan.
- Glass, B. P. and R. J. Baker. 1965. *Vespertilio subulatus* Say, 1823: proposed suppression under the plenary powers (Mammalia, Chiroptera). *The Bulletin of Zoological Nomenclature* 22:204-205.
- Glass, B. P. and R. J. Baker. 1968. The status of the name *Myotis subulatus* Say. *Proceedings of the Biological Society of Washington* 81:257-260.
- Greenbaum, I. F. 1981. Genetic interactions between hybridizing cytotypes of the tent-making bat (*Uroderma bilobatum*). *Evolution* 35:306-321.
- Greenberg, R., P. J. Cordero, S. Droege, and R. C. Fleischer. 1998. Morphological

- adaptation with no mitochondrial DNA differentiation in the coastal plain swamp sparrow. *The Auk* 115(3):706-712.
- Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 21:160-174.
- Herd, R. M. 1987. Electrophoretic divergence of *Myotis leibii* and *Myotis ciliolabrum* (Chiroptera: Vespertilionidae). *Canadian Journal of Zoology* 65:1857-1860.
- Herd, R. M. and M. B. Fenton. 1983. An electrophoretic, morphological, and ecological investigation of a putative hybrid zone between *Myotis lucifugus* and *Myotis yumanensis* (Chiroptera: Vespertilionidae). *Canadian Journal of Zoology* 61:2029-2050.
- Higginbotham, J. L., and L. K. Ammerman. 2002. Chiropteran community structure and seasonal dynamics in Big Bend National Park. *Special Publications, Museum of Texas Tech University* 44:1-44.
- Hoffman, F. G. And R. J. Baker. 2001. Systematics of the bats of the genus *Glossophaga* (Chiroptera: Phyllostomidae) and phylogeography in *G. soricina* based on the cytochrome-*b* gene. *Journal of Mammalogy* 82(4):1092-1101.
- Holloway, G. L. And R. M. R. Barclay. 2001. *Myotis ciliolabrum*. *Mammalian Species* 670:1-5.
- James, F. C. 1983. Environmental component of morphological differentiation in birds. *Science* 221:184-186.
- Jones, G. 1997. Acoustic signals and speciation: the roles of natural and sexual selection in the evolution of cryptic species. *Advances in the Study of Behavior* 26:317-354.

- Kingston, T., M. C. Lara, G. Jones, Z. Akbar, T. H. Kunz and C. J. Schneider. 2001.
Acoustic divergence in two cryptic *Hipposideros* species: a role for social selection?
Proceedings of the Royal Society of London, Series B 268(1474):1381-1386.
- Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Pääbo, F. X. Villablanca, and A.
C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals:
Amplification and sequencing with conserved primers. Proceedings of the National
Academy of Sciences USA 86:6196-6200.
- Lamb, T., C. Lydeard, R. B. Walker, and J. W. Gibbons. 1994. Molecular systematics of
map turtles (*Graptemys*): a comparison of mitochondrial restriction site versus
sequence data. Systematic Biology 43(4):543-559.
- Macey, J. R., J. A. Schulte II, A. Larson, Z. Fang, Y. Wang, B. S. Tuniyev, and T. J.
Papenfuss. 1998. Phylogenetic relationships of toads in the *Bufo bufo* group from the
eastern escarpment of the Tibetan Plateau: a case of vicariance and dispersal.
Molecular Phylogenetics and Evolution 9:80-87.
- Mayer, F. and O. von Helversen. 2001a. Sympatric distribution of two cryptic bat
species across Europe. Biological Journal of the Linnean Society 74:365-374.
- Mayer, F. and O. von Helversen. 2001b. Cryptic diversity in European bats.
Proceedings of the Royal Society of London, Series B 268(1478):1825-
1832.
- Miller, G. S., Jr., and G. M. Allen. 1928. The American bats of the genera *Myotis* and
Pizonyx. Bulletin of the United States National Museum 144:1-218.
- Moritz, C., T. E. Dowling, and W. M. Brown. 1987. Evolution of animal mitochondrial

- DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics* 18:269-292.
- Nowak, R. M. 1999. Walker's mammals of the world. Sixth edition. Volume 1. Baltimore: John Hopkins University Press. 836 pp.
- O'Farrell, M. J., and W. L. Gannon. 1999. A comparison of acoustic versus capture techniques for the inventory of bats. *Journal of Mammalogy* 80(1):24-30.
- O'Farrell, M. J., B. W. Miller, and W. L. Gannon. 1999. Qualitative identification of free-flying bats using the anabat detector. *Journal of Mammalogy* 80(1):11-23.
- Orr, M. R. and T. B. Smith. 1998. Ecology and speciation. *Trends in Ecology and Evolution* 13(12):502-506.
- Palumbi, S. 1996. Nucleic acids II: The polymerase chain reaction. Pp. 205-246 in *Molecular systematics* (Hillis, D. M., C. Moritz, and B. K. Mable, eds.). 2nd ed. Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.
- Piaggio, A. J., E. W. Valdez, M. A. Bogan, and G. S. Spicer. 2002. Systematics of *Myotis occultus* (Chiroptera: Vespertilionidae) inferred from sequences of two mitochondrial genes. *Journal of Mammalogy* 83(2):386-395.
- Piertney, S. B., R. Summers, and M. Marquiss. 2001. Microsatellite and mitochondrial DNA homogeneity among phenotypically diverse crossbill taxa in the UK. *Proceedings of the Royal Society of London, Series B* 268:1511-1517.
- Posada, D., and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817-818.
- Posada, D. and K. A. Crandall. 2001. Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution* 16(1):37-45.

- Ruedi, M. and F. Mayer. 2001. Molecular systematics of bats of the genus *Myotis* (Vespertilionidae) suggests deterministic ecomorphological convergences. *Molecular Phylogenetics and Evolution* 21(3):436-448.
- Schmidly, D. J. 1991. The bats of Texas. Texas A&M University Press, College Station, Texas, xviii+188pp.
- Seutin, G., L. M. Ratcliffe, and P. T. Boag. 1995. Mitochondrial DNA homogeneity in the phenotypically diverse redpoll finch complex (Aves: Carduelinae: *Carduelis flammae-hornemanni*). *Evolution* 49(5):962-973.
- Simpson, M. R. 1993. *Myotis californicus*. *Mammalian Species* 428:1-4.
- Smith, T. B., R. K. Wayne, D. J. Girman, and M. W. Bruford. 1997. A role for ecotones in generating rainforest biodiversity. *Science* 276:1855-1857.
- Sorenson, M. D. 1999. TreeRot, version 2. Boston University, Boston, MA.
- Spitzenberger, F., J. Pialek, and E. Haring. 2001. Systematics of the genus *Plecotus* (Mammalia, Vespertilionidae) in Austria based on morphometric and molecular investigations. *Folia Zoologica* 50(3):161-172.
- SPSS Inc. 1998. SYSTAT. Version 9. SPSS Inc., Chicago, IL.
- Sudman, P. D., L. J. Barkley, and M. S. Hafner. 1994. Familial affinity of *Tomopeas ravus* (Chiroptera) based on protein electrophoretic and cytochrome *b* sequence data. *Journal of Mammalogy* 75:365-377.
- Swofford, D. L. 2001. PAUP*: phylogenetic analysis using parsimony (* and other methods). Version 4.0b10. Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.
- Templeton, A. R., K. A. Crandall, and C. F. Sing. 1992. A cladistic analysis of phenotypic

- associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* 132:619-633.
- van Zyll de Jong, C. G. 1984. Taxonomic relationships of nearctic small-footed bats of the *Myotis leibii* group (Chiroptera: Vespertilionidae). *Canadian Journal of Zoology* 62:2519-2526.
- van Zyll de Jong, C. G. 1985. Handbook of Canadian mammals. 2. Bats. Ottawa: National Museum of Canada. 212 pp.
- Vaughan, N., G. Jones, and S. Harris. 1997. Habitat use by bats (Chiroptera) assessed by means of a broad-band acoustic method. *Journal of Applied Ecology* 34:716-730.
- Webb, N. J. and C. R. Tidemann. 1995. Hybridisation between black (*Pteropus alecto*) and grey headed (*P. poliocephalus*) flying-foxes (Megachiroptera: Pteropodidae). *Australian Mammalogy* 18(1):19-26.
- Whitaker, J. O., Jr., C. Maser, and S. P. Cross. 1981. Food habits of eastern Oregon bats, based on stomach and scat analysis. *Northwest Science* 4:281-292.
- Wilkinson, G. S. and A. M. Chapman. 1991. Length and sequence variation in evening bat D-loop mtDNA. *Genetics* 128:607-617.
- Wilkinson, G. S., F. Mayer, G. Kerth, and B. Petri. 1997. Evolution of repeated sequence arrays in the D-loop region of bat mitochondrial DNA. *Genetics* 146:1035-1048.
- Williams, D. F. and J. S. Findley. 1979. Sexual dimorphism in vespertilionid bats. *The American Midland Naturalist* 102:113-126.
- Woodsworth, G. C. 1981. Spatial partitioning by two species of sympatric bats, *Myotis californicus* and *Myotis leibii*. Master's thesis, Carleton University, Ottawa, Canada, 68 pp.

Zink, R. M. 1996. Comparative phylogeography of North American birds. *Evolution* 50(1):308-317.

Zink, R. M. and D. L. Dittmann. 1993. Gene flow, refugia, and evolution of geographic variation in the song sparrow (*Melospiza melodia*). *Evolution* 47(3):717-729.

Zink, R. M. and J. V. Remsen, Jr. 1986. Evolutionary processes and patterns of geographic variation in birds. *Current Ornithology* 4:1-69.

Figure Legends

Fig. 1. Geographic distribution of *Myotis californicus*, *M. ciliolabrum*, and *M. leibii* in North America (adapted from Best and Jennings 1997; Holloway and Barclay 2001; Simpson 1993). Dark area indicates area of sympatry between *M. californicus* and *M. ciliolabrum*.

Fig. 2. Skull profile showing angle of slope taken adjacent to top of cranium. (A) *M. californicus*, and (B) *M. ciliolabrum*.

Fig. 3. Relationship between the first two principal components (PC) derived from 11 cranial measurements. Individuals identified as *M. californicus* (o) and *M. ciliolabrum* (x).

Fig. 4. Parsimony and likelihood analyses obtained from cytochrome-*b* sequence of all taxa. A) Single most parsimonious tree (L = 251, CI = 0.829, RI = 0.811). Bootstrap values greater than 50 noted above branches based upon 1000 pseudoreplicates. Bremer support indices noted below branches. Branches without values were not well supported. B) Maximum-likelihood tree based upon the HKY + Γ model of evolution (ln L = -2312.835).

Fig. 5. Strict consensus of 10 most parsimonious trees obtained from control region sequence of the *leibii* group (L = 167 steps, CI = 0.707, RI = 0.732). Bootstrap values greater than 50 noted above branches based upon 1000 pseudo-replicates. Bremer support indices noted below branches. Branches without values were not well supported.

Fig. 6. Strict consensus of 6 most parsimonious trees obtained from combined cytochrome-*b* and control region sequence (L = 605; CI = 0.676; RI = 0.598). Bootstrap values greater than 50 noted above branches based upon 1000 pseudo-replicates. Bremer support indices noted below branches. Branches without values were not well supported.

Fig. 7. Statistical parsimony network produced by TCS from cytochrome-*b* sequence data. Parsimony connection limit (95%) = 11 steps. Filled symbols represent *M. californicus* and open symbols represent *M. ciliolabrum*. Marks on branches represent number of mutations required to connect from one haplotype to another. Dashed line represents same haplotype.

Tables

Table 1. Means, standard errors, and ranges of standard and cranial measurements for all individuals of *M. californicus* and *M. ciliolabrum* ($n = 32$). Measurements of the height of the coronoid process (HCP) for *M. californicus* (TTU 45840) and *M. ciliolabrum* (ASK 5171) are not included in the values given below because they lacked a lower mandible.

Character	<i>M. californicus</i> $n = 15$	<i>M. ciliolabrum</i> $n = 17$
Total Length (TOT)	82.00 ± 6.35 (65.00 – 93.00)	82.56 ± 4.30 (75.00 – 90.00)
Tail Length (TA)	36.80 ± 6.04 (20.00 – 44.00)	38.56 ± 4.00 (30.00 – 45.00)
Hindfoot Length (HF)	6.30 ± 1.19 (3.50 – 8.00)	7.18 ± 0.88 (6.00 – 9.00)
Forearm Length (FA)	32.02 ± 1.11 (29.74 – 35.54)	33.55 ± 1.13 (31.78 – 35.37)
Slope	28.13 ± 3.31 (23.00 – 36.00)	21.53 ± 2.90 (15.00 – 25.00)
Greatest Skull Length (GSL)	13.55 ± 0.36 (13.02 – 14.22)	13.99 ± 0.40 (13.06 – 14.74)
Rostral Breadth (RB)	5.16 ± 0.16 (4.88 – 5.42)	5.33 ± 0.16 (5.08 – 5.55)
Mastoid Width (MW)	6.93 ± 0.16 (6.71 – 7.13)	7.07 ± 0.16 (6.69 – 7.34)
Interorbital Width (IOW)	3.20 ± 0.11 (2.95 – 3.39)	3.19 ± 0.08 (3.03 – 3.35)

Rostral Width (RW)	3.12 ± 0.11 (2.93 – 3.32)	3.31 ± 0.13 (2.97 – 3.47)
Width of Upper Incisors (I3I3W)	2.16 ± 0.09 (1.94 – 2.28)	2.30 ± 0.08 (2.17 – 2.47)
Length of Maxillary Toothrow (MTL)	5.03 ± 0.15 (4.73 – 5.24)	5.27 ± 0.22 (4.96 – 5.97)
Length of P4M3 series (P4M3L)	3.58 ± 0.13 (3.35 – 3.77)	3.78 ± 0.13 (3.46 – 3.99)
Cranial Depth (CD)	5.74 ± 0.27 (5.15 – 6.27)	5.58 ± 0.25 (4.87 – 5.89)
Height of the Coronoid Process (HCP)	2.74 ± 0.18 (2.41 – 3.01)	3.05 ± 0.29 (2.77 – 3.97)

Table 2. Component loadings and percent of total variance explained from principal component analysis (PCA) of cranial characters.

	PC1	PC2
SLOPE	-0.609	0.615
GSL	0.889	0.203
RB	0.840	-0.084
MW	0.826	0.205
IOW	0.316	0.570
RW	0.854	-0.219
I3I3W	0.760	0.055
MTL	0.643	-0.165
P4M3L	0.905	-0.043

CD	0.190	0.839
HCP	0.811	0.057
Proportion (%)	53.5	14.4
Cumulative (%)	53.5	67.9

Table 3. Average genetic distances with standard errors and ranges between and within species calculated from cytochrome *b* and control region sequences. Estimates are based on the HKY + Γ (Hasegawa et al. 1995) model of DNA evolution.

	Cytochrome <i>b</i>	Control region
<i>M. yumanensis</i> versus all	19.5 ± 1.1 (17.7 – 22.8)	29.5 ± 3.7 (22.8 – 36.7)
<i>M. lucifugus</i> versus all	18.6 ± 1.1 (17.2 – 22.8)	18.0 ± 4.6 (12.7 – 36.1)
<i>M. evotis</i> versus <i>leibii</i> group	2.9 ± 0.8 (1.1 – 4.1)	18.0 ± 3.5 (11.7 – 27.4)
<i>M. leibii</i> versus <i>M. californicus</i>	3.7 ± 0.6 (3.1 – 4.8)	5.1 ± 2.5 (1.8 – 8.9)
<i>M. leibii</i> versus <i>M. ciliolabrum</i>	3.8 ± 0.9 (1.9 – 5.0)	5.0 ± 2.3 (3.0 – 9.9)
<i>M. californicus</i> versus <i>M. ciliolabrum</i>	2.3 ± 1.2 (0.3 – 4.6)	7.3 ± 3.4 (1.0 – 16.5)
Within <i>M. californicus</i>	2.1 ± 1.3 (0.0 – 4.1)	7.7 ± 3.3 (0.5 – 13.1)
Within <i>M. ciliolabrum</i>	2.5 ± 1.3	7.3 ± 3.5

(0.3 – 4.8)

(0.5 – 16.3)

APPENDIX I

Specimens examined.—List of specimens examined for both morphological and molecular analysis. Institutional abbreviations; Angelo State Natural History Collection (ASNHC), Abilene Christian University Natural History Collection (ACUNHC), University of Texas at El Paso (UTEP), Natural Science Research Laboratory (NSRL), and Museum of Southwestern Biology (MSB).

Myotis californicus caurinus.—California: El Dorado County, 6 mi E Somerset (♀, NK 576, *cyt-b*—AF#####, control region—AF#####). Humboldt County, 8 mi N, 1.5 mi E Arcata (♀, NK 599, *cyt-b*—AF#####, control region—AF#####).

Myotis californicus stephensi.—Utah: Wayne County, Capitol Reef National Park (♂, NK 34800, *cyt-b*—AF#####, control region—AF#####).

Myotis californicus californicus.—Arizona: Cochise County, 3 mi W Portal (♂, ACUNHC 744, *cyt-b*—AF#####, control region—AF#####). Pima County, 6 mi S, 8 mi E Continental Santa Rita Experimental Range Headquarters (♂, ASK 5170, *cyt-b*—AF#####, control region—AF#####). New Mexico: Hidalgo County, Animas Mountains (♂, NK 3658, *cyt-b*—AF#####, control region—AF#####). Otero County, Lincoln National Forest (♀, NK 32149, *cyt-b*—AF#####, control region—AF#####). Texas: Brewster County, Big Bend National Park (♂, ASK 3661, *cyt-b*—AF#####, control region—AF#####). Culberson County, Sierra Diablo Wildlife Management Area (♀, TK

54207, cyt-*b*—AF#####, control region—AF#####); Guadalupe National Park (♂, TTU 19939); 23 mi E NE Van Horn (♀, TTU 45840). El Paso County, El Paso (♀, UTEP 2085); 31°55'40" N, 106°29'W (♂, UTEP 3926).

Jeff Davis County, Davis Mountains (♂, TTU 9150). Presidio County, Big Bend Ranch State Natural Area (♀, TK 48149, cyt-*b*—AF#####, control region—AF#####).

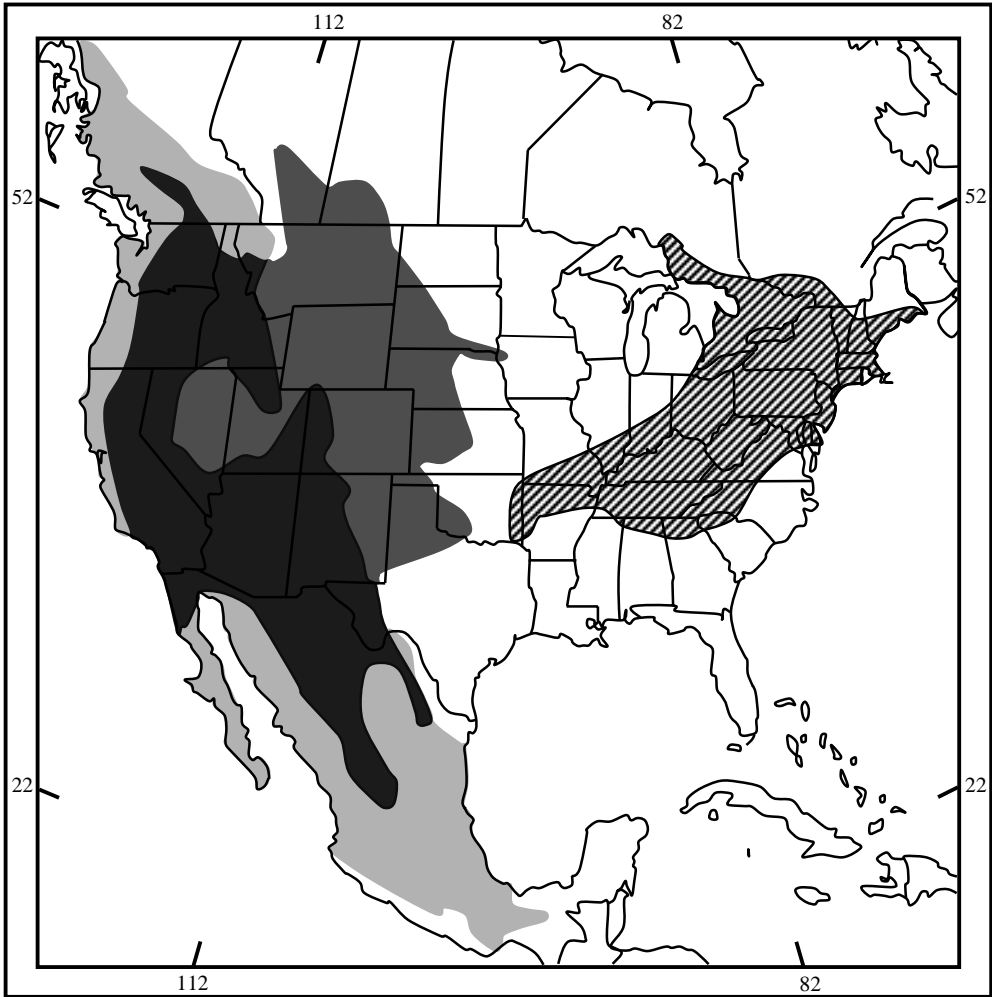
Myotis ciliolabrum melanorhinus.—Arizona: Cochise County, Stewart Creek Campground (♀, NK 37254, cyt-*b*—AF#####, control region—AF#####). Pima County, 6 mi S, 8 mi E Continental Santa Rita Experimental Range Headquarters (♂, ASK 5171, cyt-*b*—AF#####, control region—AF#####). New Mexico: Eddy County, Lincoln National Forest (♀, NK 32086, cyt-*b*—AF#####, control region—AF#####). Otero County, Lincoln National Forest (♀, NK 32128, cyt-*b*—AF#####, control region—AF#####). Sandoval County, 2.7 mi S, 4.3 mi W Jemez Springs, Guadalupe River (♂, NK 39583, cyt-*b*—AF#####, control region—AF#####). Oklahoma: Cimarron County, 2 mi E, 3 mi S Kenton (♀, NK 24872, cyt-*b*—AF#####, control region—AF#####). Texas: Brewster County, Big Bend National Park (♂, ASK 6044, cyt-*b*—AF#####, control region—AF#####). Culberson County, Sierra Diablo Wildlife Management Area (♀, TK 54207, cyt-*b*—AF#####, control region—AF#####); Guadalupe National Park (♂, TTU 19939); 23 mi E NE Van Horn (♀, TTU 45840). El Paso County, Hueco Tanks State Park (♀, UTEP 3463); Hueco Tanks State Park (♀, UTEP 3477); El Paso (♂, UTEP 6165). Jeff Davis County, 3.5 mi NE Fort Davis (♀, TTU 9166); 3.5 mi NE Fort Davis (♂, TTU 9167); 3.5 mi NE Fort Davis (♂, TTU 37221). Presidio County, Big Bend Ranch State Natural Area (♀, TK 48058, cyt-*b*—AF#####, control region—AF#####); 10 mi W SW Valentine (♂, TTU

78520). Utah: Wayne County, Capitol Reef National Park (♂, NK 34803, cyt-*b*—AF#####, control region—AF#####).

Myotis leibii.—West Virginia: Randolph County, 2.7 mi N, 4.7 mi E Bowden (., CM 82050, cyt-*b*—AF#####, control region—AF#####).

Myotis evotis.—New Mexico: Cibola County, Bureau of Land Management, W of Cerro Remdija (., NK 39900, cyt-*b*—AF#####, control region—AF#####).

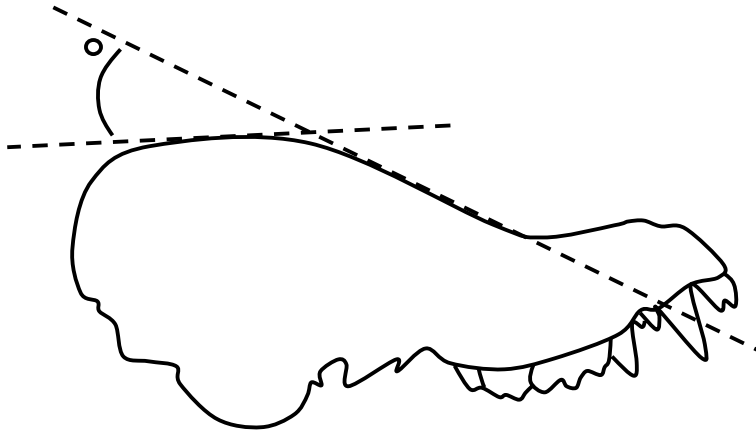
Myotis yumanensis.—Oklahoma: Cimarron County, 3 mi E, 1.5 mi S Kenton (., TK 28786, cyt-*b*—AF#####, control region—AF#####).



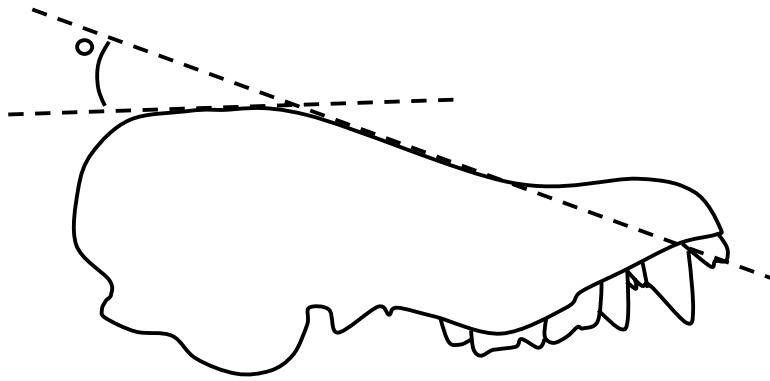
■ *M. californicus*

■ *M. ciliolabrum*

▨ *M. leibii*



A



B

