

## MITOCHONDRIAL DNA DIVERGENCE DOES NOT REFLECT MORPHOLOGICAL DIFFERENCE BETWEEN *MYOTIS CALIFORNICUS* AND *MYOTIS CILIOLABRUM*

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The California myotis, *Myotis californicus*, and western small-footed myotis, *Myotis ciliolabrum*, are 2 morphologically similar bats which 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 cytochrome-*b* and control region fragments were amplified from tissue samples ( $n = 20$ ) from Texas, Oklahoma, New Mexico, Arizona, Utah, and California to examine species boundaries and phylogenetic relationships. DNA sequences (1,184 bp) were used in parsimony and maximum-likelihood analyses. To corroborate species identification, cranial measurements from all specimens included in the molecular analysis along with additional specimens were analyzed statistically using principal components analysis (PCA). Individuals were designated either as *M. californicus* or *M. ciliolabrum*. Molecular analysis placed *M. leibii* within clades containing both *M. californicus* and *M. ciliolabrum*. In addition, sequence divergence and phylogenetic results do not recover distinct lineages for each species (*M. californicus* and *M. ciliolabrum*) as recognized by morphology. Our results suggest that these species have recently diverged, or alternatively that they are 1 phenotypically variable species. Based on network estimation of cytochrome-*b* haplotypes, phylogeographic structure is minimally represented between haplotypes with an east-west separation in the southwestern United States.

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

Species of the *Myotis 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 (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* relegated to 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, presence of free-tail, and pinnae size) 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). Bogan (1974, 1975) reported that 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 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, whereas 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*. Gannon et al. (2001) found the pinnae of *M. ciliolabrum* to be slightly larger than those of *M. californicus*. These differences are so subtle and variable that

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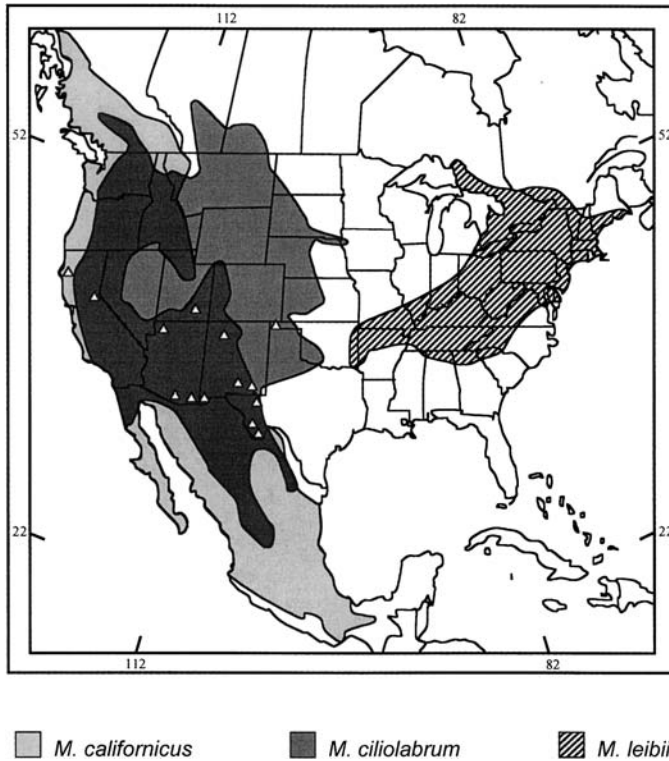


FIG. 1.—Geographic distribution of *Myotis californicus*, *M. ciliolabrum*, and *M. leibii* in North America (adapted from Best and Jennings 1997; Holloway and Barclay 2001; Simpson 1993). Dark area indicates area of sympatry between *M. californicus* and *M. ciliolabrum*. Triangles represent localities for specimens used in this study.

they rarely allow for differentiation between the 2 species in the field and have been questionable for bats captured in Texas (Higginbotham and Ammerman 2002).

Although cranial morphology seems most useful for differentiating between the species, it is not always definitive. *M. californicus* is characterized as having a globose cranium with an abruptly sloping forehead, whereas *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 2 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 2 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.

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 2 species, although for the majority of specimens *M. californicus* appeared tri-colored whereas *M. ciliolabrum* was bi-colored. Morphological descriptions were recorded along with all pertinent data from the museum label. Sexes were pooled because sexual dimorphism has not been demonstrated in these bats (Bogan 1974; Constantine 1998; van Zyll de Jong 1984; Williams and Findley 1979).

Ten cranial measurements (Table 1) adapted from van Zyll de Jong (1984) were recorded for each specimen by using digital calipers calibrated to the nearest 0.01 mm. Because both species also have been differentiated based on forehead slope, this character was taken as an additional measurement. The slope was determined by using a protractor to measure the angle between the forehead and the top of the skull from a digital image of the skull profile (Rodriguez 2002). All skulls were mounted in the same manner. Each was centered with calipers, held with mounting clay, and placed equidistant from the camera to obtain photographs that would produce consistent measurements. All 11 cranial characters were subjected to principal components analysis (PCA) using SYSTAT version 9 software (SPSS Inc., 1998) to demonstrate the ability of traditional methods to discriminate between the 2 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, because of evidence suggesting a close relationship to the *leibii* group (Herd 1987). For further outgroup comparison, *M. yumanensis* ( $n = 1$ ; Appendix I), which shows no close relation to the 4 other species, was chosen along with published sequences of *M. lucifugus* (Ruedi and Mayer 2001; Wilkinson et al. 1997).

Total genomic DNA was extracted from frozen or alcohol-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 combination of cytochrome-*b* primers known to work generally for vertebrates was used (Edwards et al. 1991; Kocher et al. 1989). This pair consists of L14841, 5'-AAAAAGCTTCCATCCAACATCTCAG-CATGATGAAA-3' (Kocher et al. 1989) and H15547, 5'-GGCAA-TAGGAAATATCATTCC-3' (Edwards et al. 1991). The following control region primers known to work specifically for bats were used: P, 5'-TCCTACCATCAGCACCCAAAGC-3' and F, 5'-GTTGCTGTT-TACGGAGGTAG-3' (Wilkinson and Chapman 1991; Wilkinson et al. 1997). Reactions were amplified for an initial cycle of denaturation at 93.0°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

**TABLE 1.**—Standard and cranial measurements (mm) for all individuals of *M. californicus* and *M. ciliolabrum* ( $n = 32$ ), given as mean and *SE*, with range in parentheses. Measurements of the height of the coronoid process for *M. californicus* (specimen number 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> $n = 17$		
	$\bar{X}$	<i>SE</i>	Range	$\bar{X}$	<i>SE</i>	Range
Total length	82.00	6.35	(65.00–93.00)	82.56	4.30	(75.00–90.00)
Tail length	36.80	6.04	(20.00–44.00)	38.56	4.00	(30.00–45.00)
Hindfoot length	6.30	1.19	(3.50–8.00)	7.18	0.88	(6.00–9.00)
Forearm length	32.02	1.11	(29.74–35.54)	33.55	1.13	(31.78–35.37)
Slope of forehead	28.13	3.31	(23.00–36.00)	21.53	2.90	(15.00–25.00)
Greatest skull length	13.55	0.36	(13.02–14.22)	13.99	0.40	(13.06–14.74)
Rostral breadth	5.16	0.16	(4.88–5.42)	5.33	0.16	(5.08–5.55)
Mastoid width	6.93	0.16	(6.71–7.13)	7.07	0.16	(6.69–7.34)
Interorbital width	3.20	0.11	(2.95–3.39)	3.19	0.08	(3.03–3.35)
Rostral width	3.12	0.11	(2.93–3.32)	3.31	0.13	(2.97–3.47)
Width of upper incisors	2.16	0.09	(1.94–2.28)	2.30	0.08	(2.17–2.47)
Length of maxillary toothrow	5.03	0.15	(4.73–5.24)	5.27	0.22	(4.96–5.97)
Length of P4–M3 series	3.58	0.13	(3.35–3.77)	3.78	0.13	(3.46–3.99)
Cranial depth	5.74	0.27	(5.15–6.27)	5.58	0.25	(4.87–5.89)
Height of coronoid process	2.74	0.18	(2.41–3.01)	3.05	0.29	(2.77–3.97)

bromide. PCR products were cloned following the manufacturer's protocol according to the TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, California) using one-fourth of the recommended amounts. 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<sup>TM</sup> DNA Sequencing Kit–LC (Epicentre, Madison, Wisconsin) using one-fourth of the amounts they recommend. Cycle sequencing of both strands was performed with dye-labeled M13 primers on an automated sequencer (LI-COR Long Read Dual Laser 4200, LI-COR Inc., Lincoln, Nebraska). Two clones were sequenced for selected samples to test for differences among clones, but sequence differences were found to be minimal and did not contradict results reported herein. Thus, single clones were sequenced from the remaining individuals.

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 Corporation 2001). Parsimony analysis 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) to further evaluate nodal support.

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, HKY +  $\Gamma$  (Hasegawa et al. 1985), 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).

Because the results of the phylogenetic analysis failed to recover lineages that correspond to current species designations, and instead suggested that there was notable gene flow between them, 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 can result in homoplasies (Posada and Crandall 2001). The computer program TCS version 1.13 (Clement et al. 2000) was used to construct a network diagram for individuals of *M. californicus* and *M. ciliolabrum*. The program 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 5th state, all haplotypes were incorporated into the network based upon a 95% parsimony connection limit.

## 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, principal component (PC) 1 accounted for 53.5% of the total variation. The 1st principal component (Table 2) was dominated by characters associated with cranial length and width, such as greatest skull length, rostral breadth, rostral width, and mastoid width. Additionally, the length of the P4–M3 series and height of the coronoid process strongly contributed to the overall variation explained by PC1. The variation explained by PC2 (14.4%) was most affected by cranial depth, interorbital width, and forehead slope. Projection of the first 2 principal components revealed a distinct separation between specimens of *M. californicus* and *M. ciliolabrum* (Fig. 2). 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 height of the coronoid process, these taxa associated with their suggested groups (not shown).

**Molecular analysis.**—Seven hundred and fifty eight base pairs of the cytochrome-*b* gene sequence were sequenced for

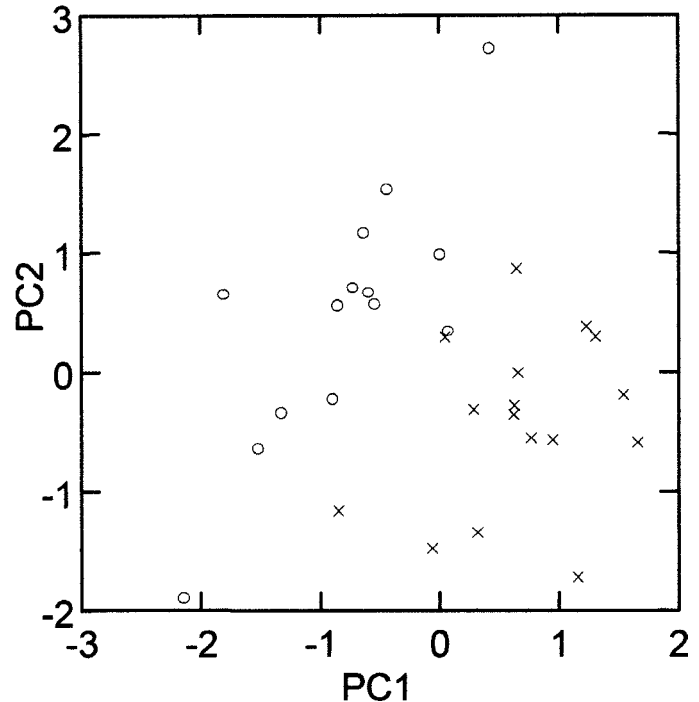
**TABLE 2.**—Component loadings and percentages of total variance explained from principal component analysis of cranial characters as shown by first 2 principal components (PC).

	PC 1	PC 2
Slope of forehead	-0.609	0.615
Greatest skull length	0.889	0.203
Rostral breadth	0.840	-0.084
Mastoid width	0.826	0.205
Interorbital width	0.316	0.570
Rostral width	0.854	-0.219
Width of upper incisors	0.760	0.055
Length of maxillary toothrow	0.643	-0.165
Length of P4–M3 series	0.905	-0.043
Cranial depth	0.190	0.839
Height of coronoid process	0.811	0.057
Proportion (%)	53.5	14.4
Cumulative (%)	53.5	67.9

each taxon (GenBank Accession No. AY460343–AY460365), of which 556 characters were constant, 130 were parsimony uninformative, and 72 were parsimony informative. Parsimony analysis of cytochrome-*b* produced a single most parsimonious tree (length = 251 steps; Fig. 3A) with a consistency index (CI) = 0.829 and retention index (RI) = 0.811. Branch-and-bound search methods produced identical topologies in all parsimony analyses. Strong bootstrap support was found for 2 monophyletic groups containing a mixture of *M. californicus* and *M. ciliolabrum* individuals. Bootstrap analysis, as well as the Bremer support index, supported the exclusion of *M. yumanensis* and *M. lucifugus* from the *leibii* group (Fig. 3A), yet failed to separate *M. evotis* from the *leibii* group.

Using the HKY +  $\Gamma$  (Hasegawa et al. 1985) model of evolution suggested by MODELTEST (Posada and Crandall 1998), genetic distances were calculated in PAUP with transition/transversion ratio = 7.065,  $\Gamma$  = 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%) to all other taxa were higher than all other species comparisons (Table 3). *Myotis evotis* compared to *M. yumanensis* and *M. lucifugus* displayed divergence values relatively similar (18.8% and 19.5%, respectively). Yet, when compared to each member of the *leibii* group, average divergences were unexpectedly low, ranging from 1.1% to 4.1%. Average 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 percentage 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 +  $\Gamma$  model of evolution produced a tree that was similar in topology to the most parsimonious tree (Fig. 3B). An exception was 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.



**FIG. 2.**—Relationship between the 1st 2 principal components (PC) derived from 11 cranial measurements. Individuals identified as *Myotis californicus* (o) and *M. ciliolabrum* (x).

For the control region, variable sequence lengths were obtained among taxa because of insertions/deletions at the 3' end, which made alignment problematic. Regions of control region sequence that could not be unambiguously aligned were excluded because they likely violated the assumption of positional homology. We took a conservative approach and only analyzed the section (from 5' end to the beginning of the questionable area) that aligned well. This resulted in a sequence of 426 base pairs with 262 constant, 90 parsimony uninformative, and 74 parsimony informative characters (GenBank accession no. AY460366–AY460388). MODELTEST identified the HKY +  $\Gamma$  (Hasegawa et al. 1985) 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  $\alpha$  = 0.4216. Average pairwise comparisons of *M. yumanensis* (34.4%) and *M. lucifugus* (22.3%) to all the other taxa produced exceptionally high divergences (Table 3). In contrast to cytochrome-*b* data, *M. evotis* sequences had higher average divergences (19.7%) compared to the *leibii* group. Average percentage distances for *M. leibii* compared to both *M. californicus* (4.8%) and *M. ciliolabrum* (4.8%) largely overlapped. Yet, these values were noticeably lower than the average distances between *M. californicus* and *M. ciliolabrum* (6.8%). Comparison of interspecific percent divergences to average values within *M. californicus* (7.1%) and within *M. ciliolabrum* (6.8%) were similar (Table 3).

Although both parsimony and maximum likelihood analyses including all outgroup taxa were performed (not shown), extensive divergence values (32.0–47.3%) for *M. yumanensis*,

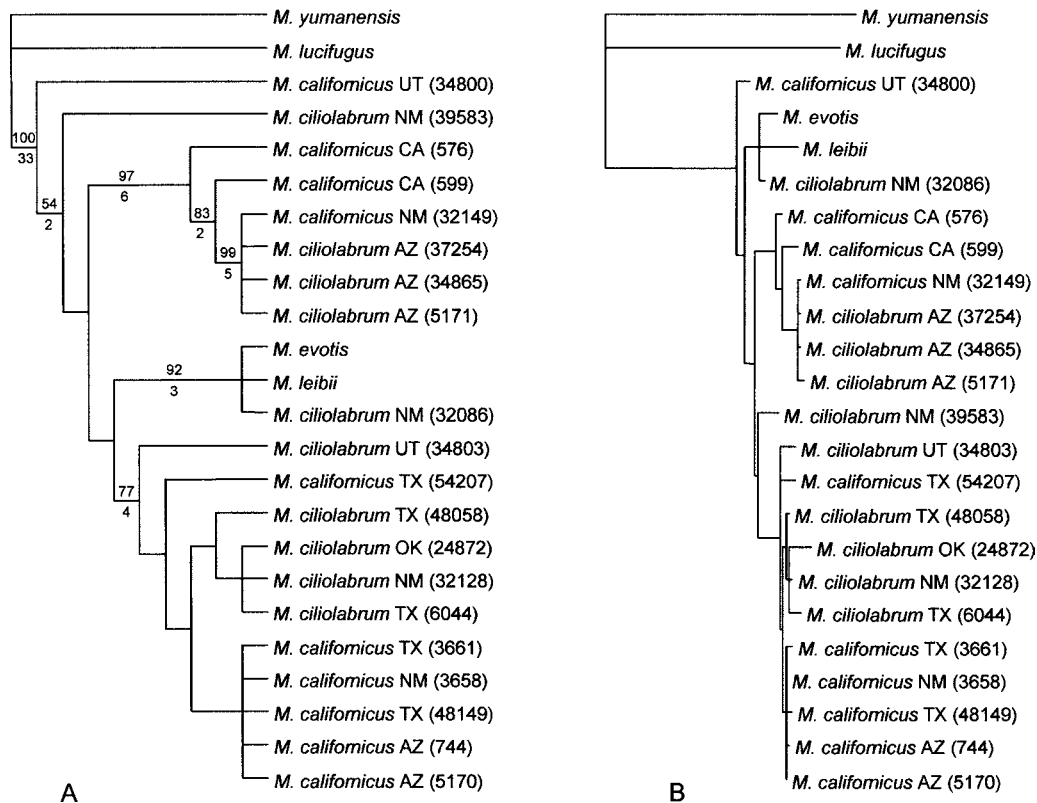


FIG. 3.—Parsimony and likelihood analyses obtained from cytochrome-*b* sequence of 6 species of *Myotis*. A) Single most parsimonious tree ( $L = 251$ ,  $CI = 0.829$ ,  $RI = 0.811$ ), with bootstrap values  $>50$  noted above branches based upon 1,000 pseudoreplicates and Bremer support indices noted below branches. B) Maximum-likelihood tree based upon the  $HKY + \Gamma$  model of evolution ( $\ln L = -2312.835$ ).

*M. lucifugus*, and *M. evotis* were found from pairwise comparisons with ingroup taxa. All outgroup taxa were excluded from additional phylogenetic analyses due to expected saturation and a high degree of homoplasy and an unrooted analysis was performed to interpret relationships within the *leibii* group. A strict consensus tree was calculated from 10 most parsimonious trees (Fig. 4). Moderate bootstrap support (71%) was found for 2 clades containing the majority of *M. californicus* and *M. ciliolabrum* taxa. These groups do not correspond to the 2 clades found from cytochrome-*b* data (Fig. 3A). Combining data from the two genes does not provide any additional insight into the relationships among the species.

In fact, the number of supported nodes is comparatively lower than when analyzed separately (results not shown).

Parsimony and maximum likelihood analyses produced no distinct structuring that differentiated between *M. californicus* and *M. ciliolabrum*. Because these results were indicative of some level of gene flow between the 2 species, patterns of gene flow were investigated by network estimation using all taxa of *M. californicus* and *M. ciliolabrum*. Network reconstruction of cytochrome-*b* haplotypes resulted in 2 groups, a western and eastern clade (Fig. 5). The 2 groups appeared to be separated in Arizona with the exception of *M. californicus* (32149) in south-central New Mexico (Otero County) belonging to the western

TABLE 3.—Genetic distances (%) between and within species calculated from cytochrome *b* and control region sequences. Estimates are based on the  $HKY + \Gamma$  (Hasegawa et al. 1985) model of DNA evolution.

	Cytochrome <i>b</i>			Control region		
	$\bar{X}$	<i>SE</i>	Range	$\bar{X}$	<i>SE</i>	Range
<i>M. yumanensis</i> compared to all	19.5	1.1	17.7–22.8	34.4	4.8	27.7–47.3
<i>M. lucifugus</i> compared to all	18.6	1.1	17.2–22.8	22.3	6.4	14.0–47.3
<i>M. evotis</i> compared to <i>leibii</i> group	2.9	0.8	1.1–4.1	19.7	3.7	13.7–32.0
<i>M. leibii</i> compared to <i>M. californicus</i>	3.7	0.6	3.1–4.8	4.8	2.1	1.8–8.1
<i>M. leibii</i> compared to <i>M. ciliolabrum</i>	3.8	0.9	1.9–5.0	4.8	2.2	2.9–9.1
<i>M. californicus</i> compared to <i>M. ciliolabrum</i>	2.3	1.2	0.3–4.6	6.8	3.0	1.0–15.7
Within <i>M. californicus</i>	2.1	1.3	0.0–4.1	7.1	2.8	0.5–11.8
Within <i>M. ciliolabrum</i>	2.5	1.3	0.3–4.8	6.8	3.1	0.5–16.1

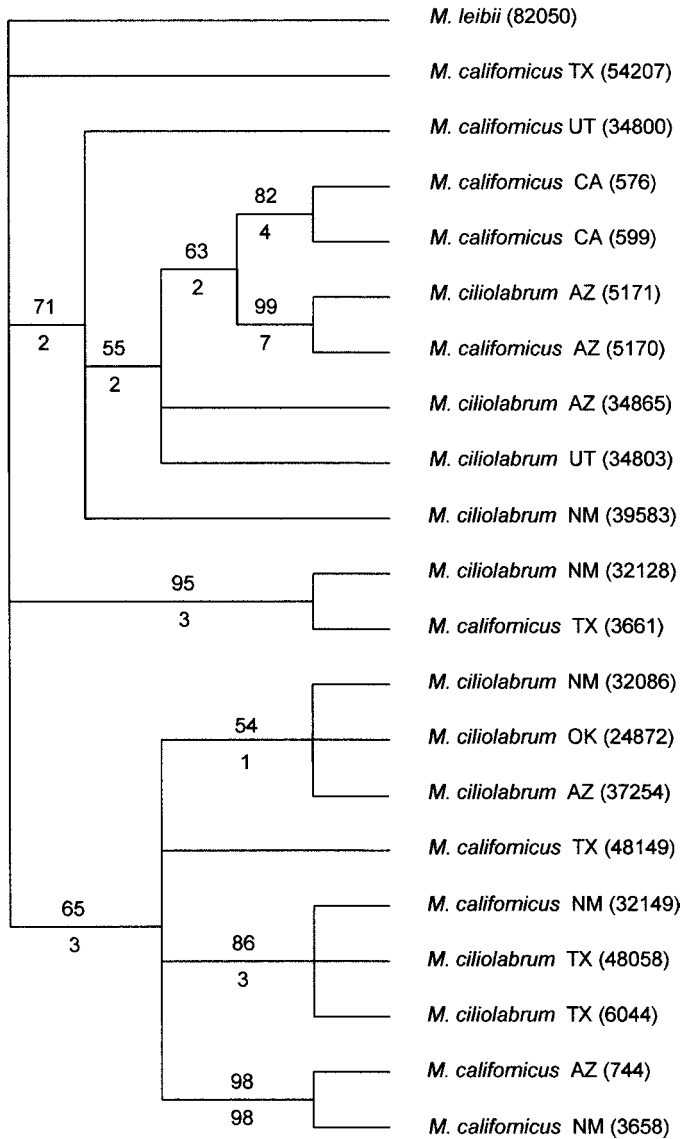


FIG. 4.—Unrooted strict consensus of 10 most parsimonious trees obtained from control region sequence of the *Myotis leibii* group (L = 167 steps, CI = 0.707, RI = 0.732). Bootstrap values >50 noted above branches based upon 1,000 pseudo-replicates; Bremer support indices noted below branches.

clade. These phylogroups also were found as distinct clades within the most parsimonious cytochrome-*b* tree (Fig. 3A). All taxa demonstrated unique haplotypes with the exception of *M. californicus* NM (3658) and *M. californicus* AZ (5170) exhibiting the same haplotype. The same 3 individuals (*M. californicus* UT [34800], *M. ciliolabrum* NM [39583], and *M. ciliolabrum* NM [32086]) that were outside any *M. californicus*-*M. ciliolabrum* clade in the most parsimonious tree did not form any connections to the network (Fig. 5). 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, 1 additional step (total of 12 steps) was required to connect with other taxa. Sixteen steps were required to connect all individuals and groups into 1 complete network. Because of

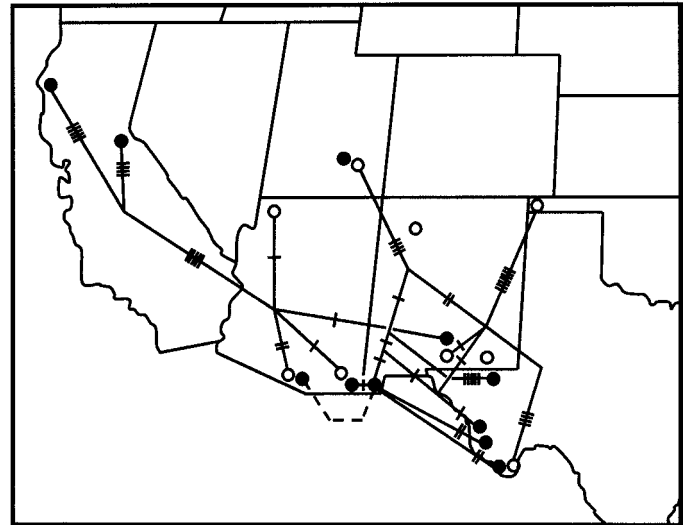


FIG. 5.—Statistical parsimony network produced by TCS computer program from cytochrome-*b* sequence data of *Myotis californicus* and *M. ciliolabrum* samples. Parsimony connection limit (95%) = 11 steps. Filled symbols (●) represent *M. californicus* and open symbols (○) represent *M. ciliolabrum*. Crossbars on branches represent number of mutations required to connect from one haplotype to another. Dashed line connects 2 localities with the same haplotype. Map shows southwestern United States, including California, Utah, Arizona, New Mexico, Texas, and Oklahoma.

extensive changes among taxa, the control region failed to form a network.

### DISCUSSION

Based on principal components analysis of cranial morphology, *M. californicus* and *M. ciliolabrum* can be distinguished from one another. This similar pattern was found in additional principal components analyses of cranial characters (Rodriguez 2002). As other authors reported, *M. californicus* generally has a smaller skull with an abrupt slope of the forehead and greater cranial depth, whereas *M. ciliolabrum* has a larger skull with a gradual slope of the forehead and lesser cranial depth (Bogan 1974; Constantine 1998; Miller and Allen 1928; van Zyll de Jong 1984). Among those characters that contributed to separation between the 2 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. Cytochrome-*b* divergences within and among *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*, whereas 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 >10.0% between species, and intraspecific values were generally

<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. This study suggests that *M. californicus* and *M. ciliolabrum* have not diverged to the same degree as other *Myotis* species and possibly should be considered a single species. The same could be suggested for *M. leibii*, but more data from this species are 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 >15.0% between species of rhinolophid bats, whereas intraspecific values were generally <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% ( $n = 4$  pairwise comparisons) and largely ranged within 3.7% to 9.9%. 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 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 warrant 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). 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.

Parsimony and likelihood analyses of cytochrome-*b* sequence did not illustrate any clear pattern of monophyly within either species (Fig. 3). These results were supported by similar analyses of control region data (Fig. 4). In fact, relationships demonstrated from topologies of each gene tree conflicted marginally; that is, taxa shown to be close relatives in 1 gene tree were distant relatives in the other tree. Additionally, according to J. Zinck (pers. comm.), data from the mitochondrial 16S ribosomal subunit gene also fail to differentiate

between the 2 species. Phylogenetic analysis using network construction of cytochrome-*b* sequence illustrated a better geographic representation of gene flow, yet produced minimal phylogeographic structure (Fig. 5).

Two explanations could be derived from the overall phylogenetic and phylogeographic patterns observed. First, it could be that *M. californicus* and *M. ciliolabrum* do not represent distinct species and instead represent a single species with a diversity of phenotypic polymorphisms. Secondly, the observed polyphyletic relationships could be explained by recent divergence and incomplete lineage sorting (Avice 2000).

The same pattern between closely related yet phenotypically different organisms has been shown (Orr and Smith 1998), especially in birds (Ball et al. 1988; Greenberg et al. 1998; Piertney et al. 2001; Seutin et al. 1995; Zink 1996; Zink and Dittmann 1993). 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). 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.

Studies of these 2 species suggest that *M. californicus* and *M. ciliolabrum* do demonstrate ecological divergence (Black 1974; Constantine 1998; Gannon et al. 2001; Woodsworth 1981). Woodsworth (1981) has observed *M. californicus* foraging mainly over or near water, whereas *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 species might be a beetle strategist, and the other a moth strategist. 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 et al. 1999). *Myotis californicus* has a characteristic frequency of 50 kHz; *M. ciliolabrum* has a characteristic frequency of 40 kHz. Although these frequency differences are apparently maintained across their range (Gannon et al. 2001), it might be possible that echolocation call differences could be correlated with 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? Perhaps these species are distinct, but very young lineages. Our results suggest the retention of ancestral polymorphisms as a result of incomplete lineage sorting. Such has been a response to discord between gene trees and the assumed species phylogeny (Maddison 1997). Patterns of polyphyly and paraphyly are indicative of early stages of speciation as predicted by the coalescent theory, in which

polyphyly is the initial step in the matriarchal ancestry (Avice 2000; Neigel and Avice 1986). Daughter populations of the ancestral pool are expected to become reciprocally monophyletic after lineage sorting has commenced over time. Thus, the polyphyletic pattern for *M. californicus* and *M. ciliolabrum* suggests that their divergence has been recent.

A phylogeographic and ecological investigation (i.e., diet analysis and spatial partitioning) spanning the entire range of *M. californicus* and *M. ciliolabrum* should be performed to make adequate assessments of the evolutionary processes at work. Sequencing of additional nuclear genes from the 2 species both in areas of allopatry and sympatry will also be important for confirming the phylogeographic patterns and distinguishing between alternative hypotheses.

### RESUMEN

*Myotis californicus* y *Myotis ciliolabrum* son dos especies de murciélagos morfológicamente similares que han tenido una compleja historia taxonómica por causa de la variación geográfica intraespecífica a través de su distribución simpátrida en el oeste de Norteamérica. Pese a varias diferencias publicadas, la identificación de estas especies en el campo sigue siendo problemática en el suroeste de Estados Unidos. Se amplificaron fragmentos del gen del citocromo *b* y de la región de control mitocondriales a partir de muestras de tejidos ( $n = 20$ ) de Texas, Oklahoma, Nuevo México, Arizona, Utah, y California, a efectos de examinar los límites entre las especies y las relaciones filogenéticas. Las secuencias de ADN (1184 pares de bases) fueron utilizadas en análisis por parsimonia y máxima verosimilitud. Para corroborar la identificación de las especies se tomaron medidas craneanas de todos los ejemplares incluidos en los análisis moleculares, así como de ejemplares adicionales; las mismas fueron examinadas estadísticamente usando análisis de componentes principales. Los ejemplares fueron designados como *M. californicus* o *M. ciliolabrum*. Los análisis moleculares ubicaron a *M. leibii* dentro de clados conteniendo tanto a ejemplares de *M. californicus* como de *M. ciliolabrum*. Además, la divergencia entre secuencias y los resultados filogenéticos no evidenciaron linajes diferentes para cada especie (*M. californicus* y *M. ciliolabrum*) tal y como se las reconoce por su morfología. Nuestros resultados sugieren que estas especies han divergido recientemente, o alternativamente que constituyen una única especie fenotípicamente variable. Sobre la base de la red de relaciones entre haplotipos del citocromo *b*, existe una mínima estructura filogeográfica entre haplotipos con una separación este-oeste en el suroeste de Estados Unidos.

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### APPENDIX I

*Specimens examined.*—List of specimens examined for both morphological and molecular analysis. Institutional acronyms: Angelo State University Natural History Collection, San Angelo, Texas (ASK); Abilene Christian University Natural History Collection, Abilene, Texas (ACUNHC); University of Texas at El Paso, El Paso, Texas (UTEP); Natural Science Research Laboratory, Texas Tech University, Lubbock, Texas (TK for tissue, TTU for museum specimen); Museum of Southwestern Biology, University of New Mexico, Albuquerque, New Mexico (NK); and the Carnegie Museum of Natural History, Pittsburg, Pennsylvania (CM). Specimens prefixed with ASK were collected for the purposes of this study following the guidelines for humane care and use of animals set forth by the Animal Care and Use Committee (1998). GenBank accession numbers are identified by AY prefix; 1st AY number is for cytochrome *b* sequence, 2nd is for control region.

*Myotis californicus caurinus.*—California: El Dorado County, 6 miles [10 km] E Somerset (NK 576, AY460350, AY460373). Humboldt County, 8 miles [13 km] N, 1.5 miles [2 km] E Arcata (NK 599, AY460351, AY460374).

*Myotis californicus stephensi.*—Utah: Wayne County, Capitol Reef National Park (NK 34800, AY460349, AY460372).

*Myotis californicus californicus.*—Arizona: Cochise County, 3 miles [5 km] W Portal (ACUNHC 744, AY460353, AY460376).

Pima County, 6 miles [10 km] S, 8 miles [13 km] E Continental Santa Rita Experimental Range Headquarters (ASK 5170, AY460355, AY460378). New Mexico: Hidalgo County, Animas Mountains (NK 3658, AY460348, AY460371). Otero County, Lincoln National Forest (NK 32149, AY460347, AY460370). Texas: Brewster County, Big Bend National Park (ASK 3661, AY460346, AY460369). Culberson County, Sierra Diablo Wildlife Management Area (TK 54207, AY460356, AY460379); Guadalupe National Park (TTU 19939); 23 miles [37 km] 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, AY460352, AY460375).

*Myotis ciliolabrum melanorhinus.*—Arizona: Cochise County, Stewart Creek Campground (NK 37254, AY460357, AY460380). Mohave County, Pipe Springs National Monument (NK 34865, AY460362, AY460385). Pima County, 6 miles [10 km] S, 8 miles [13 km] E Continental Santa Rita Experimental Range Headquarters (ASK 5171, AY460365, AY460388). New Mexico: Eddy County, Lincoln National Forest (NK 32086, AY460360, AY460383). Otero County, Lincoln National Forest (NK 32128, AY460361, AY460384). Sandoval County, 2.7 miles [4 km] S, 4.3 miles [7 km] W Jemez Springs, Guadalupe River (NK 39583, AY460358, AY460381). Oklahoma: Cimarron County, 2 miles [3 km] E, 3 miles [5 km] S Kenton (NK 24872, AY460359, AY460382). Texas: Brewster County, Big Bend National Park (ASK 6044, AY460364, AY460387). El Paso County, Hueco Tanks State Park (UTEP 3463); Hueco Tanks State Park (UTEP 3477); El Paso (UTEP 6165). Jeff Davis County, 3.5 miles [6 km] NE Fort Davis (TTU 9166); 3.5 miles [6 km] NE Fort Davis (TTU 9167); 3.5 miles [6 km] NE Fort Davis (TTU 37221). Presidio County, Big Bend Ranch State Natural Area (TK 48058, AY460354, AY460377); 10 miles [16 km] W SW Valentine (TTU 78520). Utah: Wayne County, Capitol Reef National Park (NK 34803, AY460363, AY460386).

*Myotis leibii.*—West Virginia: Randolph County, 2.7 miles [4 km] N, 4.7 miles [8 km] E Bowden (CM 82050, AY460345, AY460368).

*Myotis evotis.*—New Mexico: Cibola County, Bureau of Land Management, W of Cerro Remdija (NK 39900, AY460344, AY460367).

*Myotis yumanensis.*—Oklahoma: Cimarron County, 3 miles [5 km] E, 1.5 miles [2 km] S Kenton (TK 28786, AY460343, AY460366).