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Patterns of Genetic Diversification in a Widely Distributed Species of Bat, *Molossus molossus*

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Abstract

The taxonomy and evolutionary relationships of the Velvety Free-tailed Bat, *Molossus molossus*, from Central and South America long have been debated. Within this species, and in fact the entire genus *Molossus*, specimens have been difficult to identify and have presented several taxonomic challenges. The objective of this project was to characterize the genetic relationship among individuals representing subspecies of the widely distributed species, *M. molossus*. We tested the hypothesis that genetic patterns of diversification would reflect subspecies lineages. The mitochondrial gene cytochrome *b* (cyt*b*) was amplified and sequenced for specimens throughout its geographic range. A Bayesian analysis of 678 base pairs of the cyt*b* gene was conducted for 65 specimens with *M. alvarezi* as an outgroup. Our results showed that the subspecies *M. m. daulensis,* recognized based on morphology and geographic location, formed a statistically supported mitochondrial lineage in the phylogenetic analysis. However, not all currently recognized subspecies of *M. molossu*s were recovered by this analysis. One lineage, *M. m. tropidorhynchus* from Cuba, formed a divergent monophyletic lineage. Overall, the average divergence across all specimens was 4.7%; however the *M. m. tropidorhynchus* lineage was 7.9% divergent from the other *M. molossus* specimens. This level of divergence and the recovery of a monophyletic lineage containing all Cuban specimens was consistent with recognition of the taxon as a distinct species.

Key words: cytochrome *b*, genetic species, *Molossus molossus*, systematics, taxonomy

Introduction

The Velvety Free-tailed Bat is a widely distributed species in the family Molossidae. They reside in tropical and temperate areas of Central and South America and the Greater and Lesser Antilles Islands (Simmons 2005). Since the first molossid was described as *Vespertilio molossus* by Pallas (1766), large numbers of species and subspecies have been assigned to the genus *Molossus* (Table 1). Sexual dimorphism and high degrees of local variation in *Molossus* have confounded species definitions and groupings (Dolan 1982), and therefore the taxonomy and phylogenetic relationships of *Molossus* lineages remain highly debated.

Table 1. Summary of taxonomic history of *Molossus* species. Species names marked with the same symbol highlight synonymous groupings of the currently recognized *Molossus* species. An additional species, *M. alvarezi*, was described by González-Ruiz et al. (2011) from Yucatán, Mexico.

Miller (1913)	Dolan (1989)	Simmons (2005)	Eger (2007)
M. rufus°	*M. rufus*°	*M. rufus*°	*M. rufus*°
M. nigricans°			
*M. pretiosus**	*M. pretiosus**	*M. pretiosus**	*M. pretiosus**
M. sinaloae$^{\Delta}$	*M. sinaloae*$^{\Delta}$	*M. sinaloae*$^{\Delta}$	*M. sinaloae*$^{\Delta}$
M. currentium$^{\bullet}$		*M. currentium*$^{\bullet}$	*M. currentium*$^{\bullet}$
M. bondae$^{\bullet}$	*M. bondae*$^{\bullet}$		*M. bondae*$^{\bullet}$
M. aztecus$^{\#}$	*M. aztecus*$^{\#}$	*M. aztecus*$^{\#}$	
M. barnesi$^{\#}$		*M. barnesi*$^{\#}$	
M. coibensis$^{\#}$	*M. coibensis*$^{\#}$	*M. coibensis*$^{\#}$	*M. coibensis*$^{\#}$
M. major (*M. molossus*)$^{\$}$	*M. molossus*$^{\$}$	*M. molossus*$^{\$}$	*M. molossus*$^{\$}$
M. fulginosis$^{\$}$			
M. verrilli$^{\$}$			
M. fortis$^{\$}$			
M. debilis$^{\$}$			
M. obscurus$^{\$}$			
M. crassicaudatus$^{\$}$			
M. pygmaeus$^{\$}$			
M. tropidorhynchus$^{\$}$			

Miller (1913) recognized 18 species in the genus *Molossus* by morphologically comparing *Molossus* specimens residing in the United States National Museum. He did not recognize *M. molossus* but instead split this widespread species into nine species. Dolan (1989), in the most recent treatment of the diversity within *Molossus*, recognized seven of Miller's original 18 species. Dolan combined nine species into the single taxon *M. molossus* (Table 1). The type specimen named by Pallas (1766) remains the type specimen for the species *M. molossus* although it is a lectotype based on Husson (1962), who examined *Vespertilio molossus*. More recently, Simmons (2005) recognized eight species in the genus, whereas Eger (2007) recognized seven species. Eger (2007) placed both *M. barnesi* and *M. aztecus* in *M. coibensis*. Also, Eger (2007) recognized *M. bondae* as a separate species from *M. currentium* based on pelage differences. A recent morphometric study (González-Ruiz et al. 2011) discovered a new species from the Yucatán Peninsula in Mexico; *M. alvarezi* is similar to *M. sinaloae,* but some cranial measurements do not overlap and the two species are geographically separated (González-Ruiz et al. 2011).

Based on morphological data, Dolan (1989) concluded that species of *Molossus* are differentiated primarily on the basis of size; however, many populations within a single species also differed in size based on their geographic locality. According to Dolan (1989), "all species of *Molossus* exist in numerous, morphologically discrete populations and can be considered polytypic"; this suggests morphological data

alone is unreliable for differentiating lineages. Further, Dolan (1989) and Warner et al. (1974) were not able to uncover any interspecific variation among *M. rufus*, *M. molossus*, and *M. sinaloae* using a chromosomal approach; all three species had the same karyotype. In addition, Dolan (1989) constructed a phenogram based on electrophoretic data of 11 polymorphic allozyme loci, and these data were unable to resolve the relationship of *M. rufus* and *M. pretiosus*. Only *M. bondae* had a single species-specific marker allele. Therefore, Dolan referred to the *M. rufus*, *M. pretiosus*, and *M. bondae* clade as the *rufus* complex. *Molossus molossus* possessed two species-specific markers and was sister to the *rufus* complex. *Molossus sinaloae* was the most divergent taxon in Dolan's analysis, as it was basal to all other taxa. Dolan's results did not support a strong correlation between electrophoretic data and geographic proximity, suggesting isolated populations and possible subspecies in many of the species of *Molossus* that she examined.

Molossus molossus and its subspecies remain the most highly debated group in the genus, having about 20 synonyms (Simmons 2005). The diversity of recognized forms could be a result of the widespread geographic distribution of this taxon compared to other *Molossus* species. Complex patterns of intraspecific variation across the geographic range make resolving *M. molossus* taxonomy difficult. Eger (2007) suggested that a complete review of the entire *M. molossus* group was needed to clarify the status of the numerous available names. Genoways et al. (1981) also suggested genetic analyses would be informative and beneficial for *M. molossus*.

Nine of the original species recognized by Miller (1913) have been placed within *M. molossus* and many of these are now recognized as subspecies (Table 2). Simmons (2005) and Eger (2007) differed on recognition of subspecies of *M. molossus*. Simmons (2005) recognized seven subspecies: *M. m. molossus*, *M. m. debilis*, *M. m. pygmaeus*, *M. m. fortis*, *M. m. milleri*, *M. m. tropidorhynchus*, and *M. m. verrilli*. The four subspecies recognized by Eger (2007) were *M. m. molossus*, *M. m. pygmaeus*, *M. m. daulensis*, and *M. m. crassicaudatus*. Simmons (2005) and Eger (2007) only agreed on two subspecies, *M. m. molossus* and *M. m. pygmaeus*. Both Simmons (2005) and Eger (2007) placed all of the unresolved subspecies within *M. m. molossus*.

Genoways et al. (1981) attempted to decipher intra-island and inter-island variation of *M. molossus* from three Antillean Island populations using morphological data. Specimens from Jamaica, Guadeloupe, and Trinidad were examined to determine local versus geographic variation. From all specimens, one external and nine cranial measurements were recorded (Ge-

Table 2. Subspecies of *Molossus molossus* recognized by Simmons (2005) and/or Eger (2007) and type localities associated with each subspecies.

Subspecies	Authority	Type Locality
M. m. crassicaudatus	Geoffroy 1805	Asunción, Central Paraguay
M. m. daulensis	Allen 1916	"Daule", Los Ríos, Ecuador
M. m. debilis	Miller 1913	St. Kitts, Lesser Antilles
M. m. fortis	Miller 1913	"Luquillo", Puerto Rico
M. m. milleri	Johnson 1952	Bermuda
M. m. molossus	Pallas 1766	Martinque, West Indies
M. m. pygmaeus	Miller 1900	Netherlands Antilles
M. m. tropidorhynchus	Gray 1839	Cuba
M. m. verrilli	Allen 1908	"Samana", Dominican Republic

noways et al. 1981). They concluded that there was significant morphological variation among intra-island populations, as well as inter-island variation for populations of *M. molossus*. Genoways et al. (1981) suggested that a high degree of philopatry and inbreeding was the reason for the high levels of local geographic variation seen in this species.

Examining genetic patterns of specimens of *M. molossus* from various geographic localities should help clarify unresolved relationships. Few studies have been conducted to determine relationships among subspecies of *M. molossus*, especially any using a genetic approach. McDonough et al. (2011) used the mitochondrial gene cytochrome oxidase I (COI) to identify *Molossus* specimens but did not address subspecies relationships. Furthermore, Sudman et al. (1994) used a cyt*b* sequence from *M. molossus* to identify the familial affinity of *Tomopeas ravus*; however, there have been no further genetic analyses using DNA sequence data to determine patterns of genetic divergence within *M. molossus*. More recently, Gager et al. (2016) used 18 microsatellite markers and the mitochondrial gene COI, along with morphological and acoustic data, to distinguish between morphologically similar species of *M. bondae*, *M. molossus*, and *M. coibensis* in Panama (Gager et al. 2016). *M. bondae* was identified based on size and pelage differences, while *M. molossus* and *M. coibensis* were differentiated using the microsatellite markers and COI (Gager et al. 2016). The authors suggest using multiple approaches to determine species that are morphologically similar. The lack of published genetic data for species of *Molossus* is apparent, and further studies of the phylogenetic relationships of this genus are needed. The objective of this study was to use the mitochondrial gene cytochrome *b* (cyt*b*) to characterize patterns of diversification within *M. molossus*. We tested the hypothesis that genetic patterns of diversification would reflect subspecies lineages.

METHODS AND MATERIALS

Molecular methods.—Tissues of *M. molossus* were borrowed from the Angelo State Natural History Collection, the Field Museum of Natural History in Chicago, and the Natural Science Research Laboratory, Museum of Texas Tech University. Specimens were selected to cover the species range from Central America into northern and central South America and a few sites within the Lesser and Greater Antillean Islands (Fig. 1). A morphological key provided by Eger (2007) was used to confirm species identifications of selected specimens included in the genetic analysis. DNA was extracted from heart, kidney, liver, or muscle tissues that were either frozen or in lysis buffer using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, California) following manufacturer's protocol. The mitochondrial cyt*b* gene was amplified using the polymerase chain reaction (PCR); 12.5µL reactions contained 30–60 ng of DNA, 1 unit of Taq polymerase (New England BioLabs, Ipswich, MA), 2.5 mM of each dinucleoside triphosphate, 1X Taq buffer, 1.5–2.0 mM $MgCl_2$ and 0.16 µM of forward and reverse primers. Cyt*b* was amplified with the following thermal profile; 1 cycle at 94°C for 2 min; 39 cycles at 94°C, 48°C, and 72°C for 1 min each; and a final extension cycle at 72°C for 10 min. PCR and sequencing of each gene fragment was carried out using combinations of primer 15547 (5'- GGCAAATAGGAAATATCATTC-3'; Edwards et al. 1991), primer Gludg (5'-TGACTTGAARAACCATCGTTG-3'; Palumbi 1996), primer MVZ04 (5'-GCAGCCCCTCAGAATGATATTTGT-3'; Smith and Patton 1991), and primer MVZ05 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3'; Smith and Patton 1991). PCR products were sequenced using GenomeLab DTCS-Quick Start Mix in a Beckman Coulter CEQ8000 automated sequencer following manufacturer's protocol, except a quarter of the recommended volumes were used.

Phylogenetic analyses.—Sequencher version 5.0 (Gene Codes Corporation, Ann Arbor, Michigan) and MEGA5 (Tamura et al. 2011) were used to align the sequences, which were then refined by eye if needed. Furthermore, we confirmed that all cyt*b* fragments translated to amino acid sequences. Sequences were deposited in GenBank (Appendix). Models with the lowest Bayesian Information Criterion were used to describe the substitution pattern that best fit the data set (Tamura et al. 2011). A Maximum Likelihood (ML)

Figure 1. Map of Central and South America and Caribbean Islands with locations of specimens of *Molossus* obtained for this study. Shapes correspond to species and subspecies designation (diamond = *M. rufus*, square = *M. m. daulensis*, circle = *M. m. crassicaudatus*, and triangle = *M. m. molossus* (includes *M. m. debilis*, *M. m. fortis*, and *M. m. tropidorhynchus* according to the taxonomy recognized by Eger 2007)).

tree was generated in MEGA5. Statistical nodal support was evaluated with 1000 bootstrap pseudoreplicates.

A Bayesian analysis (BI) of cyt*b* was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Analyses consisted of two simultaneous runs, each with four Markov Chain Monte Carlo chains (three heated and one cold) run for five million generations. Convergence of the two runs was determined when convergence diagnostic <0.01. Trees were sampled every 100 generations with a 25% burn-in. A 50% majority rule consensus tree was used to calculate posterior probabilities and included the proportion of trees saved after convergence of likelihood scores was reached. Nodes in resulting trees containing ≥0.95 Bayesian posterior probabilities (BPP) were considered statistically significant (Ronquist and Huelsenbeck, 2003). FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize and draw trees generated by MrBayes.

Patterns of diversification recovered by the phylogenetic analysis were interpreted based on two different criteria. Genetic divergences between clades were evaluated for agreement with divergence levels outlined by Baker and Bradley (2006) for sister species of mammals by using the Kimura 2-parameter model without gamma correction. These divergences between clades were calculated in MEGA5. In addition, sister clades resulting from ML and BI analyses were compared using the K/θ method introduced by Birky (2013) to delimit lineages that should be recognized as species. The steps outlined in Birky (2013) were used to calculate the value of K/θ, which is the ratio of the mean pairwise sequence difference between a pair of clades (K) and the mean pairwise difference within a clade (θ). A pair of clades was considered to be different species if K/θ > 4.

RESULTS

A total of 63 novel cyt*b* sequences (Appendix) of *Molossus* species from various geographic locations were included in the phylogenetic analysis (Fig. 2). The final alignment included 678 base pairs of original sequences from 4 *M. rufus*, 2 *M. alvarezi*, 1 *M. bondae*, 2 *M. coibensis*, and 54 *M. molossus* (Appendix). Two *M. molossus* sequences from GenBank also were included in the alignment (JQ915205.1 and L19724.1). Both the Bayesian and ML methods recovered similar topologies (Fig. 2). The ML tree was generated using the model Tamura 3-parameter with gamma rate distribution (alpha = 0.1659, Log likelihood score = -1155.248). The species *M. molossus* did not form a monophyletic lineage. Four specimens of *M. molossus* from Cuba (Fig. 2, Clade D) clearly formed a separate lineage from the rest of the *M. molossus* specimens (Clade A1–3) with high BPP support (1.0). The majority of *M. molossus* specimens (Clade A2) clustered together in a large polytomy with the exception of two distinct lineages that were strongly supported—one from the western slope of the Andes in Ecuador and northern Peru (Clade A1; BPP 1.0) and the other from Cuba (Clade D; BPP 1.0). There was not significant support for resolution of branching order among clades A1, A2, A3, and B+C. Subspecies *M. m. fortis* (plus *M. m. debilis*) from Puerto Rico and St. Kitts/St. Croix, *M. m. daulensis* from western Ecuador and Peru, and *M. m. tropidorhynchus* from Cuba formed statistically supported monophyletic lineages in this phylogenetic analysis (Fig. 2). All other currently recognized subspecies (Table 2), including *M. m. crassicaudatus* from South America, did not form separate lineages.

The subspecies *M. m. daulensis* (Clade A1), from the western slope of the Andes in Ecuador and Peru, had an average genetic divergence of 2.7% from the eastern Ecuadorian specimens and an average genetic divergence of 3.7% from *M. m. molossus* specimens (Clade A2; Table 3). The clade containing the Brazil/El Salvador specimens (Clade A3) had an average genetic divergence of 6.0% from the rest of the *M. molossus* clade (Clade A1/A2; Table 3). Furthermore, a lineage containing the currently recognized Cuban subspecies *M. m. tropidorhynchus* (Clade D) had an average genetic divergence of 7.9% from the other *M. molossus* (Clade A1, A2, A3) included in the study (Table 3).

The group containing *M. rufus*/*M. bondae* (Clade B) had an average genetic divergence of 8.4% from *M. molossus* (all members of Clade A1, A2, A3). The

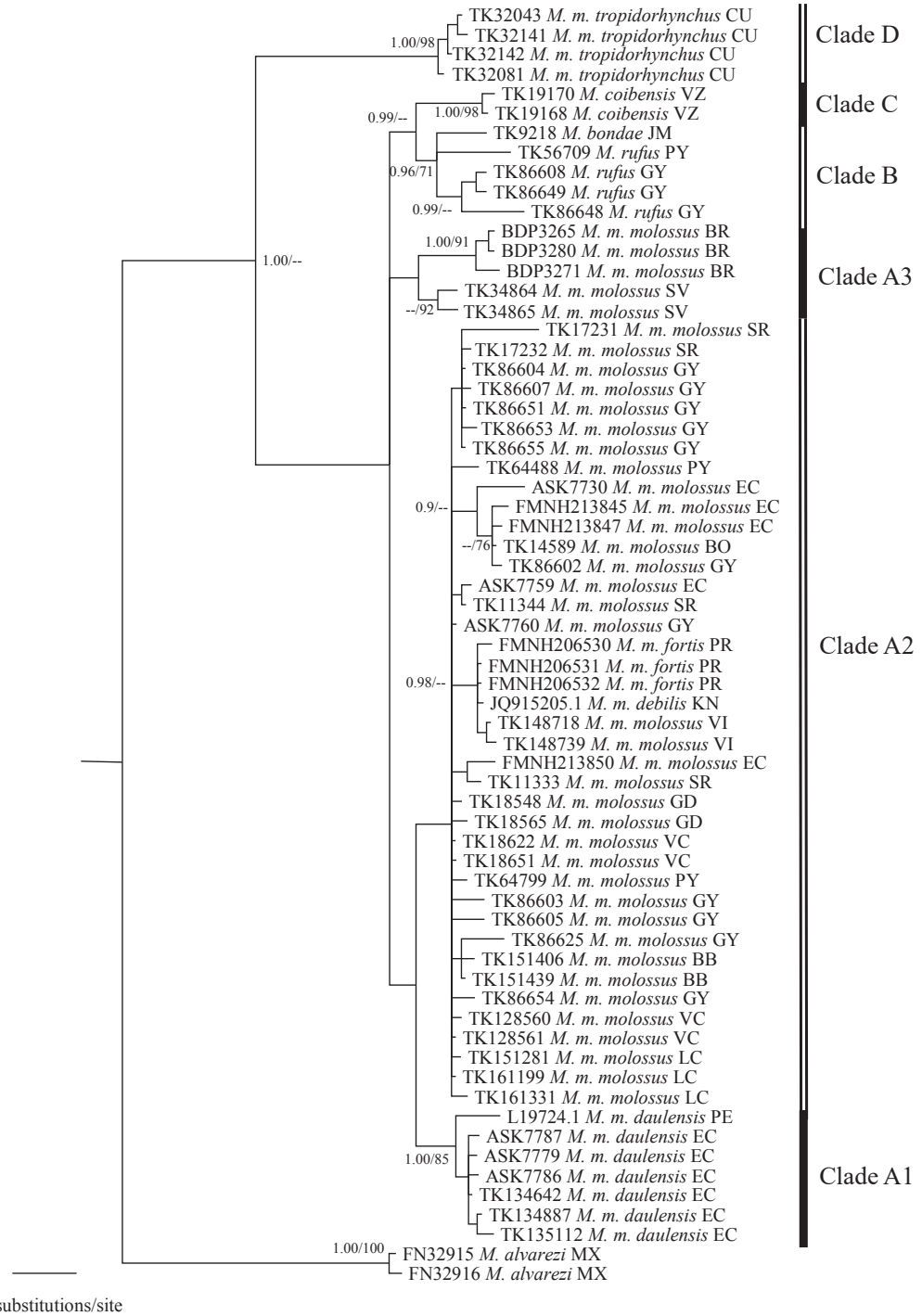

Figure 2. Bayesian phylogram of *M. molossus* specimens based on 678 base pairs of the cyt*b* gene, rooted with the outgroup *M. alvarezi*. Nodes are labeled with BPP values followed by bootstrap values (-- if BPP values <0.95 or if bootstrap values <70). Clades A1, A2, and A3 represent the species *M. molossus*. BB=Barbados, BO=Bolivia, BR=Brazil, CU=Cuba, EC=Ecuador, GD=Grenada, GY=Guyana, JM=Jamaica, KN=St. Kitts and Nevis, LC=St. Lucia, MX=Mexico, PE=Peru, PR=Puerto Rico, PY=Paraguay, SR=Suriname, SV=El Salvador, VC=St. Vincent and the Grenadines, VI=US Virgin Islands, St. Croix, VZ=Venezuela.

Table 3. Average Kimura 2-parameter distances between and within subspecies of *M. molossus* (Clade A1, A2, A3, D) and within and between species *M. rufus*/*M. bondae* (Clade B), *M. coibensis* (Clade C), and outgroup *M. alvarezi* based on 678 bases of cyt*b*. Sample sizes are listed in parentheses. Clade labels also are identified in Fig. 2.

	A1	A2	A3	B	C	D	Outgroup
Clade A1 (7)	**0.0062**						
Clade A2 (40)	0.0373	**0.0111**					
Clade A3 (5)	0.0672	0.0532	**0.0130**				
Clade B (5)	0.0942	0.0805	0.0781	**0.0246**			
Clade C (2)	0.0880	0.0554	0.0610	0.0587	**0.0047**		
Clade D (4)	0.0816	0.0752	0.0809	0.1352	0.1097	**0.0026**	
Outgroup (2)	0.1487	0.1370	0.1394	0.1164	0.1326	0.1270	**0.0030**

Venezuelan specimens (*M. coibensis*, Clade C) had an average genetic divergence of 6.8% from Clade A1, A2, and A3 combined and a genetic divergence of 5.9% from Clade B. The outgroup, *M. alvarezi*, had an average genetic divergence of 13.4% from the rest of the specimens included in this study (Table 3).

K/θ values were generated for six clade pairings using the methods described by Birky (2013) to assess species limits (Table 4). Based on criteria outlined by Birky (2013), K/θ ratios greater than 4 were considered different species. In our analysis, this measure supports species status for the Cuban clade (Clade D) separate from *M. molossus* (Clades designated by A), *M. rufus*/*M. bondae* (Clade B) separate from the Venezuelan clade of *M. coibensis* (Clade C), and *M. rufus* (Clade B) separate from *M. molossus* clade (Clades designated by A). However, the Brazil/El Salvador clade (Clade A3) does not have a K/θ ratio greater than 4, indicating that the Brazilian and Salvadoran samples should be considered part of *M. molossus* based on cyt*b* data.

Table 4. K/θ ratios used to determine species delimitation for two different clades. K/θ > 4 are considered separate species while K/θ < 4 are considered the same species (Birky 2013). Clade labels are identified in Fig. 2.

Clade 1	Clade 2	K/θ
Clade B (*M. rufus/M. bondae*)	Clade C (*M. coibensis*)	4.2*
Clade A1 (*M. m. daulensis*)	Clade A2 (*M. m. molossus*)	2.2
Clade D (*M. m. tropidorhynchus*)	Clade A1/A2/A3 (*M. molossus*)	4.1*
Brazil	El Salvador	3.3
Clade A3 (Brazil/El Salvador)	Clade A1/A2 (*M. molossus*)	2.8
Clade B (*M. rufus/M. bondae*)	Clade A1/A2/A3 (*M. molossus*)	4.3*

DISCUSSION

Phylogenetic patterns recovered from the cyt*b* analysis of *M. molossus* specimens were not consistent with all currently recognized subspecies designations. However, three lineages were consistent with currently recognized subspecies of *M. molossus*, including *M. m. daulensis* from west of the Andes in Ecuador and Peru, *M. m. tropidorhynchus* from Cuba, and *M. m. fortis*, the subspecies from Puerto Rico, with St. Croix and St. Kitts samples (*M. m. debilis*). *Molossus m. fortis* and *M. m. debilis* were recognized originally by Miller (1913) based on morphology and geographic location. Our genetic results suggest that these subspecies, limited to Puerto Rico and the Virgin Islands (Gannon et al. 2005), should be synonymized. Despite a few well-supported clades, there is very little significant phylogenetic structure to resolve branching order of the five species of *Molossus* examined in this study. These results suggest that there is some consistency between these genetic data and the currently recognized subspecies, but the cyt*b* marker was largely unable to recover a monophyletic *M. molossus* clade due to low divergence between this species and *M. rufus*, *M. bondae*, and *M. coibensis*.

M. m. tropidorhynchus should not be considered a subspecies of *M. molossus* based on our results. Instead, the Cuban specimens (Clade D) should be elevated to species level, *M. tropidorhynchus*. In 1839, Gray described a holotype, probably from Havana, Cuba, as *M. tropidorhynchus* (Carter and Dolan 1979). *M. tropidorhynchus* is reported to be somewhat smaller than *M. molossus* from Central and South America and to have an olive brown pelage (Silva-Taboada 1979). Frank (1997) described the occurrence of *M. m. tropidorhynchus* from the Florida Keys. Thus, the specimens of *M. molossus* in the United States are most likely *M. tropidorhynchus* and not *M. molossus*, although this remains to be tested. No further information on the distribution of *M. tropidorhynchus* is known. We compared forearm measurements and the second phalanx on the fourth digit of two Cuban specimens to published keys to determine they were *M. tropidorhynchus* and not another molossid species (Miller 1904; Silva-Taboada 1979).

According to Bradley and Baker (2001), cyt*b* genetic divergence values within the 2–11% range have a high probability of representing separate species of mammals. Applying the Genetic Species Concept, first proposed by Bateson (1909) and later redefined by Baker and Bradley (2006), *M. tropidorhynchus* exhibits reciprocal monophyly and a cyt*b* divergence value of 7.9% from all other *M. molossus* specimens, consistent with recognition of the taxon as a species (Baker and Bradley 2006). Additional justification for elevating this species comes from the criteria used by Birky (2013); the Cuban clade (Clade D) had a K/θ ratio greater than 4 (Table 4). Although the K/θ technique primarily has been used to determine specific relationships in asexual organisms, Birky proposed that use for determining species limits in vertebrates is also possible. Baker and Bradley (2006) suggested that, if possible, it is important to have both nuclear and mitochondrial markers to document presence or absence of species. Haplotypes of cyt*b*, like all other mitochondrial genes, represent lineages of a maternally inherited marker and should be used cautiously to represent species relationships. Furthermore, Davalos and Russell (2014) caution that sex-biased dispersal could mislead interpretations of mitochondrial patterns. However, "barcoding genes" such as COI also are mitochondrial genes and are widely used for species identification (Hajibabaei et al. 2007; Clare et al. 2011), validating our approach. Furthermore, mitochondrial genes have been used in several other mammalian species to determine very closely related species and subspecies designations. Many studies have used cyt*b* and/or other mitochondrial genes to examine very closely related species of *Peromyscus* (Harris et al. 2000; Bradley et al. 2007) and have reported similar genetic divergences among closely related rodent taxa. Piaggio et al. (2002) examined two mitochondrial markers to determine that *Myotis occultus* is not a subspecies of *Myotis lucifugus* as previously reported. Sun et al. (2008) combined cyt*b* sequences, morphological and phonic data to determine subspecies relationships of *Rhinolophus macrotis* in China. More recently, Sun et al. (2015) used the whole mitochondrial genome to examine the relationship of two subspecies of *Rhinolophus sinicus*. Hence, adding nuclear data from a rapidly evolving marker should assist in confirming our proposal that *M. tropidorhynchus* be recognized as a separate species from *M. molossus*.

Clade B and C containing *M. rufus*, *M. coibensis*, and *M. bondae* specimens unexpectedly produced a paraphyletic *M. molossus* when the Cuban specimens are considered as a subspecies of *M. molossus*. The position of Clade B/C was unresolved and therefore questions remain regarding the relationships of other *Molossus* species. Unfortunately, according to personnel at the USNM, the voucher specimens for USNM582416/TK86608, USNM582418/TK86648, and USNM582419/TK86649 have been misplaced so we were not able to examine or positively identify these three specimens. Based on their tight clustering with one known representative of *M. rufus* (TTU96091/TK56709), and the fact that *M. rufus* is known to occur in Guyana (Eger 2007), we suspect that they are *M. rufus*. The only Jamaican specimen (CM44668/TK9218) included in this study is placed in the same clade as *M. rufus* (Clade B) in the cyt*b* tree. However, according to Genoways et al. (2005), the only species of *Molossus* to occur on the island of Jamaica is *M. molossus*. We re-examined this specimen and, based on published keys (Burnett et al. 2001; Eger 2007), the specimen is *M. bondae*. Dolan (1989) placed *M. bondae* as sister to *M. rufus*; our tree depicts a similar relationship. Therefore, CM44668/TK9218 represents *M. bondae*; and, to our knowledge is the first record of *M. bondae* from Jamaica. However, given the uncertainties uncovered regarding the identity of several of our specimens, a comprehensive phylogenetic analysis of the entire genus is critically needed.

The occurrence of individuals from Brazil and El Salvador in a single, although unsupported, clade (A3) was unexpected, given that they are geographically well separated. We do not know whether to consider this clade as part of *M. molossus*. Clade A3 (Brazil + El Salvador) had a genetic divergence of 6.0% from other *M. molossus* specimens, which could be interpreted as a species level divergence (Bradley and Baker 2001). However, K/θ values do support El Salvador and Brazil (Clade A3) as the same species as the rest of *M. molossus* (Clade A1 and A2). Another possibility is that the Brazilian specimens in Clade A3 could represent *M. currentium*. This species is known from northern Paraguay and is only slightly larger than *M. molossus* (Eger 2007), making morphological identification challenging. Detailed morphological work on these specimens was not possible and therefore research on additional specimens from Brazil and El Salvador will be necessary to clarify the confusion regarding the identity of the specimens in Clade A3.

Low genetic divergence values (1.2%) were recorded for *M. molossus* specimens (Clade A2) over a wide geographic area. The low genetic divergence within *M. molossus* suggests that this group of bats evolved relatively recently. Larsen et al. (2007) observed a similar lack of geographic structuring in the Caribbean and South American species, *Artibeus planirostris*, and hypothesized rapid radiation and dispersal for this species. Rapid radiation and dispersal could account for the lack of geographic structuring within *M. molossus* as well. *Molossus molossus* have been reported to have excellent colonizing ability and a capacity for overwater dispersal (Frank 1997), as demonstrated by their colonization of Florida in recent history. Other molossids are known to forage over long distances (up to 50 mi (80 km) in *Eumops perotis*, Vaughan 1978) or to make long distance migrations (*Tadarida brasiliensis*, Glass 1958; Cockrum 1969; Russell et al. 2005), so dispersal to Caribbean islands would presumably not be difficult for *M. molossus*.

Molossus molossus is a difficult species to identify because of high morphological variation across the species range (Genoways et al. 1981; Dolan 1989). Small localized demes and environmental constraints could have played a role in increasing morphological variation. However, despite the fact that this variation is reflected in numerous recognized subspecies, genetically these bats are quite uniform (with the exception of the *M. tropidorhynchus* lineage). Phenotypic plasticity could also explain the high degree of morphological variation co-occurring with apparent genetic uniformity in *M. molossus*. Phenotypic plasticity is described as the capacity of a single genotype to exhibit variable phenotypes in different environments (Whitman and Agrawal 2009). As *M. molossus* populations expanded, the species may have adapted to different environmental factors, resulting in morphological variation that is not reflected in the mitochondrial marker examined in this study.

Biogeographically, the results of this study support two invasions into the Caribbean, as well as a separation of populations by the rise of the Andes. We hypothesize an older dispersal event into Cuba by the ancestor of *M. tropidorhynchus* and a younger dispersal

event into the Lesser Antillean Islands by *M. molossus*. Cuba, Hispaniola, and Jamaica are much older islands that originated from the Caribbean plate when South America and North America started to separate from each other (Dávalos 2009). Furthermore, smaller islands were submerged during periods of high sea levels while Cuba and Hispaniola were a single land mass (Dávalos 2009). This may explain why Cuba has many endemic species, including the bat species *Lasiurus insularis*, *Mormopterus minutus*, *Natalus primus* (Griffiths and Klingener 1988; Dávalos 2004) and now *Molossus tropidorhynchus*.

M. m. daulensis from the western slope of the Andes Mountains appears to have been separated from *M. m. molossus* on the eastern slope for a sufficient length of time to accumulate distinct genetic differences (3.7% divergence at cyt*b*). These results are similar to the divergence seen in COI sequences of *M. molossus* bats from east and west of the Andes by McDonough et al. (2011). Furthermore, other species of bats such as *Eumops wilsoni* and *E. glaucinus* show similar allopatric distribution and level of divergence based on their location on either side of the Andes (Bartlett et al. 2013). At this time, we are not proposing the elevation of *M. m. daulensis* to species status; however future work, such as a population genetic approach, might be appropriate for examining this lineage more closely.

The phylogeny of *M. molossus* generated in this study is intended to serve as a working hypothesis for future work on the hidden biodiversity in this species. Further investigation should be carried out on the remaining three subspecies in the species *M. molossus* that we were unable to include in this study. Likewise, future studies should include representatives from all recognized species of *Molossus* to create a clearer picture of the evolutionary relationships in this problematic genus. Sequence data from additional specimens and from a more rapidly evolving genetic marker, such as microsatellites (Gager et al. 2016), or high-throughput sequencing analyses, such as RADSeq (Davey and Blaxter 2010), should give a more accurate representation of the diversity within *M. molossus* across Central and South America and the Caribbean.

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APPENDIX

Species, catalog number, tissue number, locality, and GenBank accession numbers generated in the cyt*b* and analysis. ASK (tissue number), FN (tissue number), QCAZ (catalog number), and ASNHC (catalog number) = Angelo State Natural History Collection, Angelo State University; TK (tissue number) and TTU (catalog number) = Natural Science Research Laboratory, Museum of Texas Tech University; CM (catalog number) = Carnegie Museum; USNM (catalog number) = United States National Museum; BDP (tissue number) and FMNH (catalog number) = Field Museum of Natural History; KM = GenBank accession number.

Molossus alvarezi (2).—MEXICO: Yucatan; Tekax; Merida, Colegio Peninsular ASNHC7023/FN32915/KM387333; ASNHC7024/FN32916/KM387334.

Molossus bondae (1).—JAMAICA: St. Catherine Parish; 0.2 mi E Watermount CM44668/TK9218/KM387368.

Molossus rufus (4).—PARAGUAY: San Pedro; Yaguarete Forests; 1.7 km E Headquarters TTU96091/TK56709/KM387361. GUYANA: Upper Demerara-Berbice; Dubulay Ranch, Region 10, Subregion 2 USNM582416/TK86608/KM387345; USNM582418/TK86648/KM387380; USNM582419/TK86649/KM387346.

Molossus coibensis (2).—VENEZUELA: Bolivar; 12 km S El Manteco CM78716/TK19168/KM387358; CM78717/TK19170/KM387322.

Molossus molossus (5).—BRAZIL: Sao Paulo; Estaçao Biológica de Boraceia FMNH219980/BDP3265/KM387326; BDP3271/KM387327; BDP3280/KM387328. EL SALVADOR: La Paz; Playa El Zapote TTU60988/TK34864/KM387373; TTU60989/TK34865/KM387377.

Molossus molossus daulensis (6).—ECUADOR: El Oro; Manchala; Cuidad Manchala, Junin St., Hotel Mercy ASNHC14120/ASK7779/KM387366; ASNHC14121/ASK7786/KM387356; QCAZ8620/ASK7787/KM058059. ECUADOR: El Oro; Palmales, Reserva Militar Arenillas TTU102336/TK135112/KM387375. ECUADOR: Guayas; Manglares Churute; Guardiania Del Parque TTU103736/TK134642/KM387351. ECUADOR: Guayas; Bosque Protector Cerro Blanco, Centro de Visitantes TTU103300/TK134887/KM387363.

Molossus molossus molossus (39).—BARBADOS: St. Thomas Parish; Welchman Hall Gully, 0.5 km N Welchman Hall TTU109911/TK 151406/KM387364. BARBADOS: Christ Church Parish; Graeme Hall Swamp, 0.5 km N St. Lawrence TTU109888/TK151439/KM387353. BOLIVIA: La Paz; 1 mi W Puerto Linares TTU34957/TK14589/KM387336. ECUADOR: Zamora-Chinchipe; 1 km N, 0.8 km E Zamora ASNHC14140/ASK7760/KM387365; QCAZ8592/ASK7759/KM387325. ECUADOR: Morona-Santiago; north of Macas, Nueva Jerusalem ASNHC14133/ASK7730/KM387324. ECUADOR: Orellana; Estacion Cientifica Yasuni FMNH213845/BDP5170/KM387367; FMNH213847/BDP5153/KM387331; FMNH213850/BDP5175/KM387332. GRENADA: St. George; Chemin River, 0.5 km E Confer CM63415/TK18548/KM387337; CM63432/TK18565/KM387338. GRENADINES: Union Island; Big Sand Beach, 1 km N Clifton CM63270/TK18622/KM387370. GRENADINES: Union Island; 0.5 km N Clifton CM63488/TK18651/KM387371. GUYANA: Upper Demerara-Berbice; Dubulay Ranch USNM582412/TK86625/KM387344; TK86651/KM387362. GUYANA: Upper Demerara-Berbice; Dubulay Ranch, Region 10, Subregion 2 USNM582423/TK86602/KM387341; USNM582424/TK86603/KM387360; USNM582425/TK86604/KM387342; USNM582426/TK86605/KM387379; USNM582361/TK86607/KM387343; USNM582428/TK86653/KM387347; USNM582429/TK86654/KM387348; USNM582430/TK86655/KM387349. PARAGUAY: Pte. Hayes; Estancia Loma Pora TTU80400/TK64488/KM387323. PARAGUAY: Cordillera; Estancia Sombrero TTU80302/TK64799/KM387378. PUERTO RICO: Vieques Island; Green Beach Gate FMNH206530/BDP4903/KM387376. PUERTO RICO: Vieques Island; Ammunition Bunkers FMNH206531/BDP4906/KM387329; FMNH206532/BDP4907/KM387330. ST. VINCENT AND THE GRENADINES: Bequia; 2.3 km NE Port Elizabeth TTU105217/TK128560/KM387350; TTU105218/TK128561/KM387374. SURINAME: Paramaribo; Paramaribo CM64421/TK11333/KM387335; CM64432/TK11344/KM387357. SURINAME: Saramacca; Raleigh Falls TTU35731/TK17231/KM114224; TTU35732/TK17232/

APPENDIX (CONT.)

KM387369. UNITED STATES: St. Croix; West End Quarter; Brugall Rum Factory; 0.45 km E, 0.9 km N Frederiksted TTU111461/TK148718/KM387381. UNITED STATES: St. Croix; West End Quarter; Estate Jolly Hill; 0.35 km E, 0.25 km S Jolly Hill TTU111464/TK148739/KM387352. ST. LUCIA; Castries; Union Nature Trail, 0.6 km N, 0.5 km W TTU109924/TK151281/KM387382. ST. LUCIA: Dennery; Dennery River, 0.25 km S, 2 km W Dennery TTU109943/TK161331/KM387355. ST. LUCIA: Micoud; Troumassee River, 1.3 km W Micoud TTU109945/TK161199/KM387354.

Molossus molossus tropidorhynchus (4).—CUBA: Guantanamo Province; Guantanamo Bay Naval Station TTU52669/TK32043/KM387339; TTU52666/TK32081/KM387359; TTU52648/TK32141/KM387340; TTU52649/TK32142/KM387372.

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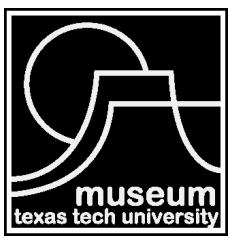

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