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## Speciation within Bonneted Bats (Genus *Eumops*): The Complexity of Morphological, Mitochondrial, and Nuclear Data Sets in Systematics

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# SPECIATION WITHIN BONNETED BATS (GENUS *EUMOPS*): THE COMPLEXITY OF MORPHOLOGICAL, MITOCHONDRIAL, AND NUCLEAR DATA SETS IN SYSTEMATICS

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We phylogenetically analyze 705 base pairs of the cytochrome-*b* gene and 351 amplified fragment length polymorphism (AFLP) bands from populations of the karyotypically variable Wagner's bonneted bat, *Eumops glaucinus*, and the Florida bonneted bat, *Eumops floridanus* (Chiroptera: Molossidae). Three karyotypes have been documented across the range of *E. glaucinus*, and we report that the karyotype from Cuba is morphologically similar to that from Jamaica. A 4th karyotype is present in specimens from western Ecuador. Three distinct lineages are present in both the cytochrome-*b* and AFLP trees. One lineage is restricted to western Ecuador and exhibits cytochrome-*b* divergence values comparable to the values seen between recognized species of *Eumops*, suggesting that this lineage represents a distinct species. The other 2 lineages are distributed in disjunct areas: Paraguay and Venezuela; and Mexico, the Caribbean, and the United States. Specimens of *E. floridanus* are morphologically distinct from *E. glaucinus*, but cannot be distinguished by examination of cytochrome-*b* or AFLP DNA data. We conclude that there are 4 species in the *E. glaucinus* complex—*E. glaucinus* (South America east of the Andes), *E. ferox* (Caribbean, Mexico, and Central America), *E. floridanus* in south Florida, and an unnamed taxon in western Ecuador. Speciation is a complex process and no single mechanism, model, concept, or definition is likely to cover all the diverse patterns observed.

Key words: amplified fragment length polymorphism, bonneted bat, cytochrome *b*, *Eumops floridanus*, *Eumops glaucinus*, Genetic Species Concept, karyotype, Molossidae

The use of modern molecular tools in combination with morphological and ecological data sets provides insight for species delineation and quantifying biodiversity. Agreement between morphological and molecular data sets is expected, but speciation events in bats have been documented that are not accompanied by obvious morphological changes, leading to the discovery of cryptic species (Baker 1984; Barrat et al. 1995; Hoffmann and Baker 2003; Hulva et al. 2004). An opposite pattern has been documented, particularly in avifauna, that includes significant morphological changes that are incongruent with historical patterns observed using conventional molecular markers (Greenberg et al. 1998; Questiau et al. 1999; Seutin

et al. 1995; Zink and Dittmann 1993). Mitochondrial introgression, differential evolutionary rates of genetic markers, and nuclear-mitochondrial translocations have the potential to create disparity among data sets (Avice 1994; Cathey et al. 1998; Triant and DeWoody 2007). A critically evaluated, holistic approach to resolving phylogenetic patterns within a species complex can provide a deeper understanding of evolutionary and speciation processes.

Examining morphological and molecular patterns in wide-ranging species provides a mechanism to address cryptic biodiversity and contributes to our understanding of how ecological and geological constraints affect the speciation process (Avice 2000; Baker and Bradley 2006). This is true even for volant mammals, which would be expected to have high rates of dispersal and therefore high amounts of gene flow (Hoffmann and Baker 2001; Hoffmann et al. 2003). The bonneted bat genus *Eumops* (Molossidae) is an example of a widely distributed genus with the potential for unidentified biodiversity.

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The most widely distributed species in *Eumops*, Wagner's bonneted bat (*E. glaucinus*), ranges from Mexico through Central America, the Caribbean, and the northern two-thirds of South America (Best et al. 1997; Timm and Genoways 2004). Koopman (1971) recognized 2 subspecies of *E. glaucinus*, the wide-ranging nominate form and *E. g. floridanus*, restricted to southern Florida. A recent comprehensive morphological study of the 2 subspecies elevated the Florida population to specific status, *Eumops floridanus* (Timm and Genoways 2004), and brought the number of species in the genus to 11. As currently understood, *E. floridanus* occurs in only 4 counties in southern Florida (Timm and Genoways 2004). Additionally, fossil records document the presence of *E. floridanus* in Florida since at least the late Pleistocene (Allen 1932; Ray et al. 1963).

Chromosomal, morphological, and ecological evidence indicates that there is extensive diversity within *E. glaucinus* (Eger 1977; Timm and Genoways 2004; Warner et al. 1974). Three distinct karyotypes have been described in this bonneted bat, whereas little karyotypic variation is observed within the 3 other species of *Eumops* that have been karyotyped (*E. auripendulus*, *E. perotis*, and *E. underwoodi*—Morielle-Versute et al. 1996; Warner et al. 1974). *E. glaucinus* from Costa Rica, Honduras, Jamaica, and Mexico has a diploid number (2n) of 38 and a fundamental number (FN) of 64. However, within the 2n = 38 karyotype, forms from Costa Rica and Honduras vary in the placement of the X-chromosome centromere (Genoways et al. 2005; Warner et al. 1974). A 3rd distinctive karyotype has been described from Colombia (2n = 40, FN = 64—Warner et al. 1974). Significant intraspecific karyotypic variation is uncommon in molossid bats (Warner et al. 1974). Karyotypic descriptions are lacking for *E. floridanus*.

Within its extensive geographic range, *E. glaucinus* can be found in a variety of habitats. In the northern part of the range, Wagner's bonneted bat inhabits tropical forests and dry or moist subtropical forests; in the southern part of the range it inhabits deserts, scrublands, and montane forests (Best et al. 1997). In addition to chromosomal differences and ecological diversity, 2 previous morphological studies of *E. glaucinus* documented significant variation among populations (Eger 1977; Timm and Genoways 2004).

Both Eger (1977) and Timm and Genoways (2004) demonstrated that bonneted bats from Florida are significantly larger in all cranial measurements than any other population. Eger (1977) also documented that individuals from Venezuela and Colombia have longer and wider mandibles than those from Mexico, Costa Rica, Guyana, Peru, and Cuba. Moreover, individuals of *E. glaucinus* from Mexico, Costa Rica, and Cuba have longer skulls, greater canine–canine widths, and narrower braincases than individuals from Colombia, Venezuela, Guyana, and Peru. Eger (1977) found statistical support for *E. glaucinus* as a polytypic species consisting of several isolated populations and she proposed that the Sierra Madre del Sur separates northern and southern populations. In the Caribbean, *E. glaucinus* occurs in the Greater Antilles but not in the Lesser Antilles. Baker and Genoways (1978) concluded that this distribution was best explained by an invasion from the north or west. Eger (1977) and Genoways et al. (2005)

hypothesized that Caribbean populations are more closely related to Central American and Mexican populations than to South American populations.

Given the extensive range, karyotypic variation, and cranial polymorphisms of *E. glaucinus*, we predicted that distinct DNA-defined phylogroups are present in this taxon. Herein, we test the monophyly of *E. glaucinus* by analyzing nucleotide sequences of the mitochondrial cytochrome-*b* gene and amplified fragment length polymorphisms (AFLPs) of the nuclear genome. Using these data, we investigate patterns of genetic divergence across the range of this species. We also examine our results for compatibility with geographic hypotheses (Baker and Genoways 1978; Eger 1977; Genoways et al. 2005) regarding the origin of Caribbean populations. Finally, we discuss our findings in light of the Genetic Species Concept as recently applied to mammals (Baker and Bradley 2006; Bradley and Baker 2001).

## MATERIALS AND METHODS

*Taxonomic sampling for molecular analysis.*—Individuals of *E. floridanus* from Florida and *E. glaucinus* from Mexico, Cuba, Jamaica, Venezuela, Ecuador, and Paraguay were included in cytochrome-*b* and AFLP analyses (Appendix I). Additionally, specimens of *E. perotis* and *E. underwoodi* were included as outgroup taxa based on previous morphological studies (Eger 1977); these taxa have never been considered members of the ingroup in question. Vouchers for all specimens are deposited at the museums of Angelo State University, Texas Tech University, and the University of Kansas (Appendix I). All animal handling protocols were in accordance with the guidelines of the American Society of Mammalogists (Gannon et al. 2007).

*DNA sequencing.*—Total genomic DNA was extracted from frozen liver tissue or liver preserved in lysis buffer (Longmire et al. 1997). Tissues were extracted using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, California) following manufacturer's protocol. Cytochrome-*b* sequences were amplified using conserved vertebrate primers: L14841, 5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA AA-3' (Kocher et al. 1989), and H15547, 5'-GGC AAA TAG GAA ATA TCA TTC-3' (Edwards et al. 1991). DNA template was amplified using Eppendorf Taq polymerase (5 U/μl; Eppendorf, Westbury, New York) in a total volume of 12.5 μl following a standard reaction protocol (Palumbi 1996). Thermal cycling was performed using the following thermal profile: 1 cycle at 94°C for 3 min; 39 cycles at 94°C, 48°C, and 72°C for 1 min each; 1 cycle at 72°C for 3 min. Polymerase chain reaction products were gel purified by excising bands from 1% low-melt agarose gels and cloning into a polymerase chain reaction 2.1-TOPO TA plasmid vector following manufacturer's protocols, except we used one-fourth of the reagent volumes (Invitrogen Corporation, Carlsbad, California). The plasmids containing the polymerase chain reaction inserts were purified using the QIAprep Spin Miniprep Kit (QIAGEN Inc.) and 2 clones from each sample were sequenced. Cycle sequencing (Thermo-sequenase, USB Corporation, Cleveland, Ohio) was performed

**TABLE 1.**—Primers used for selective amplification of amplified fragment length polymorphism bands and number of scored bands per primer pair. An asterisk (\*) indicates the fluorescently labeled primer.

Name	Sequence	Scored bands
<i>EcoRI</i> -CAT*	5'-GACTCGCTACCAATTCAT-3'	
<i>AseI</i> -TAC	5'-GATGAGTCCTCACTAATTAC-3'	47
<i>AseI</i> -TAG	5'-GATGAGTCCTCACTAATTAG-3'	55
<i>AseI</i> -TCT	5'-GATGAGTCCTGAGTAATTCT-3'	62
<i>AseI</i> -TGC	5'-GATGAGTCCTGAGTAATTGC-3'	44
<i>AseI</i> -TGT	5'-GATGAGTCCTGAGTAATTGT-3'	57
<i>AseI</i> -TGA	5'-GATGAGTCCTGAGTAATTGA-3'	50
<i>AseI</i> -TGG	5'-GATGAGTCCTGAGTAATTGG-3'	36

on both strands of DNA using M13 dye-labeled primers and analyzed on an automated LI-COR Long Read Dual Laser 4200 sequencer with e-Seq version 3 DNA analysis software (LI-COR Inc., Lincoln, Nebraska).

**Amplified fragment length polymorphism methods.**—The AFLP protocol followed Phillips et al. (2007) with slight modifications. The thermal profile for selective reactions was modified to the following: 94°C for 2 min; 12 cycles of denaturation at 94°C for 50 s, annealing at 66°C for 1 min (0.5°C reduction each cycle), extension at 72°C for 2 min; 23 cycles of denaturation at 94°C for 50 s, annealing at 60°C for 1 min, extension of 72°C for 2 min; final 72°C for 10 min. The selective *EcoRI* primer was labeled with the 6FAM fluorophore (Table 1; Applied Biosystems, Foster City, California). Labeled fragments were detected using an ABI 3100-*Avant* genetic analyzer, scored for presence or absence using GeneMapper version 4.0 software (Applied Biosystems), and converted into a binary data matrix using GenAlEx version 6 software (Peakall and Smouse 2006). DNA fragments were automatically sized and compared with the Genescan-400HD ROX Size Standard (Applied Biosystems). Seven selective primer pairs (Table 1) produced distinct scorable fragments within a 50- to 400-base pair (bp) region.

**Phylogenetic analyses.**—Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, Michigan) and MacClade software version 4.05 (Maddison and Maddison 2000) were used to align and translate nucleotide sequences. Sequences were exported into PAUP\* version 4.0b10 (Swofford 2002) for neighbor-joining, maximum-parsimony, and maximum-likelihood (Felsenstein 1981) analyses. Parsimony criteria were only used to calculate nodal support using 1,000 bootstrap replicates (Felsenstein 1985). Fifty-six maximum-likelihood models were analyzed in MODELTEST version 3.7 (Posada and Crandall 1998) in order to determine the appropriate model of evolution for both maximum-likelihood and Bayesian analyses. Based on the Akaike information criterion we used the general time reversible (GTR+G) model of evolution and the following parameters for likelihood analysis: assumed nucleotide frequencies using maximum-likelihood estimates were A = 0.2965, C = 0.2975, G = 0.1445, T = 0.2615; proportion of invariable sites was 0; and a gamma distribution parameter of 0.3067. Bayesian analysis of cytochrome-*b* sequence data was performed with MrBayes version 3.1.2 (Ronquist and

Huelsenbeck 2003) and consisted of 2 simultaneous runs each with 4 Markov chain Monte Carlo chains (1 heated and 3 cold) run for  $2 \times 10^6$  generations. GTR+G models were applied and all model parameters were estimated. Trees were sampled every 100 generations for a total of 40,000 trees (pooled from the 2 replicate runs) sampled. Stationarity was evaluated by the convergence of log-likelihoods. We excluded the first 10% of trees sampled and therefore calculated the Bayesian posterior probabilities and generated a 50% majority-rule consensus tree from 36,000 trees. The average standard deviation of split frequencies (variance between the 2 independent runs) was 0.01, indicating that the sampling of the posterior distribution was adequate.

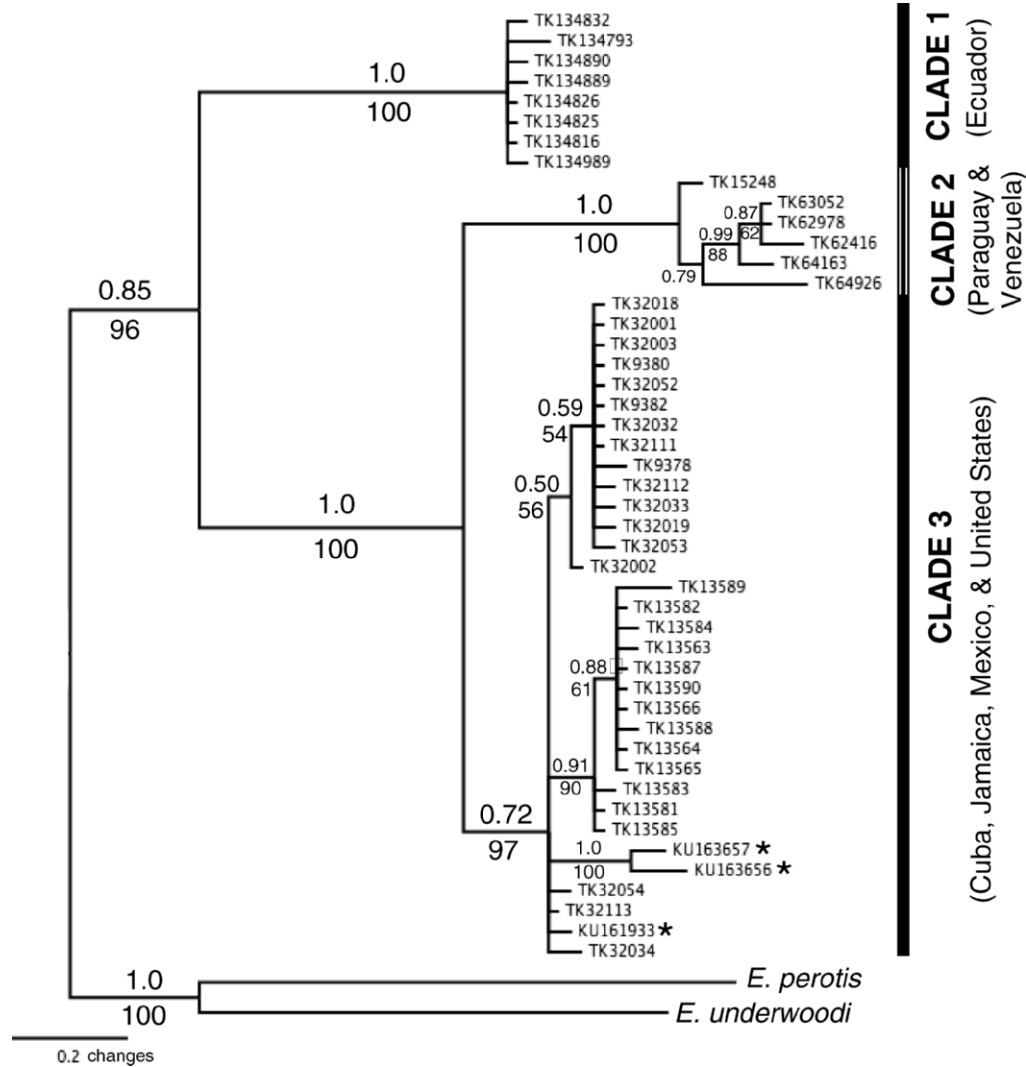
Average genetic distances were calculated in PAUP\* using the Kimura 2-parameter model of nucleotide substitution (Kimura 1980) and the GTR+G model. Kimura 2-parameter distances computed in this study were compared to documented divergences between known sister taxa of bats and evaluated within the framework of the Genetic Species Concept (Baker and Bradley 2006; Bradley and Baker 2001).

The binary AFLP matrix was analyzed using PAUP\* version 4.0b10 software (Swofford 2002). Neighbor-joining and maximum-parsimony analyses were used to infer phylogenies. A neighbor-joining distance tree was generated using the restriction site model of Nei and Li (1979). Maximum-parsimony analysis was performed using heuristic searches, 100 replicates of the random taxon-addition option, each with random starting trees, and tree-bisection-reconnection branch-swapping. For bootstrap support values, 250 replicates were conducted using the heuristic search criterion.

**Karyotypic preparation.**—Specimens were karyotyped using bone marrow after 1 h of in vivo incubation with the mitotic inhibitor Velban (Sigma-Aldrich, St. Louis, Missouri), following the methods described in Baker et al. (2003). No yeast stress was employed and animals were karyotyped the morning after capture from buildings or with mist nets the previous night. Karyotypes were visualized using an Olympus BX51 microscope. Images were photographed using an Applied Imaging camera and captured using the Genus System 3.7 from Applied Imaging Systems (San Jose, California).

**Morphological analysis.**—Thirty-four *E. glaucinus* and 2 *E. floridanus* that were included in the genetic analysis were examined to both confirm identification and to test for congruence between morphological and molecular data sets. Specimens of *E. glaucinus* and *E. floridanus* from the following countries were examined: Cuba ( $n = 13$ ), Ecuador ( $n = 7$ ), Jamaica ( $n = 1$ ), Honduras ( $n = 1$ ), Mexico ( $n = 6$ ), Paraguay ( $n = 5$ ), United States ( $n = 2$ ), and Venezuela ( $n = 1$ ). Eight cranial measurements were taken using digital calipers calibrated to the nearest 0.01 mm following the measurements of Timm and Genoways (2004): greatest length of skull, condylobasal length, zygomatic breadth, postorbital constriction, mastoid breadth, palatal length, breadth across upper molars, and length of maxillary toothrow. A principal component analysis was performed with SYSTAT version 11 software (SYSTAT Software Inc., Richmond, California). Eighty-five percent of the individuals ( $n = 33$ ) used in the





**FIG. 1.**—Bayesian tree generated from 705 base pairs of the cytochrome-*b* gene of 44 *Eumops glaucinus*, 3 *E. floridanus* (indicated by asterisks [\*]), 2 *E. perotis*, and 1 *E. underwoodi*. Numbers above branches are Bayesian posterior probabilities and below are bootstrap values generated from 1,000 bootstrap replicates (only values > 50 are shown).

morphological analysis were included in the molecular analysis (Appendix I).

**RESULTS**

*Phylogenetic analyses of cytochrome b.*—Seven hundred five base pairs of the cytochrome-*b* gene were sequenced from 44 *E. glaucinus*, 3 *E. floridanus*, 2 *E. perotis*, and 1 *E. underwoodi* and submitted to GenBank (Appendix I). Of the 705 base pairs sequenced, 175 were parsimony-informative characters (when including outgroup taxa). Parsimony, maximum-likelihood, and Bayesian analyses resulted in 3 well-supported phylogroups (Fig. 1): a monophyletic phylogroup (clade 1) of South American specimens containing samples of *E. glaucinus* from western Ecuador (Bayesian posterior probability: 1.0, parsimony bootstrap: 100); a phylogroup of South American specimens collected east of the Andes (clade 2) including individuals from Venezuela and Paraguay (Bayesian posterior probability: 1.0, bootstrap: 100); and a phylogroup of

Cuban, Jamaican, and Mexican specimens, as well as *E. floridanus* (clade 3; Bayesian posterior probability: 0.72, bootstrap: 97; Fig. 1). Within clade 3, Mexican specimens (TK 13563–13566, 13581–13585, and 13587–13590) form a clade supported by a bootstrap value of 90. There also is significant statistical support (Bayesian posterior probability: 1.0, bootstrap: 100) for the sister relationship between clades 2 and 3.

The 3 clades contained 49 autapomorphic characters, of which 33 fixed changes were unique to clade 1, 11 were unique to clade 2, and 5 were unique to clade 3. These nucleotide changes resulted in 2 amino acid changes that were present in all specimens of *E. glaucinus* except for members of the Ecuador clade.

The average level of sequence divergence between bats from western Ecuador and those from all other populations sampled ranged from 7.1% (between Ecuador and Mexico) to 8.4% (between Ecuador and Paraguay; Table 2). Low levels of sequence divergence were present between *E. floridanus* and

**TABLE 2.**—Average Kimura 2-parameter (cytochrome-*b*; above diagonal) and Nei–Li (amplified fragment length polymorphism [AFLP]; below diagonal and boldface along diagonal) distances between and within populations of *Eumops floridanus* (United States [USA]) and *E. glaucinus* (Cuba [CUB], Jamaica [JAM], Mexico [MEX], Venezuela [VEN], Paraguay [PAR], and Ecuador [ECU]) based on 705 base pairs of the cytochrome-*b* gene and 351 AFLP bands. Sample sizes are number of individuals used for cytochrome-*b* (1st number) and AFLP (2nd number) analyses.

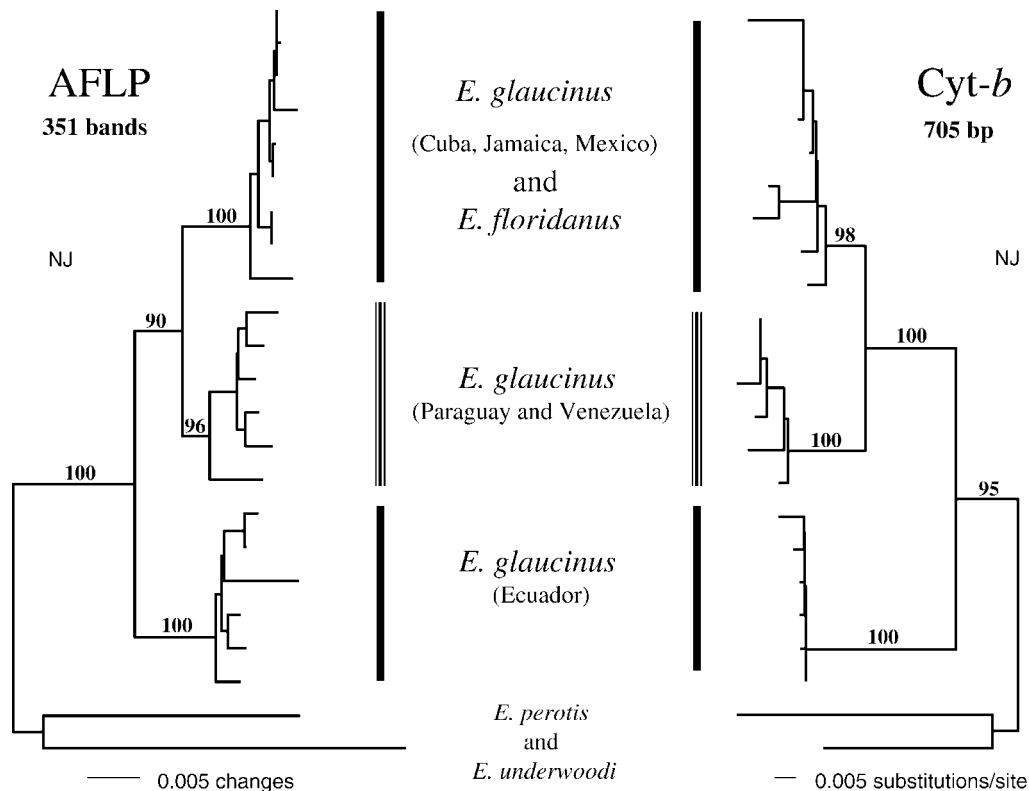
	1	2	3	4	5	6	7
USA ( <i>n</i> = 3, 3)	0.83/ <b>0.92</b>	1.30	1.78	2.00	3.96	4.68	7.56
CUB ( <i>n</i> = 14, 4)	0.78	<b>0.29/0.44</b>	0.40	1.28	3.37	4.04	7.19
JAM ( <i>n</i> = 3, 1)	1.12	0.81	0.94/—	1.50	3.70	4.38	7.75
MEX ( <i>n</i> = 13, 1)	0.69	0.33	0.55	0.48/—	3.92	4.53	7.09
VEN ( <i>n</i> = 1, 1)	3.86	3.77	4.04	3.51	—	1.09	7.59
PAR ( <i>n</i> = 5, 5)	3.90	3.66	4.21	3.67	2.23	<b>0.89/1.09</b>	8.35
ECU ( <i>n</i> = 8, 6)	5.59	5.59	6.31	5.67	5.45	5.53	<b>0.37/1.35</b>

other members of clade 3 (1.3–2.0%). Genetic distances between members of clades 1 and 2 and clades 2 and 3 averaged 8.0% and 4.0%, respectively. The divergence between the 2 outgroup species *E. underwoodi* and *E. perotis* was 9.9%.

*Phylogenetic analyses of AFLPs.*—Three hundred one AFLP bands were scored from 23 individuals: 18 *E. glaucinus*, 3 *E. floridanus*, 1 *E. perotis*, and 1 *E. underwoodi* (see Appendix I). Of the 351 total scored bands, 271 (~77%) were polymorphic including outgroup taxa, and 142 (~40%) were polymorphic within *E. glaucinus*. Three distinct clades were identified within the *E. glaucinus* complex (Fig. 2). Neighbor-joining and maximum-parsimony analyses resulted

in identical trees with well-supported topologies similar to those from cytochrome-*b* analyses (neighbor-joining tree shown in Fig. 2). Within *E. glaucinus* average genetic distance values ranged from 6.3% (Jamaica versus Ecuador) to 0.3% (Cuba versus Mexico; Table 2).

*Karyotypic data.*—New data on karyotypes are provided for specimens from western Ecuador and Cuba. The karyotype (Figs. 3A and 3B) of *E. glaucinus* from western Ecuador has 2*n* = 38 and FN = 54. The largest 9 pairs of autosomes are a graduated series of biarmed elements, all of which are either metacentric or submetacentric. The 9 smallest pairs of autosomes are a graduated series of acrocentrics. In some spreads, a small 2nd arm is visible on 2 of the largest pairs. In



**FIG. 2.**—Neighbor-joining trees based on 351 amplified fragment length polymorphism bands (left) and 705 base pairs of the cytochrome-*b* gene (right) for *Eumops glaucinus* and *E. floridanus*. Bootstrap support values (percentage of 250 iterations) are from maximum-parsimony analyses. *E. perotis* and *E. underwoodi* were used as outgroups for both data sets.

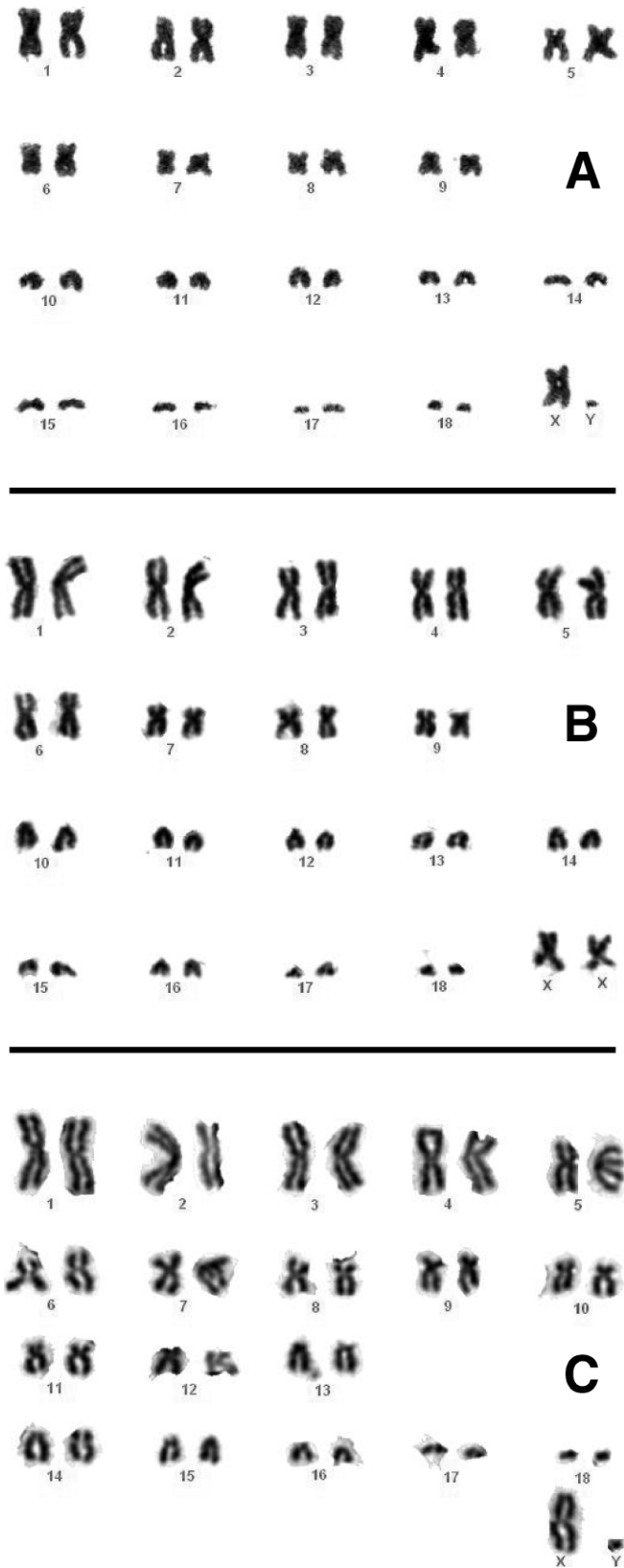


FIG. 3.—Karyotypes of *Eumops glaucinus*. A) Ecuador, Cerro Blanco, male, TK 134825. B) Ecuador, Cerro Blanco, female, TK 134832. C) Cuba, Guantanamo Bay, male, TK 32018.

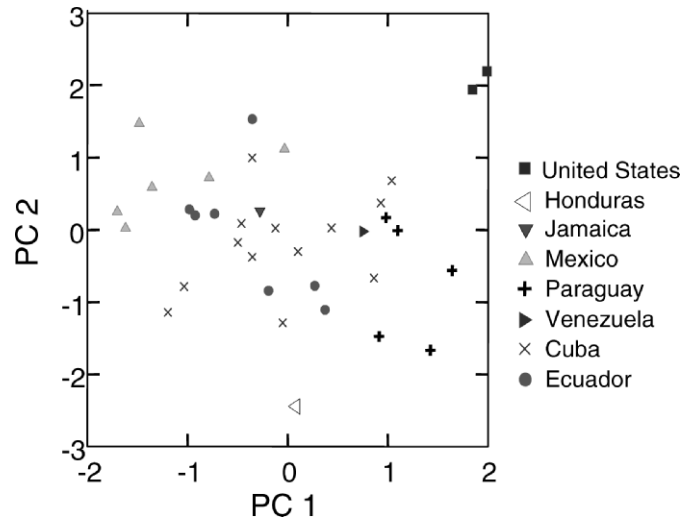


FIG. 4.—Principal component analysis based on 8 cranial measurements of 34 specimens of *Eumops glaucinus* from 7 countries (Cuba, Ecuador, Jamaica, Honduras, Mexico, Paraguay, and Venezuela) and 2 specimens of *E. floridanus* (United States).

molossid bats, determining the number of small arms present on acrocentrics or near-acrocentrics is a persistent problem (Warner et al. 1974). In some cases the small 2nd arms have been counted as part of the fundamental number; if counted as such in the karyotype of *E. glaucinus* from western Ecuador, the fundamental number would be as high as 58 (see Fig. 3B). The X is a medium-sized submetacentric and the Y is the smallest acrocentric. We examined 5 specimens from Cerro Blanco, Ecuador (west of the Andes). No populational variation was detected except for the sex chromosomes, which appear to be the typical XX/XY sex-determining system. A single male from Isla Puná, Ecuador (west of the Andes), has a karyotype indistinguishable from the karyotype of 2 males from Cerro Blanco.

The karyotype of specimens from Guantanamo Bay, Cuba (Fig. 3C), is similar to those reported for Jamaica and may be identical (Genoways et al. 2005). The number of short arms on some subtolocentrics or acrocentrics is variable within and between individuals, making it difficult to accurately describe the fundamental number. Without evidence to the contrary, we assign the Cuban specimens to the same fundamental number that was assigned to Jamaican specimens (FN = 64), although we note that the karyotype figured in Genoways et al. (2005) for a Jamaican bat generally would be described as having FN = 62.

*Morphological analyses.*—Results of the principal component analysis are similar to those found in previous studies of *E. glaucinus* and *E. floridanus* (Eger 1977; Timm and Genoways 2004), demonstrating that larger size distinguishes individuals from Florida from those from all other localities (Fig. 4). The first 3 principal components (PCs) explain 90.7% of the total variation (PC1, 67.9%; PC2, 15.4%; PC3, 7.4%). All cranial characters have a positive loading for PC1, of which greatest length of skull has the highest value with a loading of 0.945. In addition to separation of *E. floridanus* along PC1, specimens



from Paraguay and Venezuela are larger than *E. glaucinus* from other regions, although there is minor overlap with specimens from Cuba. Specimens from Cuba, Ecuador, Jamaica, Mexico, and a single individual from Honduras overlap along PC1. PC2 contains both positive and negative values, of which postorbital constriction has the highest value with a loading of 0.691 and palatal length has the highest negative loading of 0.539. Individuals from Florida were separated along PC2, indicating that those specimens have a large postorbital constriction and short palatal length. The single specimen from Honduras is highly separated from other localities on PC2, indicating that it has a small postorbital constriction and a long palatal length. There is overlap among all other *E. glaucinus* along PC2 (Fig. 4).

## DISCUSSION

A significant problem in describing biodiversity and planning conservation is identifying species limits. Increasingly, genetic characteristics are being used to define species (Baker and Bradley 2006). For the *E. glaucinus* complex, there is substantial variation in 3 genetic (chromosomal, AFLP, and mitochondrial), as well as classical morphological data sets. Below, we assess the effectiveness, congruence, and conflicts of multiple data sets in defining species and discuss the effect of different species concepts on our conclusions.

*Variation within E. glaucinus.*—Examination of both nuclear and mitochondrial DNA (mtDNA) sequence data sets confirm 3 well-supported lineages within what was regarded as *E. glaucinus* (Eger 1977; Koopman 1971; Figs. 1 and 2). The status of clade 1 is the most easily resolved. The phylogroup from western Ecuador (clade 1) exhibits a unique karyotype ( $2n = 38$ ,  $FN = 54$ ; Figs. 3A and 3B), a high level of cytochrome-*b* sequence divergence (7.1–8.4%), and AFLP band divergence (5.5–6.3%) compared to all other populations of *E. glaucinus* examined herein. Clades 1 and 2 + 3 are reciprocally monophyletic in both nuclear and mitochondrial trees (Fig. 2), a feature that has been proposed as an operational criterion for species recognition (da Silva and Patton 1998). Given this evidence, we conclude that the populations west of the Andes currently assigned to *E. glaucinus* should be recognized as a distinct species. This new taxon will be described elsewhere.

*Status of clades 2 and 3.*—The phylogenies recovered with cytochrome *b* and AFLPs indicate a sister relationship between eastern South American (clade 2) and Caribbean–Mexican (clade 3) lineages. Divergence between these lineages is 3.4–4.7% and 3.6–4.0% based on cytochrome *b* and AFLPs, respectively. Karyotypes are unknown for specimens from Venezuela and Paraguay, but the karyotype  $2n = 40$ ,  $FN = 64$  is shared by individuals from distant locations in Colombia (Warner et al. 1974) and eastern Brazil (Morielle-Versute et al. 1996). We hypothesize that this is the karyotype for South American populations east of the Andes, distinguishing them from populations in the Caribbean and Central America (for which  $2n = 38$ ,  $FN = 64$ —Genoways et al. 2005; Warner et al. 1974; Fig. 3C). We interpret this evidence as indicating that clade 2 and clade 3 merit specific status. The name *E.*

*glaucinus*, from the type locality in Mato Grosso, Brazil, is the senior synonym for the South American populations east of the Andes. Members of clade 3 (including *E. floridanus*) exhibit a low level of intraclade genetic divergence (<2% cytochrome *b*; 0.40% AFLP) and there is no resolution among geographic regions (Cuba, Jamaica, Florida, and Mexico) in either cytochrome-*b* or AFLP data sets (Fig. 2). However, members of clade 3 do exhibit some karyotypic polymorphism with a primary karyotype of  $2n = 38$ ,  $FN = 64$ . Variation in the number of short arms or acrocentrics in chromosomes of *Eumops* has been discussed in previous publications (Genoways et al. 2005; Morielle-Versute et al. 1996; Warner et al. 1974), and is present in the samples from Cuba, Jamaica, and Mexico. The karyotype of *E. floridanus* is unknown. The senior synonym available from the geographic areas outside of those present in clade 2 is *Eumops ferox* (Gundlach 1861); Gundlach's type is from Cuba. Even if *E. floridanus* is synonymized with Caribbean and Mexican populations, the name *E. ferox* will be the senior synonym. The exact boundaries of *E. ferox* in Mesoamerica cannot be determined at present because there are no genetic data available from these populations. Moreover, specimens from Florida do not show specific morphological affinities with *E. ferox*, *E. glaucinus*, or the taxon from western Ecuador.

The relatively high genetic divergence (mtDNA and AFLP) among populations of *E. glaucinus* on either side of the Andes is likely the result of genetic isolation due to uplift of the Andes 2–5 million years ago (Gregory-Wodzicki 2000). The phylogroups reported herein are consistent with the biogeographical hypothesis of Eger (1977) and Genoways et al. (2005) that Caribbean populations are most closely related to Mexican populations. However, Eger (1977) reported distinct northern and southern populations in Mexico that are separated by the Madre del Sur. We are unable to test this hypothesis with genetic data because all specimens from Mexico used in this study are from the Yucatan.

*Variation within E. floridanus.*—*Eumops floridanus* was originally discovered and described based on fossil material (Allen 1932), and is now documented from 4 late Pleistocene sites in southern Florida. Recent specimens are identified as conspecific with subfossil *E. floridanus* based on size, cusp patterns, and overall body proportions (Ray et al. 1963; Timm and Genoways 2004). The presence of subfossil records of *E. floridanus* from the Rancholabrean North American Land Mammal Age (10,000–0.3 million years ago) provides evidence that the establishment of morphological features (probably local adaptation) distinguishing Floridian populations from Caribbean and Mexican populations occurred before 10,000 years ago.

The low level of genetic divergence between *E. floridanus* and both Mexican and Caribbean populations of *E. glaucinus* (Figs. 1 and 2; Table 2) does not resolve the origin of *E. floridanus* and is most compatible with the hypothesis that these populations have not been genetically isolated for a substantial amount of time. In the absence of morphological data, there would be no justification for according *E. floridanus* specific status.

In fact, however, recognition of *E. floridanus* is not based only on size, but also on the unique shapes of its basisphenoid pits, glenoid fossa, and baculum (Timm and Genoways 2004). It is our interpretation that the morphological basis for recognition of *E. floridanus* is similar to that of many species found in authoritative lists such as Wilson and Reeder (2005).

*Systematic data sets and the application of species concepts.*—The systematic data sets reported here reveal species boundaries in some cases and not in others. For example, *E. floridanus* is defined by morphology but not by nuclear (AFLP) or mtDNA (cytochrome-*b*) characteristics. In contrast, populations of *Eumops* from western Ecuador form a statistically supported phylogroup defined by mtDNA and nuclear markers, but are not easily distinguished by morphological features. The western Ecuadorian populations probably would never have been recognized as a distinct species if only morphology had been examined, whereas *E. floridanus* would not be recognized if only mtDNA and AFLP nuclear data were used.

Cases such as *Eumops* frustrate attempts at consistent application of species concepts and the definition of species limits using combined morphological, mitochondrial, and nuclear data. The inability to establish a genetically defined threshold for species recognition is not restricted to mammals, having been particularly well documented in birds and fishes (Ball et al. 1988; Meyer et al. 1990; Zink and Dittmann 1993). Speciation is a complex process and no single mechanism, model, concept, or definition is likely to cover all the diverse patterns observed, even among mammals. The relative significance of overall morphological differentiation, genetic divergence as predicted by the Bateson–Dobzhansky–Muller model, or ecological specialization in one or a few phenotypic characters seems to vary considerably among mammals. Species concepts that emphasize each of these models—morphological species (Corbet 1997), genetic species (Baker and Bradley 2006), and ecological species (Rundle and Nosil 2005), respectively—are expected to vary in their applicability to specific cases, sometimes yielding conflicting conclusions. For example, adherence to the Genetic Species Concept would relegate *E. floridanus* to conspecific status with *E. glaucinus*. Just as clearly, morphological and ecological concepts call for the recognition of *E. floridanus* as a distinct species.

What is a species? This question is and has been difficult to answer. The *floridanus*–*glaucinus* complex presents a unique opportunity to study the process of speciation using new techniques from the emerging field of genomics. Baker and Bradley (2006) and Storz and Hoekstra (2007) predicted that it will soon be possible to empirically study the genetic basis of speciation and better understand the speciation process as outlined in Coyne and Orr (2004) and Gavrillets (2004). Examination of mtDNA, nuclear AFLP, karyotypic, and morphological data within the *E. glaucinus* complex suggests that morphological distinction in *E. floridanus* has preceded establishment of either mitochondrial or nuclear distinction. The significance of this observation is that it documents fluctuating tempos of evolution across multiple character sets (mtDNA, nuclear, and morphological) that typically are used to define species. Production of species lists of mammals will

be forced to accommodate such diversity and evolutionary scenarios.

## RESUMEN

Analizamos filogenéticamente secuencias del citocromo-*b* y amplified fragment length polymorphisms (AFLPs) de poblaciones cariotípicamente variables del murciélago de bonete de Wagner *Eumops glaucinus* y el murciélago de bonete floridano *Eumops floridanus* (Chiroptera: Molossidae). Tres formas cariotípicas han sido documentadas a lo largo del rango de *E. glaucinus*, nosotros reportamos la similitud morfológica existente entre las formas cariotípicas de Cuba y Jamaica. Un cuarto cariotipo está presente en los especímenes del occidente de Ecuador. Tres linajes distintos están presentes tanto en los árboles construidos a partir de datos del citocromo-*b* como de AFLP. Uno de los linajes identificados está restringido al occidente del Ecuador y exhibe divergencias comparables a aquellas observadas entre especies reconocidas de *Eumops*, sugiriendo que este linaje representa una nueva especie. Los otros 2 linajes identificados están distribuidos en áreas disjuntas: Paraguay y Venezuela; y México, el Caribe, y los Estados Unidos. Los especímenes de *E. floridanus* son morfológicamente distintos de *E. glaucinus*, pero no pueden ser distinguidos por nuestros datos de DNA del citocromo-*b* y AFLP. Concluimos que hay 4 especies en el complejo *E. glaucinus*—*E. glaucinus* (vertiente oriental de los Andes en Sur América), *E. ferox* (Caribe, México, y Centro América), *E. floridanus* en el sur de la Florida, y un taxón aun sin nombre en el occidente de Ecuador. La especiación es un proceso complejo y no es probable que un único mecanismo, modelo, ó definición cubra toda la diversidad de los patrones observados.

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- and karyotype (K) analyses. ASK (tissue number) and ASNHC (catalog number) = Angelo State Natural History Collection, Angelo State University; CM = Carnegie Museum; RMT (tissue number) and KU (catalog number) = University of Kansas; TK (tissue number) and TTU (catalog number) = Natural Science Research Laboratory, Texas Tech University (an asterisk [\*] indicates a tissue sample where the location of the corresponding voucher specimen is unknown).
- Eumops glaucinus*.—Paraguay: Alto Paraguay; Estancia Tres Marias TK62416, TTU79823, EU350000 (M, C, A). Boquerón; Base Naval Pedro P. Peña TK63051, TTU79956, EU350002 (M); TK63052, TTU79957, EU350003 (M, C, A); TK62978, TTU79955, EU350001 (M, C, A). Concepción; Parque Nacional Serranía de San Luis TK64163, TTU80255, EU350005 (M, C, A). Presidente Hayes; Estancia Samaklay TK64926, TTU80542, EU35004 (C, A). Venezuela: Guarico; 48 km S Calabzo TK15248, TTU33408, EU35006 (M, C, A).
- Eumops ferox*.—Cuba: Guantanamo Province; Guantanamo Bay Naval Station TK32001, TTU52635, EU350007 (C, A); TK32002, TTU52642, EU350008 (M, C); TK32003, TTU52643, EU350009 (M, C); TK32111, TTU52626, EU350018 (C); TK32112, TTU52627; EU350019 (M, C); TK32113, TTU52628, EU350020 (M, C); TK32017, TTU52636 (M); TK32018, TTU52612, EU350010 (M, C, K); TK32019, TTU52637, EU350011 (M, C, A); TK32020, TTU52638 (K); TK32032, TTU52639, EU350012 (M, C); TK32033, TTU52613, EU350013 (M, C); TK32034, TTU52640, EU350014 (M, C, A); TK32052, TTU52617; EU350015 (M, C, A); TK32053, TTU52618, EU350016 (M, C); TK32054, TTU52619, EU350017 (M, C). Honduras: Cisco Morazan; 10 miles N Tegucigulpa TTU13470 (M). Jamaica: Queenhythe; St. Ann Parish TK9378, CM44612, EU350022 (C, A); TK9380, CM44614, EU350021 (C); TK9382, CM44616, EU350023 (C); TK8166, TTU22081 (M). Mexico: Merida; Campestre Country Club \*TK13563, EU350027 (C); \*TK13564, EU350028 (C); \*TK13565, EU350029 (C); \*TK13566, EU350030 (C); \*TK13581, EU350032 (C); \*TK13582, EU350033 (C); \*TK13583, EU350034 (C); \*TK13584, EU350035 (C); TK13585, TTU47519, EU350036 (M, C, A); \*TK13587, EU350038 (C); TK13588, TTU47520, EU350039 (M, C); TK13589, TTU47521, EU350040 (M, C); TK13590, TTU47522, EU350041 (M, C); TTU29075 (M); TTU29076 (M).
- Eumops floridanus*.—United States: Florida; Lee County, North Fort Myers RMT4610, KU163656, EU350024 (C, A); RMT4611, KU163657, EU350025 (M, C, A). Dade County, Miami, Miami MetroZoo Asian elephant house RMT4618, KU163658, EU350026 (M, C, A).
- Eumops sp.*—Ecuador: Guayas Province; Bosque Protector Cerro Blanco TK134816, TTU103278, EU349993 (M, C, A, K); TK134825, TTU103281, EU349994 (M, C, A, K); TK134889, TTU103302, EU349997 (M, C, A, K); TK134890, TTU103303, EU349998 (M, C); TK134793, TTU103255, EU349992 (C); TK134826, TTU103282, EU349995 (M, C, A, K); TK134832, TTU103286, EU349996 (M, C, A, K). Isla Puna TK134989, TTU103466, EU349999 (M, C, A, K).
- Eumops underwoodii*.—Nicaragua: Boaco; 14 km S Boaco TK12366, TTU29311 (C, A).

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## APPENDIX I

Species, locality, tissue and catalog number, and GenBank accession number (EU) for specimens used in the morphometric (M), cytochrome-*b* (C), amplified fragment length polymorphism (A),