



# OCCASIONAL PAPERS

## MOLECULAR DIVERSITY WITHIN *MELANOMYS CALIGINOSUS* (RODENTIA: ORYZOMYINI): EVIDENCE FOR MULTIPLE SPECIES

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### ABSTRACT

Nucleotide sequences from the mitochondrial DNA cytochrome-*b* gene were used to infer phylogenetic relationships and estimate genetic distances from 10 individuals of *Melanomys caliginosus* and to explore the hypothesis that this taxon is comprised of multiple species. Individuals of four geographic populations of *M. caliginosus* from Central America (Nicaragua and Costa Rica), Panama, Venezuela, and Ecuador, respectively, were included in this analysis. Topologies obtained from maximum parsimony and Bayesian inference analyses were identical and produced clades referable to each of the geographic populations. Genetic distances between any pair-wise comparisons of the four groups (except between Panamanian and Venezuelan samples) were comparable to values estimated from comparisons of sister species in the closely related genus *Nectomys*. Distances between samples from Panama and Venezuela were greater than those of samples within the Ecuadorian and Central American clades, but less than that between species of *Nectomys*. Based on results from the sequence data, it appears that all four of the populations should be elevated to species level; however, additional data are needed to resolve the nomenclature of the Panamanian and Venezuelan populations.

Key words: *Melanomys caliginosus*, *Melanomys chrysomelas*, *Melanomys columbianus*, *Melanomys idoneus*, molecular phylogenetics, Oryzomyini

### INTRODUCTION

*Melanomys caliginosus* originally was described as a species of *Hesperomys* by Tomes (1860) and later aligned with *Akodon* (Allen 1891, 1899), *Zygodontomys* (Bangs 1902), or *Oryzomys* (Thomas 1894, 1902, 1903; Trouessart 1905; Miller 1924). Allen (1913) elevated *Melanomys* to generic level and included nine species (Fig. 1). Subsequent studies (Glydenstolpe 1932; Tate 1932) followed this arrangement; however,

Goldman (1918) reduced the number of recognized species by placing *chrysomelas* and *idoneus* in synonymy with *caliginosus*. Cabrera (1961) relegated five of Allen's seven remaining species (Allen 1912, 1913) to subspecies of *M. caliginosus*, resulting in *Melanomys* containing three species (*caliginosus*, *robustus*, and *zunigae*).

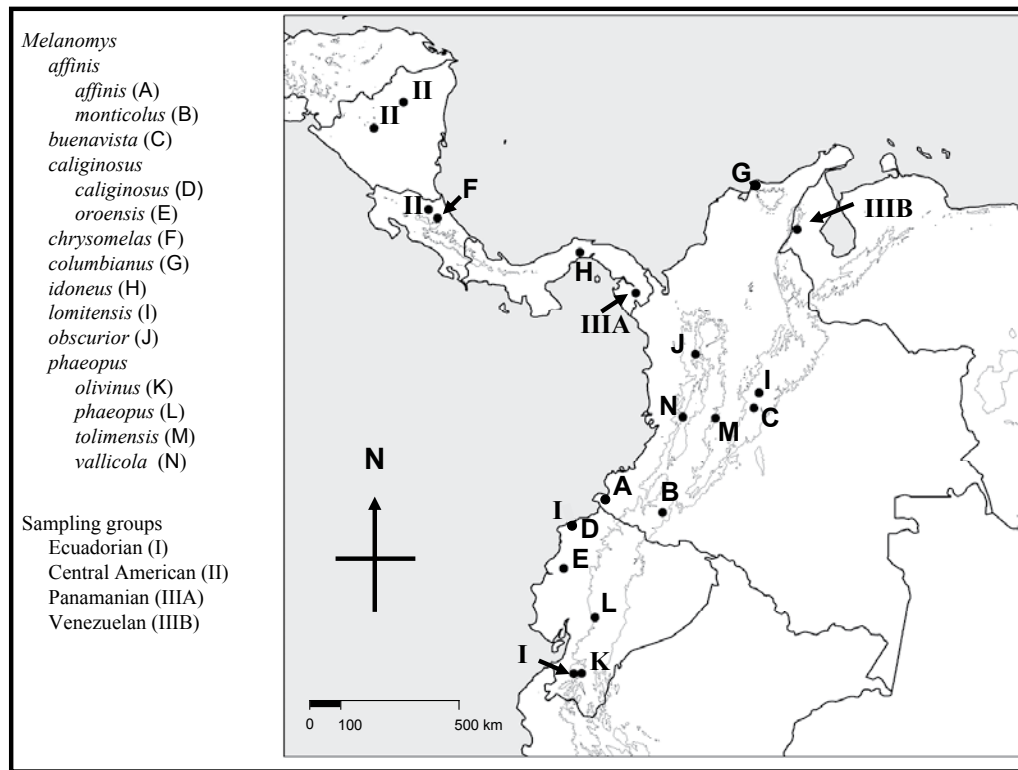


Figure 1. Map of type localities for *Melanomys* taxa (species and subspecies) based on Allen's (1913) classification, as well as location of samples used in analyses. Type localities are listed alphabetically by species, then subspecies, and designated on the map with capital letters (A–N). Sampling localities are listed by geographic groups and designated on the map with Roman numerals (I–III). Topographic line represents 1,500 m elevation. Type locality D (*caliginosus*) is the same as for one of the Ecuadorian sample localities.

*Melanomys caliginosus* as recognized by Musser and Carleton (2005) ranges from Ecuador to the highlands of Nicaragua. This arrangement contains nine taxa (*affinis*, *buenavista*, *caliginosus*, *chrysomelas*, *columbianus*, *idoneus*, *lomitensis*, *obscurior*, and *phaeopus*; Musser and Carleton 2005) recognized as species or subspecies by previous authors (Allen 1912, 1913; Goldman 1912; Glydenstolpe 1932; Tate 1932; Cabrera 1961). However, morphological variation across its geographic range, as recognized by previous authors (Allen 1913; Glydenstolpe 1932; Tate 1932), suggests that *Melanomys caliginosus* may be comprised of multiple species-level taxa, although to date no genetic data (nucleotide sequence or karyotypic) is available to test this supposition.

The purpose of this study was to examine genetic variation, using nucleotide sequences from the

mitochondrial cytochrome-*b* gene (*Cytb*), among populations of *M. caliginosus*, to determine whether phylogroups (Avise and Walker 1999) exist within this species as currently recognized and if potential phylogroups merit species status (Baker and Bradley 2006). These populations were chosen because they represent groups historically recognized as species within *Melanomys* and because of their representation of both the extreme ends and the middle of the geographic distribution of *M. caliginosus*. Additionally, sampling was restricted by availability of specimens (all populations with known available tissue samples were examined). The utility of *Cytb* as an informative genetic marker for examining species level questions in Oryzomyini rodents is well documented (Myers et al. 1995; Bonvicino and Moreira 2001; Langguth and Bonvicino 2002), and therefore, is an appropriate genetic marker to address the questions proposed herein.

## MATERIALS AND METHODS

*Samples.*—Ten individuals of *Melanomys caliginosus* were collected from natural populations, following methods approved by the American Society of Mammalogists Animal Care and Use Committee (Gannon et al. 2007), or were borrowed from museum collections (Appendix). Samples of the four geographic populations (Fig. 1) were examined as follows: Ecuador ( $n = 3$ ), Central America ( $n = 4$ ), Venezuela ( $n = 1$ ), and Panama ( $n = 2$ ).

*DNA Extraction, PCR Amplification, and DNA Sequencing.*—Genomic DNA was isolated from approximately 0.1 g of liver or muscle tissue, using a Qiagen extraction kit (Qiagen, Inc.; Valencia, California). The complete *Cytb* gene (1,143 bp) was amplified using polymerase chain reaction methods (PCR—Saiki et al. 1988) with GoTaq (Promega Corp.; Madison, Wisconsin), and primers MVZ05 (Smith and Patton 1993) and CB40 (CCACTAYCAGCACCCAAAGC). Reaction concentrations (50  $\mu$ l volume) included:  $\leq 300$  ng genomic DNA, 0.07 mM dNTPs, 2.86 mM MgCl<sub>2</sub>, 5  $\mu$ l 10X buffer, 1.25 U enzyme, and 0.286  $\mu$ M of each primer. PCR thermal profiles included an initial denaturation at 95°C (2 min), 30–40 cycles with denaturation at 95°C (45 sec), annealing 47°C (45 sec), extension at 72°C (1 min 30 sec), and a final extension cycle of 72°C (8 min).

Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Inc.; Valencia, California) and sequenced using ABI Prism Big Dye Terminator v3.1 ready reaction mix (Applied Biosystems; Foster City, California). The primers used for PCR amplification also were used with internal primers (O400R, GCCCTCAGAAGGATATTGTCTCATGG; O700H, GGAAATATCATTCTGGTTAATATGTGC; O870R, ATGGAGCGTAGRATRGCGTAGGC; F1, Whiting et al. 2003; 400F, Tiemann-Boege et al. 2000; and 700L, Peppers and Bradley 2000) for cycle sequencing at 95°C (30 sec) denaturing, 50°C (20 sec) annealing, and 60°C (3 min) extension. Following 25–30 cycles, reactions were precipitated in isopropanol. Purified samples were sequenced using an ABI 3100-*Avant* automated sequencer. Sequencher 4.1 software (Gene Codes Corp.; Ann Arbor, Michigan) was used to align and proof nucleotide sequences and chromatograms

were examined to verify any discrepancies. MEGA 4.0 (Tamura et al. 2007) was used to check for stop codons and the presence of pseudogenes. Nucleotide sequences were deposited in GenBank and accession numbers are listed in the Appendix.

*Data analyses.*—Nucleotide sequence data were evaluated using three methods. First, a maximum parsimony analysis, implemented in PAUP\* version 4.0b10 (Swofford 2002), was conducted on *Cytb* sequences to evaluate phylogenetic relationships. Nucleotide positions were treated as equally-weighted, unordered, discrete characters with four possible states: A, C, G, and T. Uninformative characters were excluded from parsimony analyses. Optimal trees were estimated using the heuristic search method with tree bisection-reconnection branch swapping and stepwise addition sequence options. Nodal support was assessed by using non-parametric bootstrapping (Felsenstein 1985) with 1,000 pseudo-replicates.

Second, the software MrModeltest (Nylander 2004) identified the GTR+ $\Gamma$ +I as the best fit model for Bayesian inference. Parameters estimated from the model are as follows: substitution rate matrix: 6.7890 (AC), 15.1377 (AG), 7.1674 (AT), 1.3367 (CG), 76.8337 (CT), and 1 (GT);  $\Gamma = 0.9254$ ; I = 56%. Bayesian analysis was performed using MRBAYES 3.1 software (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). In the Bayesian inference analysis, sequences were partitioned by codon with a site-specific gamma distribution and the following options: four Markov-chains, 10 million generations, and sample frequency every 1,000 generations. Four independent runs of one million generations each were performed to confirm the stabilization of likelihood scores. Based on this analysis, the first 1,000 trees were discarded as “burnin” and the remaining trees were used to estimate a maximum likelihood tree. Nodal support for topologies was estimated by using clade probabilities estimated with MRBAYES 3.1 (Huelsenbeck and Ronquist 2001).

Third, mean pairwise genetic distances were estimated using the Kimura 2-parameter (Kimura 1980) model of evolution. A priori groups were established

based on the four subspecific delineations for comparison of genetic distances. *Sigmodontomys alfari*, *Holochilus chacarius*, *Nectomys squamipes*, *N. apicalis*,

*Aegialomys xantheolus*, and *Oryzomys palustris* were used as outgroup taxa in all analyses.

## RESULTS

Nucleotide sequences from the 1,143 bp of the *Cytb* gene were obtained for 10 individuals representing four populations of *M. caliginosus*. Overall, nucleotide frequencies were A = 31.8%, C = 27.3%, G = 11.8%, and T = 29.2%. Comparisons of nucleotide substitutions revealed that transitions were 3.76 times more common than transversions.

In the parsimony analysis, 265 informative characters were used to generate two equally most-parsimonious trees (length = 813 steps, consistency index = 0.4588, and retention index = 0.5510). A strict consensus tree (not shown) produced three major clades (I–III) of *Melanomys*. Clade I contained three individuals from Ecuador, clade II contained four individuals from Central America, and clade III contained individuals from Panama (IIIA) and Venezuela (IIIB). A fourth clade (IV) containing *Sigmodontomys* was placed within the large clade containing all samples of *Melanomys*. All major clades (I–IV), as well as the

clade containing all *Melanomys* samples, had moderate (76) or strong (100) bootstrap support.

The Bayesian analysis generated a topology (Fig. 1) that was identical to the parsimony topology. All major clades were supported with posterior probabilities >95%; however, no support (bootstrap or posterior probabilities) was obtained for relationships between clades.

The genetic distance (Table 1) between specimens of the benchmark species, *N. apicalis* and *N. squamipes*, was 7.36%. In comparison, genetic distances between clade I and the other clades (II and III) were 6.76% and 7.64%. Genetic distances between clade II and the other clades (I and III) were 6.95% and 7.64%. Genetic distances of individuals within clades I and II were less than 2.0%, whereas the distance between the two subgroups (IIIA and IIIB) comprising clade III was 4.50%.

## DISCUSSION

Phylogenetic analyses of the *Cytb* data produced topologies that were identical in parsimony and Bayesian analyses (Fig. 2). Samples from each of the four populations form reciprocally monophyletic groups that were moderately or strongly supported, and followed previously proposed relationships (Allen 1913; Glydenstolpe 1932; Tate 1932). Additionally, genetic distances between these taxa (Table 1) were similar to the genetic distance between *N. apicalis* and *N. squamipes*, members of a closely related genus.

The first group (clade I) corresponded to Ecuadorian specimens and was strongly supported by statistical support and genetic distances (Table 1) as distinct from the other samples. Clade II, composed of specimens from Central America, was strongly supported as distinct from other clades. The genetic distances (Table 1) between clade II and the other taxa were similar to that

between the benchmark species (*Nectomys squamipes* and *N. apicalis*). Clade III was comprised of two samples from Panama (IIIA) and one from Venezuela (IIIB). Members of this clade were distinct from the other clades and a comparison of genetic distances between this group and other taxa was similar to that observed between the benchmark species. The genetic distance (Table 1) between the samples from Panama and the sample from Venezuela, although less than the distance between other geographic groups, was greater than the genetic distance (Table 1) within other taxa. Additionally, strong clade support values and historical recognition of two species based on larger body size of the Venezuelan samples (Allen 1913) raises additional questions regarding relationships between these two taxa and the remaining ingroup taxa. More individuals of both subspecies from a broader geographic range will be needed to better address these questions.

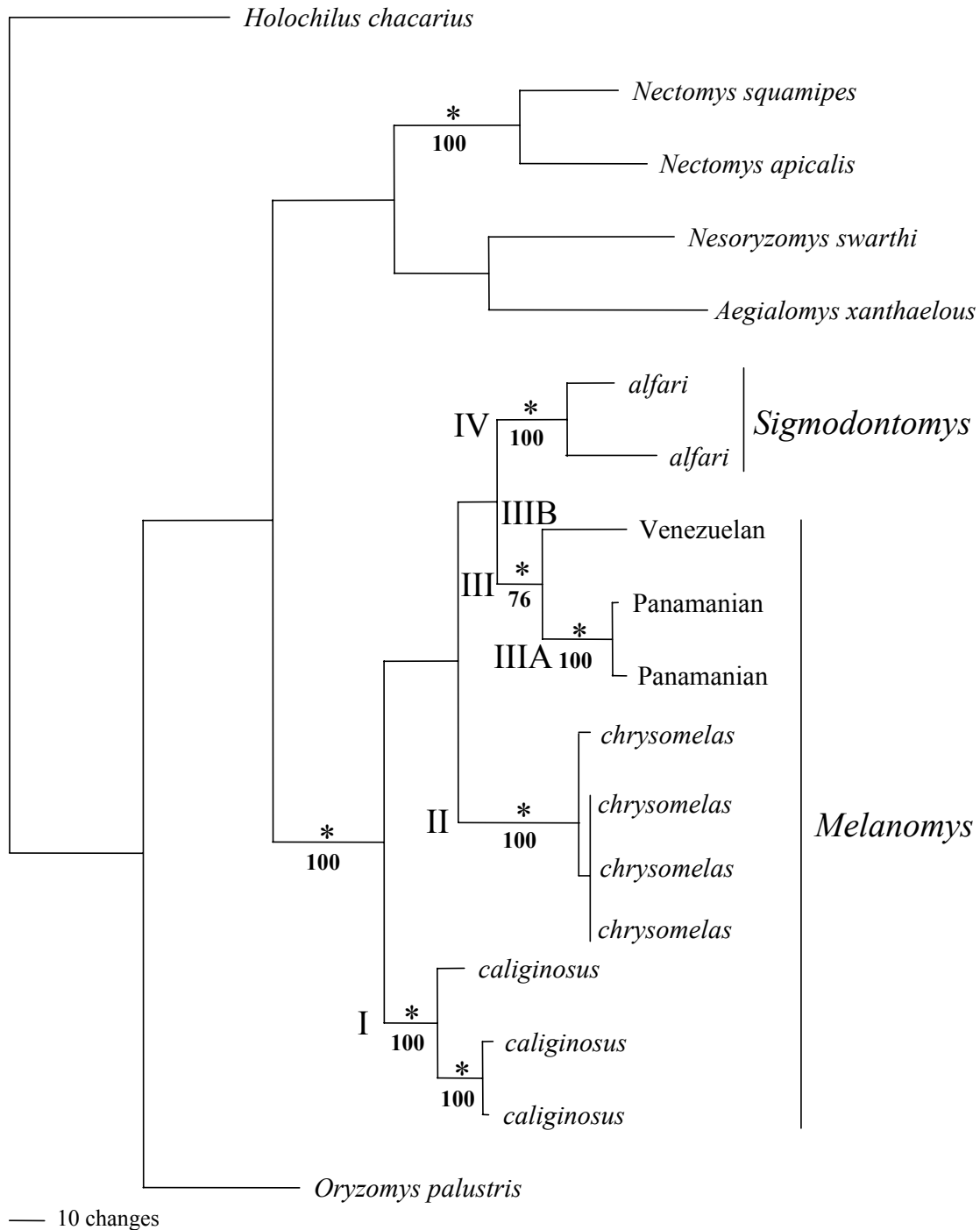


Figure 2. Phylogram constructed from nucleotide sequences obtained from the cytochrome-*b* gene of four taxa of *Melanomys* using Bayesian inference (GTR+ $\Gamma$ +I model of evolution). Roman numerals (I-IV) depict major clades as described in text. Asterisks above branches represent Bayesian posterior probabilities  $\geq 95\%$ . Numbers below branches are parsimony bootstrap support values. Branches without support values had Bayesian posterior probabilities  $< 95\%$  and bootstrap values less than 50.

Table 1. Kimura 2-parameter (Kimura 1980) genetic distances from the cytochrome-b gene showing average percent differences between and among groups. Clade designations from text are listed in parentheses.

Between <i>Nectomys apicalis</i> and <i>N. squamipes</i>	7.52% ± 0.30%
Within clade I	1.84% ± 1.29%
Within clade II	0.27% ± 0.23%
Within clade III	3.23% ± 2.57%
Between clade I and clade II	7.64% ± 0.08%
Between clade I and clade III (Venezuela)	7.19% ± 0.05%
Between clade I and clade III (Panama)	6.54% ± 0.19%
Between clade II and clade III (Venezuela)	7.02% ± 0.12%
Between clade II and clade III (Panama)	7.01% ± 0.18%
Between clade III (Venezuela) and clade III (Panama)	4.54% ± 0.30%

There are three options to explain the phylogenetic topology of *Melanomys* clades (I–III) reconstructed herein. The first option would treat all samples as a single species. This would be congruent with current usage (Musser and Carleton 2005), but minimizes genetic differences identified among clades, as well as morphological differences identified by previous researchers (e.g. Allen 1913). The second option would be to recognize three unique species (corresponding to clades I, II, and III). This option, although not in agreement with the current taxonomy, emphasizes genetic distances observed between clades I, II, and III, is congruent with historical arrangements (Allen 1912, 1913; Glydenstolpe 1932; Tate 1932), and follows the genetic species concept as discussed by Bradley and Baker (2001) and Baker and Bradley (2006). However, this option fails to resolve the genetic variation within clade III. The third option would be to recognize four species (one species each from clades I and II and two species from clade III). This option would agree with historical classifications (Allen 1912, 1913; Glyden-

stolpe 1932; Tate 1932), and would address the genetic variation present within clade III.

It is our recommendation that the third option (four species) is the most appropriate option based on data available at this time. These data unequivocally support recognizing clades I–III as species level entities, which agrees with morphological differences identified by previous authors (Allen 1913; Glydenstolpe 1932; Tate 1932) to justify multiple species. Additionally, the genetic divergence between specimens from Panama and Venezuela suggests that two species exist within clade III.

Clade I contains individuals from Ecuador, including two from Esmeraldas which is thought to be the type locality (Allen 1913) for *M. caliginosus* (Tomes 1860), and this is the name assigned to individuals in this clade. The only available name for members of clade II is *M. chrysomelas* (Allen 1897), for which the type locality is Suerre, near Jimenez, Limon, Costa Rica,

which is approximately 38 km from a sample included in the analyses herein. Additionally, in his discussion of *chrysomelas*, Allen (1913) noted that the samples of *chrysomelas* from Nicaragua were identical to samples from Costa Rica, a relationship recovered herein as well. The most appropriate name for the Panamanian members of clade III (IIIA) is *idoneus* (Goldman 1912), described from Cerro Azul, near the headwaters of the Chagres River, Panama. Specimens sequenced herein were compared to the original description as well as to the comparison between *chrysomelas* and *idoneus* performed by Allen (1913). Samples of *chrysomelas* we examined had a broader interorbital breadth than samples from Panama; additionally, the Panamanian individual was paler, with more yellow and less rufus than *chrysomelas*. These two characters, described by Allen (1913) further support the use of *idoneus* for the Panamanian individuals of clade III. For the Venezuelan member of clade III (IIIB), three valid names exist; *columbianus* (Allen 1899), *lomitensis* (Allen 1913), and *buenavista* (Allen 1913). Unfortunately, we were unable to examine the specimen included here to compare it with the type specimens or the type descriptions of *columbianus*, *lomitensis*, or *buenavista*. However, the most appropriate name for this group appears to be *M. columbianus* (Allen 1899). Described from a specimen collected at 914 m in the Santa Marta district of northeastern Colombia (Allen 1899), *columbianus* is close geographically to the origin of the sample from Venezuela (approximately 170 km). The next closest taxon of *caliginosus* is described as *lomitensis* from a specimen collected at 1,524 m in eastern Colombia (Allen 1913). In addition to the elevational difference and geographical distance (~689 km), the Andes Mountains presumably present a significant barrier between the type locality of *buenavista* and the sample from Venezuela used herein. Although we recommend that the Venezuelan sample represents a distinct species within the genus *Melanomys*, determination of which name is most appropriate should be withheld until the sample sequenced herein can be compared to the type

specimens for *buenavista*, *lomitensis*, and *columbianus*. Because of the small sample size and uncertainty in nomenclature for the Venezuelan sample we recognize the two groups as different species, but further examination of additional Panamanian and Venezuelan samples, as well as the inclusion of Colombian individuals, will be necessary before a definitive conclusion can be drawn regarding the relationship and nomenclature of these two groups.

A fourth clade (clade IV), containing samples referable to *Sigmodontomys alfari*, was observed within the clade containing samples referable to *Melanomys*. Examinations of sequences and chromatograms found no evidence of contamination or human error that could have generated this result. It appears that *Melanomys* and *Sigmodontomys* are not reciprocally monophyletic (although the actual relationship was represented by a polytomy). Although placement of *Sigmodontomys* within *Melanomys* may be an artifact of taxon bias due to other members of *Melanomys* (*zunigae* and *robustus*) not being represented, the data possibly suggest that the genus *Sigmodontomys* is not a valid genus. The taxonomic status of *Sigmodontomys* is not the focus of this study, but it does represent a question that should be pursued further.

The current taxonomic arrangement of *Melanomys caliginosus* as a single species does not recognize the levels of genetic variation between taxa placed under this name. Phylogenetic topologies, as well as genetic distances, support recognition of four species; *caliginosus*, *chrysomelas*, and two of undetermined nomenclature. This arrangement is congruent with previous morphological examinations of the species (Allen 1913; Glydenstolpe 1932; Tate 1932). An examination of other populations previously assigned to *M. caliginosus* and the two other species in the genus (*robustus* and *zunigae*) are needed to better elucidate the taxonomic relationships within this group.

## ACKNOWLEDGMENTS

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## APPENDIX

*Specimens examined.*—Specimens included in this study are listed below by species. Museum acronyms follow Hafner et al. (1997). Specific identification numbers or catalogue numbers, followed by GenBank accession numbers, are in parentheses. Abbreviations for identification numbers are as follows: Angelo State Natural History Collections (ASNHC); Field Museum of Natural History (FMNH); Museo Historia Natural de La Salle (MNHLS); Museum of Vertebrate Zoology (MVZ), Robert Timm (RMT) Voucher at University of Kansas Museum of Natural History; Royal Ontario Museum (ROM); Museum of Texas Tech University (TTU); and Smithsonian Museum of Natural History (USNM).

*Aegialomys xantheolus.*—ECUADOR: Guayas; Bosque Protector Cerro Blanco (TTU103309, EU340015).

*Holochilus chacarius.*—PARAGUAY: Pte Hayes; Estancia Loma Pora (TTU104423, DQ227455).

*Melanomys caliginosus.*—ECUADOR: El Oro; Zaruma, Cerro Urcu (TTU102727, EU340019). ECUADOR: Esmeraldas; Comuna San Fransisco de Bogota (TTU102819, EU340020; TTU102975, EU340021).

*Melanomys chrysomelas.*—COSTA RICA: Heredia; La Selva Biological Station, La Guaria (RMT4658, EU665204). NICARAGUA: Atlantico Norte; Rosa Grande, Siuna (TTU100324, EU340017; TTU100309, EU340018; TTU100313, EU074633).

*Melanomys* sp. 1.—VENEZUELA: Zulia; Mision Tukuko (MHNLS7698, EU340022).

*Melanomys* sp. 2.—PANAMA: Darien; Cana (ROM116303, EU340034; TTU39150, EU340024).

*Nectomys apicalis.*—PERU: Kiteni; Rio Urubamba (MVZ166700, EU340013).

*Nectomys squamipes.*—BRAZIL: Sao Paulo; Ilha do Cardoso (FMNH141632, EU340012). PARAGUAY: Paraguari; Parque Nacional Ybycui (TTU108150, EU074634).

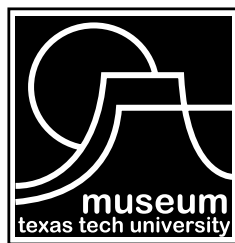
*Nezoryzomys swarthi.*—ECUADOR: Galapagos; Isla Santiago (ASNHC10003, EU340014).

*Oryzomys palustris.*—UNITED STATES: Texas; Galveston, Virginia Point (TTU82920, DQ185382).

*Sigmodontomys alfari.*—PANAMA: Boca del Toro; Isla San Cristobal (USNM449895, EU074635). ECUADOR: Esmeraldas; Estacion Experimental “La Chiquita” (TTU103047, EU340016).

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