

Molecular Phylogenetic Relationships Among Crested-tailed Mice (Genus *Habromys*)

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Published online: 13 January 2007
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Abstract We examined genealogical relationships among six of seven species of crested-tailed mice (*Habromys chinanteco*, *H. delicatulus*, *H. ixtlani*, *H. lepturus*, *H. lophurus*, and *H. simulatus*) using DNA sequence data from the cytochrome-*b* gene. Gene trees based on maximum likelihood, Bayesian inference and maximum parsimony were largely congruent in that *H. lepturus* and *H. ixtlani* were closely related and formed the sister group to *H. lophurus*. All analyses also arranged *H. chinanteco* and *H. simulatus* as sister taxa. These results are concordant with the phenetic groupings of Carleton et al. (2002) based on morphology. Our unweighted maximum parsimony trees did not resolve placement of *H. delicatulus* relative to other taxa. However, analyses using weighted maximum parsimony, maximum likelihood and Bayesian inference optimality criteria recovered a sister group relationship between *H. delicatulus* and the clade comprised of (*H. lepturus* *H. ixtlani*)(*H. lophurus*). This relationship differs from the overall phenetic similarity of *H. delicatulus* with *H. simulatus* and *H. chinanteco*, influenced by the small size of these three taxa, but is consistent with some derived features of the phallus (Carleton et al., 2002). Based on our sequence data, a specimen from Michoacán, México, recently assigned to *Peromyscus sagax* likely was inadvertently misidentified (Tiemann-Boege et al., 2000) and actually represents a new locality record for *H. delicatulus*. Finally, we comment on the conservation status of species of *Habromys*.

Keywords Phylogenetic relationships · *Habromys* · Cytochrome-*b* · Mesoamerica · Phylogeography · *Peromyscus sagax*

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Introduction

In his revision of the genus *Peromyscus*, Osgood (1909) determined that the three species of crested-tailed mice known at the time (*P. lepturus* (Merriam, 1898), *P. lophurus* (Osgood, 1904), and *P. simulatus* (Osgood, 1904)) formed a closely allied group. Based largely on the morphology of the glans penis, Hooper and Musser (1964) placed these three taxa in a new subgenus (*Habromys*) within *Peromyscus*. Goodwin (1964) described a fourth species, *P. ixtlani*, but Musser (1969) relegated *P. ixtlani* to subspecific status under *P. lepturus*. Later, *P. chinanteco* was described from the Sierra de Juárez mountains in central Oaxaca, Mexico, by Robertson and Musser (1976). In 1980, Carleton evaluated a suite of morphological characters and chose to underscore synapomorphies possessed by *Habromys* by assigning this taxon generic rank. Carleton et al. (2002) revised the genus based on an analysis of craniodental characters. They described a sixth species (*H. delicatulus*), presented morphological evidence that *H. l. ixtlani* was specifically distinct from *H. lepturus*, and provided a working hypothesis of phenetic relationships among species in the genus. Based on their analysis of 19 craniodental characters, Carleton et al. (2002) proposed that the six species in the genus *Habromys* were separable into two phenetic groups, based in part on body size. One was a large-to medium sized group consisting of the sister taxa *H. ixtlani* and *H. lepturus*, together with *H. lophurus*. The small bodied group was comprised of comprised the sister taxa *H. chinanteco* and *H. simulatus*, and *H. delicatulus*. With the exceptions of *H. ixtlani* and *H. lepturus*, all species are known from only a few museum voucher specimens, making characterization of species limits problematic for *H. chinanteco*, *H. delicatulus*, *H. lophurus*, and *H. simulatus*. The purpose of this study was to use mitochondrial DNA (mtDNA) sequence data to examine phylogenetic relationships within the genus and to test the systematic arrangement proposed by Carleton et al. (2002). To accomplish this, we generated sequence data from the cytochrome-*b* (*cyt-b*) gene for all recognized species-level taxa with the exception of a seventh, recently described species (*H. schmidlyi*; Romo-Vázquez et al., 2005), which was not included in our study.

Materials and methods

Amplification and sequencing

Twenty-seven specimens were examined representing five *Habromys* species (*H. chinanteco*, *H. ixtlani*, *H. lepturus*, *H. lophurus* and *H. simulatus*) and three allied specimens (*incertae sedis*), which herein are assigned to *H. delicatulus*. One of these samples (LAF, 1801) was taken from a specimen in the original type series of *H. delicatulus*. However, the tissue number (LAF, 1801) was not recorded on the voucher label and it is no longer possible to associate this tissue sample with a specific specimen within the series (F.A. Cervantes, pers. comm.). Therefore, we obtained partial *cyt-b* sequence data (see below) from a museum voucher skin from the type series of *H. delicatulus* to confirm the identification of LAF 1801. The third sample is of a sequence previously identified as *Peromyscus sagax* (Teimann-Boege et al., 2000), but which, based on the results of this analysis, clearly is allied to and conspecific with *H. delicatulus*. Sequences for additional outgroup taxa (sensu Watrous and Wheeler, 1981; Miller and Engstrom, unpublished) included *Megadontomys cryophilus*, *Peromyscus boylii*, *P. crinitus*, *P. leucopus*, *P. megalops*, *P. truei*, *Neotomodon alstoni*, *Reithrodontomys creper* and *Scotinomys xerampelinus* (see Appendix).

Total genomic DNA was extracted from liver, either frozen or preserved in 95% ethanol, following Fetzner (1999), or using the QIAGEN Dneasy™ Tissue Kit for all taxa except for

Table 1 Primers designed specifically for this study

Primer name	Primer sequence	Direction
JML2 ^a	AACATTCGRAAAWACACCCACTACTC	forward
JML4 ^a	TACTACGGCTCATAYACATTAC	forward
B1F ^a	ACCCTAACCCGATTCTTYGCATTC	forward
B4F ^a	CTCCSACGCAGAYAAAATYCCRTTC	forward
B5F ^a	ATAGCAACAGCATTYATAGGYTA	forward
T3F ^a	CAGATATTCTYGGAGAYCCGG	forward
T4F ^a	TCAAAAACAACGAGGACTTAYATTC	forward
T1F ^b	GATAYATACAYGCAAACGAGGAGC	forward
T4F ^b	CAAACYTCAAAAACAACGAGGACTTAYATTC	forward
JMH6R ^a	CCTGCAATGGGYATTAGGATGATRATA	reverse
JMH4R ^a	CCTARTTTRTTGGGGATRGAGCGTA	reverse
B1R ^a	GAATGCRAAGAATCGGGTTAGGGT	reverse
B4R ^a	GAAYGGRATTTTRTCTGCGTSGGAG	reverse
B6R ^a	TGGYTAAATRTGTGCTGGAGTGT	reverse
T1R ^a	GCTCCTCCGTTTGCRGTGTATRTATCG	reverse
T4R ^a	AATRTAAGTCCTCGTTGTTTGGARGTTTG	reverse
T3R ^a	CCGGRTCTCCRAGAATATCTG	reverse
T1R ^b	GCTCCTCCGTTTGCRGTGTATRTAT	reverse
T4R ^b	GAATRTAAGTCCTCGTTGTTTGGARGTTTG	reverse

^aMiller and Engstrom, unpublished.

^bFunk (2005).

H. chinanteco and a specimen *H. delicatulus*, the former because preserved tissue samples were unavailable and the latter to confirm species identification of an unvouchered tissue sample of *H. delicatulus*. Therefore, DNA was extracted and amplified from museum skins representing the original type series of these two species (KU 124131 and CNMA 22442, respectively).

The entire mitochondrial *cyt-b* gene was amplified using the primers L14724 with H15915 (Irwin et al., 1991) and MVZ-05-M with MVZ-14-M (Smith and Patton, 1993). Light and heavy strand primers used to amplify a portion of the *cyt-b* gene included WDRAT 400F (Edwards et al., 2001); L15162 and H15149 (Irwin et al., 1991); 700L (Peppers and Bradley, 2000); F1 (Whiting et al., 2003); CBH3 (Palumbi, 1996); as well as MVZ-04, MVZ-05, and MVZ-16 (Smith and Patton, 1993). In addition, several new primers were developed specifically for this study (Funk, 2005; see Table 1).

Thermal profiles for the majority of PCR reactions were: 2–4 min at 94°C, 35–40 cycles (1 min at 94°C, 1 min at 46°C, and 1 min at 72°C) plus 5 min at 72°C. Purified DNA was sequenced using the Perkin Elmer ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). Excess dye terminator was removed using a separation column with Sephadex 50G (3 g/50 ml H₂O) or by using Millipore MultiscreenTM filter plates. Both strands of each DNA fragment were sequenced using either a Perkin-Elmer ABI Prism 377 automated sequencer or an ABI 570 Genetic Analyzer (Applied Biosystems, Inc.) housed at Brigham Young University.

Obtaining DNA from museum skins is problematic due to degradation and carryover from chemical treatments. This leads to low copy recovery and impeded PCR reactions. Extraction of *H. chinanteco* and *H. delicatulus* DNA therefore required stringent protocols to minimize the risk of contaminants and optimize amplification results. A small (2 × 2 mm) skin sample was digested using the QIAGEN QIAAMPTM DNA minikit (Cat. No. 51306) and incubated for 30 h.

Positive and negative controls were employed during extraction and the subsequent product was concentrated to 50 μ l.

Primers designed for specificity (Table 1) allowed higher annealing temperatures to be used in the PCR reaction. A PCR cocktail containing 1.0–2.0 μ l of template, 2.5 μ l Erika Hagelberg Buffer (EH), 1.0 μ l forward primer, 1.0 μ l reverse primer, 1.0 μ l dNTPs (5 mM), 0.2 μ l Taq polymerase (QIAGEN), 0–0.60 μ l MgCl₂ (20 mM), and 17.5–18.3 μ l ddH₂O was used with good results.

A two-step touchdown thermal profile was employed for PCR (an initial 2–3 min denaturing at 94°C, then 40 s at 94°C, 40 s at 58–60°C, and 1 min 45 s at 72°C for 15–17 cycles; 40 s at 94°C, 40 s at 56°C, and 1 min 45 s at 72°C for 21–26 cycles) with both annealing and extension temperatures decreased for reluctant products. A five minute final extension at 72°C was tailed to either protocol.

Entire 25 μ l amplicons were electrophoresed across a 1.5% agarose gel, the bands extracted and purified using a QIAGEN QIAQUICK™ gel extraction kit. Products were concentrated by alcohol precipitation. Direct cycle-sequencing was performed using the DYNAMIC™ Direct Cycle Sequencing kit (Amersham, Cat.No. US79535). Removal of unincorporated reagents and small fragments was facilitated by alcohol precipitation, but provided no appreciable improvement in sequence quality. Sequencing was done on a LICOR LongReader 4200 analyzer, at the Royal Ontario Museum. All *cyt-b* sequences were edited and aligned using Sequencher version 4.1.1 (Gene Codes, 2000).

Phylogenetic analyses

The overall pattern of the nucleotide data matrix was inspected using the software program MEGA (Kumar et al., 1993). Base composition across taxa was subjected to a Chi-square goodness of fit test and inspected for heterogeneity, a factor which can bias analytical inference (Yang and Roberts, 1995) and influence the model of evolution selected.

We employed Bayesian inference (BI), Maximum likelihood (ML) and Maximum parsimony (MP) approaches. Although BI is similar to ML, it differs in its use of posterior probabilities to interpret statistical support. These probabilities are based on a selected model of evolution and are used to estimate the probability of finding a particular tree conditional to the character of the data and the model applied.

The models of evolution most appropriate for our data were selected using MODELTEST v3.7 (Posada and Crandall, 1998). Both the model of evolution and model parameters were determined through hierarchical likelihood ratios (hLRT) and associated AIC criterion (Akaike, 1974), partitioning by codon position. These corresponded to the Tamura-Nei model (TrN) with an allowance for a proportion of invariant sites (I) and a gamma distribution (Γ) of rates by both hLRT and AIC criteria for 1st positions; TrN + I + Γ by rLRT criteria and a general time reversible (GTR) model + I + Γ by AIC for 2nd positions; and the Hasagawa-Kishino-Yano (HKY) model + Γ by hLRT and TrN + I + Γ by AIC for 3rd positions (Gu et al., 1995; Hasegawa et al., 1985; Rodriguez et al., 1990; Tamura and Nei, 1993; Yang, 1994). These were approximated by allowing GTR + I + Γ , a model under which the assumptions of each independent model is nested, to run over each partition independently (see Swofford et al., 1996; Posada, 2003; Geuten et al., 2004), with all model parameters unlinked and therefore estimated for each partition. The resulting log-likelihood values were compared between both partitioned and unpartitioned data in the Bayesian analyses.

A Metropolis-coupled Markov-chain Monte Carlo (MCMC) method of sampling was performed (Yang and Rannala, 1997; Larget and Simon, 1999) running 2 \times 4 chains in parallel (2 cold, 6 hot) for 10 \times 10⁶ generations using MrBayes 3.1 (Huelsenbeck and Ronquist, 2003;

see also Metropolis et al., 1953 and Atelkar et al., 2004). Trees were sampled every 1000 generations. Stationarity was assessed through log-likelihood plots and conservatively, 50 trees were discarded as burnin (the first 5000 generations). Group support was measured by posterior probabilities based on 19749 saved trees for the partitioned model.

Bayesian posterior probabilities can potentially overestimate actual nodal support (Suzuki et al., 2002) and non-parametric bootstrapping is still of value, albeit a conservative estimator of support (Sanderson and Shaffer, 2002; Soltis and Soltis, 2003). We therefore further analyzed the unpartitioned dataset under Maximum Likelihood using the program PHYML (Guindon et al., 2003), obtaining a topologically identical tree as the Bayesian analyses and generating nonparametric likelihood bootstrap support percentages. We used the GTR + I + Γ model with parameters estimated by the program.

Maximum parsimony analyses, both unconstrained and weighted, were performed using PAUP* 4.0b10 (Swofford, 2002). These analyses employed a heuristic search, characterized by TBR branch-swapping and random sequence addition and included only informative characters. The application of weight constraints, depending on the nature of substitution at each base position, has been recommended (Irwin et al., 1991; Collins et al., 1994; Yang, 1996b; Barker and Lanyon, 2000; Farias et al., 2001; Huchon et al., 2002). Codon positions were therefore inversely weighted against the substitution rates observed at each base position. Nodal support was calculated using 2000 bootstrap pseudoreplicates with heuristic searches at each replication (10 random addition sequence replicates). Tree sets produced under these constraints were compared with both consensus and likelihood trees produced by BI methods.

Although some authors advocate removal of 3rd position sequence where saturation is suspected (e.g. Farias et al., 2001; Huchon et al., 2002; Ericson and Johansson, 2003), synonymous substitutions at third positions have been shown to be phylogenetically informative, even in moderately saturated sequences (Yoder et al., 1996; Yoder and Yang, 2000; Poux and Douzery, 2004). This is especially true in closely related taxa (Hästad and Björklund, 1998; Björklund, 1999; Pereira et al., 2002). Given our assessment of the data, employing both weighted and unweighted analyses, all codon positions were included.

Hypothesis testing

Alternative phylogenetic hypotheses were tested with both MP and ML approaches. Tree searches were conducted with constraints designed to match tree topologies for each hypothesis. Differences in tree scores between all equally optimal trees from constrained searches were compared to optimal trees overall using the Kishino - Hasegawa test (K-H; Kishino and Hasegawa, 1989) for trees generated using the MP criterion and the Shimodaira - Hasegawa test (S-H; Shimodaira and Hasegawa, 1999) with restricted ML. Both the K-H and S-H tests were conducted using PAUP* 4.0b10 (Swofford, 2002).

Results

Sequence characterization

We obtained the complete *cyt-b* sequence (1143 nucleotides) for 35 individuals representing 16 putative taxa (*Mus* positions 14139-15282, GenBank Accession No. J01420, Bibb et al., 1981). A total of 26 samples represented all *Habromys* species (with the exception of *H. schmidlyi*) including six *H. ixtlani*, nine *H. lepturus*, three *H. lophurus*, two *H. delicatulus*, five *H. simulatus*, and one *H. chinanteco* sequence. The remaining gene sequences are from nine species of

Peromyscus and peromyscine taxa used as outgroups. In addition, we obtained partial *cyt-b* sequence from a skin sample of *H. delicatulus* (CNMA 22442) to verify the identity of LAF 1801. We obtained a nearly complete sequence of 915 bp, with three fragments of 260, 455, and 194 bp in length (positions 1–260, 303–758, 949–1143, respectively). These fragments were aligned to the *Habromys* matrix, and compared to the LAF 1801 sequence. The sequence representing CNMA 22442 differed from LAF1801 by only six nucleotides, which was equivalent to or less than within-species differences for other *Habromys* species (17/1143 nucleotide differences within *H. lepturus*, 37/1143 within *H. lophurus* and 8/1143 within *H. ixtlani*). In addition, the clade including CNMA 22442, LAF 1801 and *Peromyscus sagax* differed from other species of *Habromys* by four autapomorphic characters (C → T, A → G, A → G, and A → G at positions 109, 115, 369 and 1111, respectively). Therefore, we are confident that LAF 1801 represents *H. delicatulus* and we used the complete LAF 1801 *cyt-b* sequence in all analyses.

Of the 1143 bp sequenced, 723 (63.3%) were conserved across species. Among variable sites, 97 (8.5%) were autapomorphic, resulting in 323 (28.3%) phylogenetically informative sites. The majority of substitutions occurred at 3rd positions, including the vast majority of phylogenetically informative sites. Base composition was fairly evenly distributed at 1st positions, demonstrating a positive T and negative G bias at 2nd position sites and a marked negative G bias at 3rd positions. Third positions also were characterized by strong positive A and C biases, while 2nd positions were relatively pyrimidine rich. The overall ti:tv ratio value R was 2.8, but also varied predictably by base position, with fewer transversions at both 1st and 2nd positions. Although base composition demonstrated predictable bias, its distribution was uniform across species ($\chi^2 = 22.922$, $df = 102$, $p = 1.000$). Overall nucleotide substitution was similar to that reported elsewhere for the majority of mammals (Irwin et al., 1991; Honeycutt et al., 1995; Yoder et al., 1996).

Large genetic divergences can indicate approach to saturation (Yoder et al., 1996). Uncorrected pairwise nucleotide differences in this data set range from less than 0.1% to 1% within species, to roughly 10–15% among genera (*Habromys* or *Peromyscus*). When *Habromys* species are compared with *Reithrodontomys creper* and *Scotinomys xerampelinus*, differences were 16–19% (Table 2). Only in these latter two outgroup taxa do nucleotide distances approach 15–20%, which is the level purported to indicate 3rd position saturation (Meyer, 1994). This translates to overall amino acid differences that range from 0–12% across all taxa, which on average is 1.67 times lower than the nucleotide distances and increases to 3 times lower when considering all *Peromyscus* and *Habromys* to the exclusion of these two outgroups. Plots of transitional/transversional change against distance were generated using DAMBE (Xia, 2000; Xia and Xie, 2001) and were characterized by increased rates of transitional change in third positions (not shown). Our data suggest that although this gene was not completely saturated at the principal level of taxonomic inquiry central to our analyses, there was sufficient evidence across taxa to warrant the models employed.

The sequence matrix was assessed for homogeneity of evolutionary rate by constraining both the Bayesian and MP trees to be clocklike, and comparing these likelihood scores with those of the unconstrained trees. A likelihood ratio test was done assuming a χ^2 distribution, with degrees of freedom set to the number of taxa–2 (Huelsenbeck and Bull, 1996; Posada, 2003). *Habromys* and its neotomine relatives do not seem to evolve in a clock-like manner ($\chi^2 = 92.706$, $df = 33$, $P < .05$) across all taxa equally. We then used The MEGA™ sub-routine for Tajima's Relative Rates Test (Tajima, 1993), using a Bonferroni correction, to assess the general constancy of evolutionary rates among sequences relative an outgroup (*Reithrodontomys creper*).

Selected tests were based on the assumption of rate constancy within *Habromys*, and between *Habromys* and *Megadontomys*, *Neotomodon* and *Peromyscus*. The relative rate of molecular change is generally uniform across taxa, with few exceptions. These exceptions tend to include

Table 2 Pairwise nucleotide distances among selected taxa^a

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 <i>S. xerampelinus</i>		[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.013]	[0.012]	[0.012]	[0.012]
2 <i>R. creper</i>	0.174	[0.011]	[0.012]	[0.011]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]	[0.012]
3 <i>M. cryophilus</i>	0.166	0.137	[0.010]	[0.010]	[0.011]	[0.010]	[0.010]	[0.011]	[0.010]	[0.010]	[0.010]	[0.011]	[0.011]	[0.010]	[0.010]	[0.011]	[0.011]	[0.011]	[0.010]	[0.011]
4 <i>N. alstoni</i>	0.164	0.145	[0.011]	[0.010]	[0.011]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.011]	[0.010]	[0.010]	[0.010]	[0.010]
5 <i>P. trui</i>	0.180	0.162	0.147	0.129	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.012]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.012]	[0.011]	[0.011]	[0.011]
6 <i>P. leucopus</i>	0.167	0.152	0.111	0.120	0.146	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.010]
7 <i>P. megalops</i>	0.165	0.134	0.125	0.110	0.141	0.132	[0.011]	[0.011]	[0.011]	[0.011]	[0.010]	[0.011]	[0.011]	[0.010]	[0.010]	[0.011]	[0.011]	[0.010]	[0.010]	[0.010]
8 <i>P. crinitus</i>	0.178	0.161	0.125	0.111	0.135	0.130	0.139	[0.011]	[0.011]	[0.011]	[0.011]	[0.010]	[0.010]	[0.010]	[0.010]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]
9 <i>P. boylii</i>	0.181	0.157	0.122	0.124	0.151	0.149	0.123	0.145	[0.011]	[0.012]	[0.011]	[0.010]	[0.010]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]	[0.011]
10 <i>H. chinanteco</i>	0.175	0.169	0.130	0.121	0.152	0.139	0.131	0.144	0.162	[0.011]	[0.011]	[0.010]	[0.010]	[0.010]	[0.010]	[0.011]	[0.011]	[0.011]	[0.011]	[0.009]
11 <i>P. sagax</i>	0.167	0.160	0.134	0.114	0.129	0.128	0.119	0.140	0.144	0.124	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]
12 <i>H. lepCNMA</i> 34869	0.173	0.162	0.128	0.106	0.130	0.140	0.126	0.122	0.136	0.115	0.112	0.112	[0.000]	[0.006]	[0.006]	[0.009]	[0.010]	[0.010]	[0.010]	[0.010]
13 <i>H. lep CMC</i> 73	0.173	0.162	0.128	0.106	0.130	0.140	0.126	0.122	0.136	0.115	0.112	0.000	[0.006]	[0.006]	[0.006]	[0.009]	[0.010]	[0.010]	[0.010]	[0.010]
14 <i>H. ixt CNMA</i> 29845	0.179	0.157	0.122	0.099	0.130	0.130	0.115	0.114	0.139	0.106	0.109	0.034	0.034	[0.002]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]
15 <i>H. ixt CNMA</i> 29842	0.180	0.160	0.125	0.102	0.130	0.133	0.119	0.118	0.142	0.107	0.110	0.033	0.033	0.003	[0.009]	[0.010]	[0.010]	[0.010]	[0.010]	[0.010]
16 <i>H. lep ROM</i> 98379	0.185	0.179	0.139	0.125	0.149	0.142	0.139	0.140	0.148	0.138	0.121	0.091	0.091	0.098	0.097	[0.006]	[0.010]	[0.010]	[0.011]	[0.011]
17 <i>H. lep ROM</i> 99834	0.192	0.176	0.137	0.119	0.153	0.142	0.138	0.137	0.146	0.143	0.122	0.098	0.098	0.105	0.106	0.034	[0.010]	[0.010]	[0.011]	[0.011]
18 <i>H. delicatulus</i>	0.169	0.163	0.134	0.111	0.129	0.127	0.122	0.140	0.142	0.123	0.012	0.110	0.110	0.109	0.108	0.119	0.120	[0.010]	[0.010]	[0.010]
19 <i>H. sim</i> BYU 16062	0.167	0.154	0.121	0.105	0.142	0.119	0.116	0.131	0.138	0.080	0.104	0.105	0.105	0.099	0.100	0.124	0.126	0.102	[0.010]	[0.012]
20 <i>H. sim</i> BYU 16063	0.171	0.156	0.125	0.109	0.146	0.121	0.120	0.135	0.141	0.084	0.108	0.109	0.109	0.103	0.104	0.128	0.130	0.106	0.004	[0.010]

^aComplete scientific names and voucher information are given in the Appendix). Distance values appear in the lower diagonal, with standard errors in the upper diagonal. Complete distance table available on request. *H. lep* = *H. lepturus*, *H. ixt* = *H. ixtlani*, *H. lep* = *H. lophurus*, *H. sim* = *H. simulatus*.

rates of several *H. lepturus* sequences. Furthermore, these apparent differences in rates of change become statistically insignificant ($p > .10$) when transversions are considered in isolation. Therefore, we believe that it was reasonable to assume uniform evolutionary rates among species.

The observed rates of substitution at the three codon positions matches the expected frequency (3rd position > 1st position > 2nd position) for mammalian mitochondrial genes (Kimura, 1983; Irwin et al., 1991; Yang, 1996a). In addition, fragment lengths were as anticipated for the primers we used and the sequences generated were not characterized by unexpected stop codons, or indels. Therefore, we are confident that none of our sequences represent nuclear pseudogenes.

Phylogenetic analyses

Trees were rooted using a member of the tribe Baiomyini (*Scotinomys xerampelinus*) as the outgroup inasmuch as this taxon hypothesized to be the sister group to the remaining taxa, all of which are members of the tribe Peromyscini (Reeder et al., 2006). Phylogenetic reconstructions based on BI, ML and MP optimality criteria all confirm that *Habromys* is monophyletic (Figs. 1 and 2). Tree topologies based on ML and BI (Fig. 1) were more resolved than those based on MP (Fig. 2). In all analyses, “*P. sagax*” (AF155404) and *H. delicatulus* (LAF 1801), were sister taxa. These latter two taxa formed a consistent clade with strong posterior probability (PP) support (PP = 1.00). Given that the 1.2% nucleotide distance between “*P. sagax*” and *H. delicatulus* represents the low end of variability identified within each of the other *Habromys* species, and that four autapomorphic substitutions are shared between these three samples (including the partial *cyt-b* for a second *H. delicatulus*), we believe that this specimen has been misidentified in the literature and also represents *H. delicatulus*. Genetically, *Habromys* is divided into two major clades consisting of (*H. chinanteco* – *H. simulatus*), and (*H. delicatulus* (*H. lophurus* (*H. ixtlani*/*H. lepturus*))).

Bayesian inference and maximum likelihood

Tree topologies are identical for both unpartitioned and partitioned GTR + I + Γ models in BI analyses (Fig. 1). However, there are differences in both the overall likelihood scores and in nodal support. The likelihood score for the best “cold” chain state is –6691.07 in the unpartitioned model, with a marginal likelihood score of –6733.93 (harmonic mean). Flat priors were incorporated in this model, with substitution rates accorded a Dirichlet distribution. The model therefore used a proportion of invariable sites equal to 0.539 and a shape parameter (α) for the Γ distribution = 1.0266. Base frequency estimates under Modeltest were 0.327, 0.310, 0.116, and 0.246, for A, C, G and T, respectively. Log likelihood was determined using $-\ln L = 6678.389$ and the Akaike Information Criterion (AIC) = 13376.778. Likelihood scores improved in the partitioned model, with the best state for the “cold” chain as –6200.47 and the marginal likelihood score as –6312.05 (harmonic mean). Modeltest (Posada and Crandall, 1998) for the ML analyses indicated that the GTR + I + Γ model was the most appropriate model of nucleotide substitution overall for our unpartitioned data. Resulting ML tree topologies (not shown) were identical to those generated under the BI criterion.

Parameterization of the partitioned GTR + I + Γ model was more complex, with independent estimations of invariant site proportion, substitution rate variation and base composition determined by codon position. To control for the possibility of representation bias, a reduced taxonomic set representing only the major divisions was run under the same assumptions of either GTR + I + Γ model. This produced a robust ML tree (not shown) supporting relationships within *Habromys* depicted in Fig. 1 and also supporting the allied relationship of *N. alstoni*,

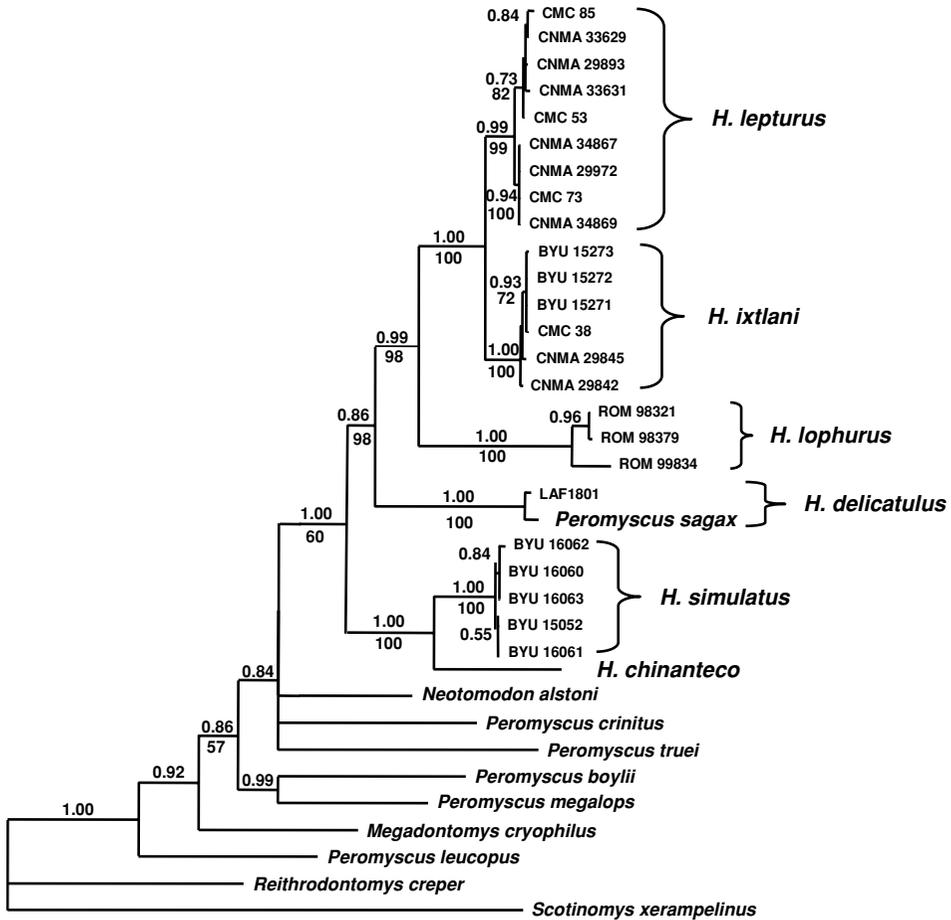


Fig. 1 Phylogram depicting phylogenetic relationships among *Habromys* and outgroup taxa based on unpartitioned maximum likelihood analysis of cytochrome-*b* sequence data. Posterior probabilities derived from Bayesian analyses with data partitioned by codon position are shown above nodes. Maximum likelihood non-parametric bootstrap support values (500 pseudoreplicates) appear below nodes

albeit as an outlying polytomy with *P. truei*. These relationships and their improved posterior probability supports are reproduced in a similarly reduced partitioned model (not shown). The reduced taxonomic set allows removal of basal ambiguity among *Peromyscus* (sensu stricto), suggestive of homoplasy at the relative depths of these diversification events, particularly at 4-fold degenerative sites.

Maximum parsimony

Heuristic searches under the unconstrained (equally weighted) parsimony model recovered six equally parsimonious trees (Fig. 2) with a tree solution of fewer steps than generated under weighted models [tree length (TL) = 1137, consistency index (CI) = 0.4037, retention index (RI) = 0.6896, excluding uninformative characters]. Topological relationships were strongly concordant with those recovered under ML and BI, with the exception of *H. delicatulus* removed

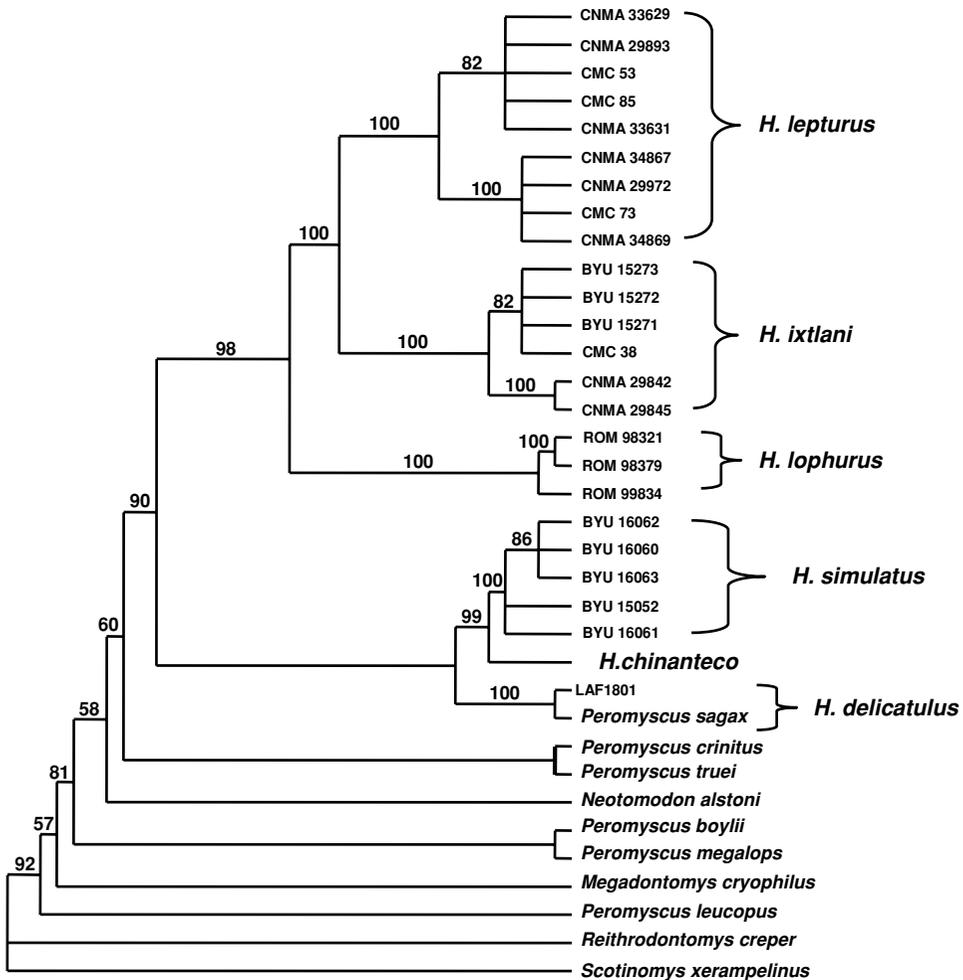


Fig. 2 Maximum parsimony consensus cladogram (50% majority rule) of the six most parsimonious trees (1137, CI = 0.4037, RI = 0.6896) of cytochrome-*b* sequence data. Non-parametric bootstrap support (2000 pseudoreplicates) appears above nodes. Character optimization employs ACCTRAN

to a sister relationship to the (*H. chinanteco* and *H. simulatus*) clade. *Neotomodon* forms the sister group to the *Habromys* clade, but this relationship is not supported with ML and BI approaches. Relationships among outgroups are poorly supported by bootstrap pseudoreplication.

Weighted MP analyses produced a topology consistent with the results of the Bayesian model, although more poorly resolved (not shown). Tree statistics were strongest under the constraints of base position weighting (either 2:3:1 or 5:10:1) and ti:tv biases empirically determined from the data. Heuristic searches resulted in 12 equally parsimonious trees under the first model and 24 under the second. Although stronger weighting improved overall tree statistics, it also resulted in a longer tree (TL = 22554 steps, CI = 0.4572, RI = 0.6995, excluding uninformative characters). A weight assignment of 2:3:1 yields an identical consensus topology that is similarly supported, but with weaker tree statistics (CI = .4238, RI = 0.6906), and shorter tree length (TL = 1713 steps). Both models recover trees that indicate a sister relationship between

H. delicatulus and the (*H. lophurus* (*H. lepturus* and *H. ixtlani*)) clade, congruent with ML and BI topologies. This solution differs from the unweighted model, which relegates *H. delicatulus* to the (*H. chinanteco* and *H. simulatus*) clade (Fig. 2). Among the equally parsimonious trees, topographic variability represents only minor alterations in the relationships among individuals within species of *Habromys*. Alternative, less constrained MP weighting scheme did not affect tree topology, nor significantly alter bootstrap support. Relationships among the remaining species of *Peromyscus* (sensu lato) and *Habromys* remain unresolved by consensus under MP analysis.

Statistical tests

Using MP (K-H test) and ML (S-H test) optimality criteria, we tested the hypothesis that the genus *Habromys* was monophyletic relative to its sister clade. Except for the unweighted MP consensus tree which implicated *Peromyscus polionotus* and *P. truei* as outgroups to the genus *Habromys*, all other analyses (weighted MP, ML and BI) arranged the genus *Neotomodon* as the sister group to *Habromys*. We constrained *Neotomodon* (ML topology – S-H test) and the *P. polionotus/P. truei* clade (MP topology – K-H test) by forcing these outgroup taxa to fall within the genus *Habromys*. The resulting MP tree was not significantly longer (6 steps, $P < 0.109$). However, the constrained ML tree had a significantly larger log likelihood score (6666.257 versus 6679.027, $P < 0.029$).

The placement of *H. delicatulus* differed among the unweighted MP results (Fig. 2) and the trees obtained using ML and BI optimality criteria (Fig. 1), but nodal support for these arrangements is not strong. We tested these alternatives by constraining the MP tree to resemble the BI topology and the vice versa. The resulting differences in tree length (4 steps) and tree score (3.76860) were not significant ($P < 0.346$ and $P < 0.234$, respectively).

Discussion

Genealogical relationships

All analyses provided support for the monophyly of *Habromys*. The S-H test indicated that trees arranging all species of crested-tailed mice as monophyletic were significantly more likely, although the K-H test was non-significant. Within *Habromys*, we find strong evidence for (1) a sister group relationship between *H. chinanteco* and *H. simulatus* and (2) a clade with *H. lophurus* as the sister group to *H. ixtlani* + *H. lepturus*. The position of *H. delicatulus* remains equivocal. There is moderate to strong nodal support for association of *H. delicatulus* with the ((*H. lophurus* (*H. ixtlani*/*H. lepturus*)) clade under ML and BI optimality criteria, but weak nodal support for grouping *H. delicatulus* with *H. chinanteco* and *H. simulatus* (MP analyses). However, we found no significant difference between these alternatives.

Based on phenetic analysis of craniodental variables, Carleton et al. (2002), identified a cluster of “large” bodied species (*H. ixtlani*, *H. lepturus* and *H. lophurus*) and a second group consisting of morphologically smaller species (*H. chinanteco*, *H. delicatulus* and *H. simulatus*). Romo-Vázquez et al. (2005) also hypothesized that the genus *Habromys* is divided into two phylogenetic groups. One consists of *H. ixtlani*, *H. lepturus* and *H. lophurus* and the other is formed by *H. chinanteco*, *H. delicatulus*, *H. schmidlyi* and *H. simulatus*. Our *cyt-b* data support the second grouping, but we did not evaluate genealogical relationships of *H. schmidlyi*. However, we find no statistical support for the former as a phyletic clade. Resolution among outgroup taxa is not clear, possibly reflecting the practical limits of *cyt-b* sequence data with

increased phylogenetic depth (Graybeal, 1993; Meyer, 1994). Depth and age of branches among outgroups are reflected in the contrast between pairwise nucleotide differences among species and genera, as compared to within-species distances (see Table 2). Despite the lack of resolution in outgroup relationships using *cyt-b*, *Neotomodon alstoni* is consistently placed close to the root of the *Habromys* clade, or as its sister taxon. In fact, we find statistical support for this arrangement based on the S-H test. This is suggestive of similarly allied relationships identified by Carleton (1980, 1989). However, determination of relationships among these taxa awaits a comprehensive analysis of the tribe Peromycini.

Like Carleton et al. (2002), we recovered a close and highly significant relationship between *Habromys ixtlani* and *H. lepturus*, and the association of *H. simulatus* and *H. chinanteco* as sister taxa. Carleton et al. (2002) questioned whether *H. chinanteco* was specifically distinct from *H. simulatus*, and suggested that its status merited further investigation. Nucleotide distances between these taxa (0.074–0.085) however, are consistent with their recognition as distinct species, and are equal-to or greater-than the genetic distances between, for instance, *H. ixtlani* and *H. lepturus* (0.034), or *H. lophurus* and *H. lepturus* (0.091). Likewise, the genetic distance between *H. delicatulus* exceeds 0.100 in all pairwise comparisons with other *Habromys*, except the specimens identified as *Peromyscus sagax* (pairwise distance of 0.012, or approximately 1%). Genetically, the specimens assigned to *P. sagax* by Bradley et al. (1996) and as applied in subsequent treatments and analyses (Bradley and Baker, 2001; Bradley et al. 2004; Durish et al. 2004; Teimann-Boege et al. 2000;) clearly is allied to, and conspecific with, *H. delicatulus* and is herein assigned to that species. Reassignment of this specimen results from a probable misidentification and does not indicate synonymy of *P. sagax* and *H. delicatulus*. Unfortunately, we have been unable to examine the museum voucher for this specimen. Relationships estimated by our *cyt-b* trees are not wholly consistent with the phenetic relationships identified by Carleton et al. (2002), which corresponded loosely to size divisions. Some synapomorphies identified by Carleton et al. (2002, p. 505) also indicate a relationship of *H. delicatulus* with the large-bodied group, such as phallic similarities held in common with *H. lepturus* and *H. lophurus*.

Biogeographic implications

Crested-tailed mice occur in highland forests in southern Mexico, Guatemala and El Salvador (Fig. 3) at sites ranging from 1,830 to 3,150 m in elevation (Carleton et al., 2002). Habitat descriptions at collecting sites typically emphasize “cloud forest” associations in conjunction with lush understories (Goldman, 1951; Musser, 1969; Carleton and Huckaby, 1975; Robertson and Musser, 1976; León-Paniagua and Romo-Vázquez, 1993). As a result, members of the genus *Habromys* occur in disjunct mountain ranges with suitable wet slopes as follows: *Habromys delicatulus* (Cordillera Neovolcanica), *H. chinanteco* (Oaxacan Highlands), *H. simulatus* (Sierra Madre Oriental), *H. ixtlani* and *H. lepturus* (the Sierra de Jurez and Cerro Zempoaltepec mountains in the Oaxacan Highlands) and *H. lophurus* (Chiapan and Central American Highlands).

The phyletic pattern recovered for the “large” *Habromys* clade, identified by Carleton et al. (2002), suggests that the Isthmus of Tehuantepec has formed a barrier to gene flow between (*Habromys ixtlani*- *H. lepturus*) and *H. lophurus* members of this lineage (Fig. 3a). These findings are congruent with those of Hooper (1952), Baker (1963), Choate (1970), Engstrom et al. (1981), Sullivan et al. (1997, 2000), Woodman and Timm (1999), Edwards and Bradley (2002), Arellano et al. (2005), all of whom implicated the Isthmus of Tehuantepec as a major geographic barrier for other small, montane mammals as discussed at length by Carleton et al. (2002). In particular, the studies by Sullivan et al. (2000), Edwards and Bradley (2002) and Arellano et al. (2005) document *cyt-b* divergence values for species samples on either side of the isthmus of approximately 10%. Given the magnitude and consistency of these values, we

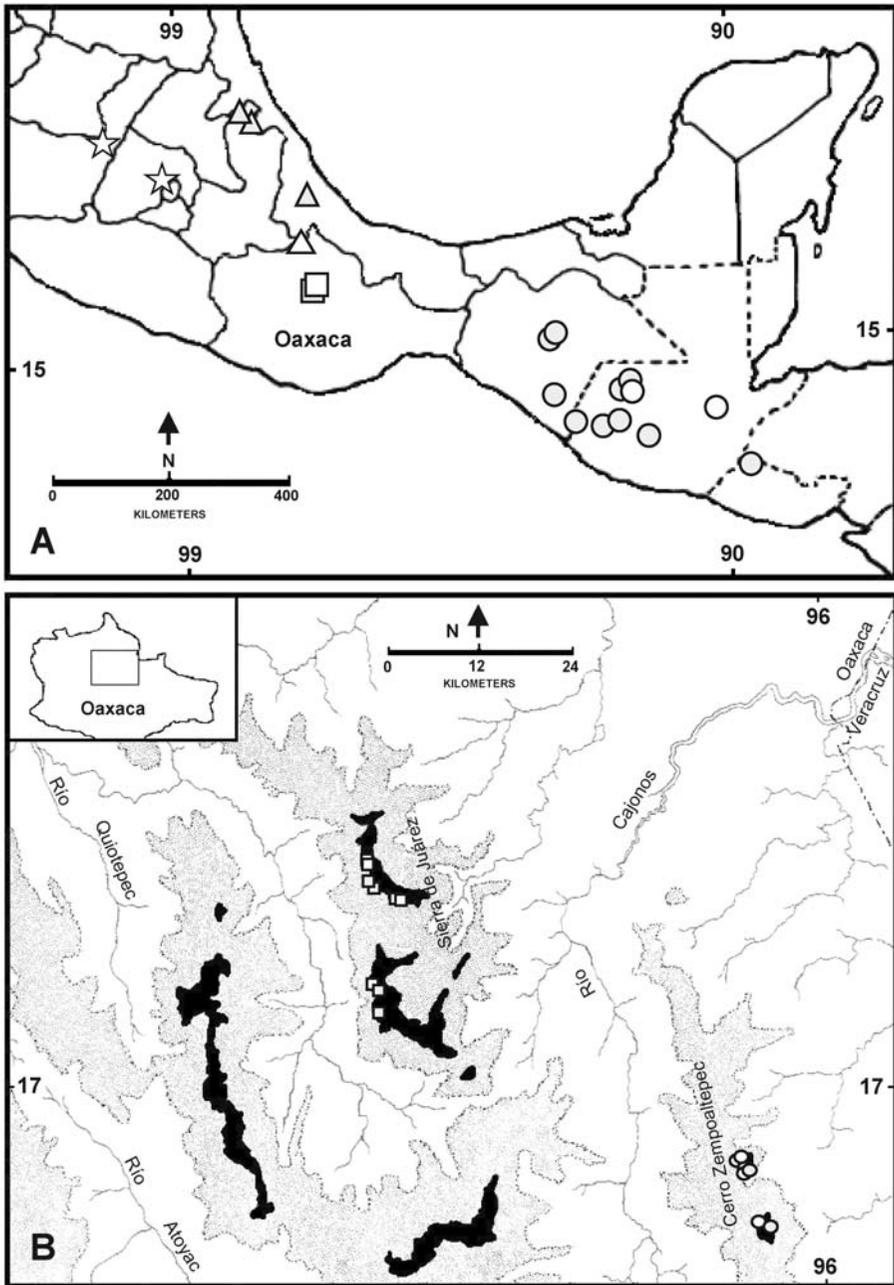


Fig. 3 A. Map of south-central México and northern Middle America illustrating the geographic location of collecting localities for *Habromys chinanteco* (squares), *H. delicatulus* (stars), *H. lophurus* (circles), and *H. simulatus* (triangles). Gray symbols indicate museum records whereas white symbols represent localities from which specimens used in this study were collected. B. Inset map of north-central Oaxaca, México, indicating the geographic position of the Sierra de Juárez and Cerro Zempoaltepec (redrawn from Musser, 1969). Areas in gray are above 2,000 m whereas areas in black are above 3,000 m elevation. Squares and circles are collecting localities of *H. ixtlani* and *H. lepturus*, respectively. Symbol colors are as in 3A

hypothesize that the vicariant event(s) separating montane taxa on either side of the isthmus predates the Pleistocene and may have affected all small mammal taxa in the same manner.

The Sierra Madre Oriental of eastern Mexico is implicated elsewhere as an important area for diversification of Mexican small mammals. *Habromys simulatus* (Fig. 3a) is endemic to this mountain range as is *Peromyscus furvus* (Harris et al., 2001; Carleton et al., 2002) and an undescribed species of *Reithrodontomys* (Arellano et al., 2005). In addition, for taxa that are more widely distributed in Mexico and Middle America (the *Peromyscus aztecus* assemblage and *R. sumichrasti*), the Sierra Madre Oriental typically houses a major genetic subdivision within those species (Sullivan et al., 2000). Certainly, the role that the Sierra Madre Oriental and other associated mountain ranges in southern Mexico have played in shaping the modern distributions of small mammals and other codistributed taxa is worthy of continued investigation.

Our results as well as those of earlier workers (Musser, 1969; Carleton et al., 2002) arrange *Habromys ixtlani* and *H. lepturus* as sister taxa. These two taxa occur in cloud forests of the Sierra de Jurez and Cerro Zempoaltepec, respectively, in central Oaxaca, Mexico (Fig. 3b). These cloud forests are separated from one another by a relatively low, dry river valley (Rio Cajonos). Both species of *Habromys* co-occur with essentially the same set of rodent taxa at each site (*Oryzomys chapmani*, *Peromyscus aztecus*, *P. melanocarpus*, *R. mexicanus*, and *R. sumichrasti* and *Microtus* sp.). However, all these species have a considerably broader distribution in southern Mexico and Central America with the exception of *P. melanocarpus*, which co-occurs with *H. ixtlani* and *H. lepturus* in the Sierra de Jurez and Cerro Zempoaltepec, respectively. Interestingly, the response to the barrier posed by the Rio Cajonos is identical for these two co-distributed taxa. Both *H. ixtlani* and *H. lepturus* and the two populations of *P. melanocarpus* are well differentiated genetically (approximately 7% *cyt-b* divergence – González-Cózatl et al., unpublished) from one another, and we hypothesize that the Rio Cajonos has served as an effective barrier to gene flow for these two taxa.

Conservation issues

We have demonstrated that all species-level taxa of crested-tailed mice are distinct genetically. Unfortunately, all species with the exception of *Habromys lophurus* are of some conservation concern given that annual deforestation rates in Mexico have been greater than 1% per year since the 1970s (Arriaga et al., 2000; Mas et al., 2004). *Habromys simulatus* and *H. lepturus* are listed as endangered and lower risk, near threatened, respectively (Baillie, 1996a,b). According to Sánchez-Cordero et al. (2005), these taxa have lost about 75% and 21%, respectively, of their habitat due to deforestation. Given the dramatic habitat loss sustained by *H. simulatus* over the past 35 years, Sánchez-Cordero et al. (2005) consider this species as threatened with extinction. The status of *H. lepturus* is problematic because Sánchez-Cordero et al. (2005) and the IUCN listing did not recognize *H. ixtlani*, a systematic change that effectively reduces the geographic range and population size of *H. lepturus* by one-half.

Although *Habromys chinanteco* is not listed, this species is known from only a few locations in the Sierra de Juaréz of Oaxaca and has not been collected in the wild since the late 1970's. The status of *H. delicatulus* and *H. schmidlyi* also are of concern from a conservation standpoint. Our study demonstrates that these two species are presently known from only two and three sites, respectively, in the Cordillera Transvolcánica. Given that all *Habromys* species occur in cloud forest or high elevation forested habitats that have suffered an overall 30% decrease in land area due to deforestation and habitat fragmentation since 1970 (Challenger, 1998; Arriaga-Cabrera et al., 2000; Mas et al., 2004), the long term outlook for these taxa in terms of their genetic variability (Garner et al. 2005) and long-term persistence in Mexico is not encouraging.

Appendix

Specimens examined are listed by taxon, collecting locality, museum acronym and voucher number and GenBank accession number. Museum acronyms (Hafner et al., 1997) are as follows: BYU = Monte L. Bean Life Science Museum, Brigham Young University; CMC = Colección de Mamíferos, del CEAMISH (Centro de Educación Ambiental e Investigación Sierra de Huautla), Universidad Autónoma del Estado de Morelos; CNMA = Colección Nacional de Mamíferos, Universidad Nacional Autónoma de México; LAF = Los Angeles County Museum; ROM = Royal Ontario Museum.

Habromys chinanteco: MEXICO; Oaxaca, N slope of Cerro Pelon, 31.6 km S Vista Hermosa, 2,650 m (KU 124131 – DQ861380).

Habromys delicatulus: MEXICO; México, Municipio Jilotepec, Dexcaní Alto, 2 km E and 3.5 km S Jilotepec, Cañada de la Ermita, 2,570 m (19°56' N, 99°30' W)(LAF 1801 – DQ861399, CNMA 22442–EF043236); Michoacán, Puerto Garnacia, 9,200 ft (AF155404*).

Habromys ixtlani: MEXICO; Oaxaca, Atepec, Llano de las Flores, Km 132 Carretera Tuxtepec-Oaxaca, 2,750 m (CNMA 29842 – DQ861391, CNMA 29845 – DQ861390); Distrito de Ixtlani, 28 km SW (by road) La Esperanza, 2,950 m (BYU 15271 – DQ861395, 15272 – DQ861394 and 15273 – DQ861393); Distrito de Ixtlani, 28 km SW (by road) La Esperanza, 17°35'08" N, 96°30'41" W, 2,950 m (CMC 56 – DQ861392).

Habromys lepturus: MEXICO; Oaxaca, Municipio Tlahuitoltepec, 16 km E Tlahuitoltepec, 2,750 m (CNMA 29893 – DQ861386); Municipio Tlahuitoltepec, Santa María Yacochi, 3 km N Cerro Zempoaltepec, 2,450 m (CNMA 34867 – DQ861382 and 34869 – DQ861381); Municipio Tlahuitoltepec, Santa María Yacochi, 4.5 km N Cerro Zempoaltepec, 2,450 m (CNMA 33629 – DQ861384 and 33631 – DQ861383); Municipio Tlahuitoltepec, Santa María Yacochi, 3.5 km N Cerro Zempoaltepec, 2,750 m (CNMA 29972 – DQ861385); Municipio Tlahuitoltepec, vicinity Santa María Yacochi, 17°15'00" N, 96°00'45" W, 2,400 m (CMC 38 – DQ861387 and 85 – DQ861388); Municipio Tlahuitoltepec, vicinity Santa María Yacochi, 17°09'30" N, 96°01'00" W, 2,300 m (CMC 73 – DQ861389).

Habromys lophurus: GUATEMALA; Huehuetenango, 12 km NW of Santa Eulalia (ROM 98321 – DQ861396 and 98379 – DQ861398); Zacapa, 2 km N of San Lorenzo (ROM 99834 – DQ861397).

Habromys simulatus: MEXICO; Oaxaca, Municipio Teotitlán, 1.5 km S Puerto de la Soledad, 18°09.742' N, 96°59.852' W, 2,600 m (BYU 15052 – DQ861404, 15053 – DQ861401, 16060 – DQ861403, 16061 – DQ861402 and 16063 – DQ861400).

Outgroup taxa

Megadontomys cryophilus: MEXICO; Oaxaca, Municipio Teotitlán, 1.5 km S Puerto de la Soledad, 18°09.742' N, 96°59.852' W, 2,600 m (BYU 16076 – DQ861373).

Neotomodon alstoni: MEXICO; Morelos, Huitzilac, Lagunas de Zempoala, 3 km W Huitzilac, 19°02.020' N, 99°17.626' W (BYU 15513 – DQ861374).

Peromyscus boylii: USA; Utah, Kane Co., Smoky Hollow, 37°09.41' N, 111°32.10' W, 1,270 m (BYU 19433 – DQ861379).

Peromyscus crinitus: USA; Utah, Kane Co., 59 km E, 25 km N Kanab, 1,450 m (BYU 18029 – DQ861378).

Peromyscus leucopus: MEXICO; San Luis Potosí, Municipio Xilitla, Ejido Aguayo, 6.2 km N Xilitla, 1,000 m (BYU 15919 – DQ861376).

Peromyscus megalops: MEXICO; Guerrero, 6.1 km N (by road) Omiltemi, 17°32.950' N, 99°43.260' W, 2,490 m (BYU 20755 – DQ861377).

Peromyscus truei: USA; Utah, Kane Co., Wolverine Petrified Forest, 37°48.0902' N, 111°12.3641' W, 1,840 m (BYU 20324 – DQ861375).

Reithrodontomys creper: COSTA RICA; Cartago, Rio Birris, 12 km N of Porter (ROM 97321 – DQ861372).

Scotinomys xerampelinus: COSTA RICA; Cartago, Parque Nacional Volcán Irazu (ROM 116812 – DQ861371).

*Listed as *Peromyscus sagax* in GenBank.

Acknowledgments We thank the following persons for their help in obtaining specimens or tissue loans that formed the basis of this report: E. Arellano, F.A. Cervantes, J. Dines, F.X. González-Cózatl, D.J. Harris, J. Martínez and T. Maxwell. Permits for field work in Mexico were issued by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT). R.M. Timm and T. Holmes kindly allowed us to sequence DNA from the skin of a *Habromys chinanteco* housed in the University of Kansas, Museum of Natural History. F.A. Cervantes generously allowed us to sequence a skin sample of a specimen from the type series of *Habromys delicatulus* housed in the Colección Nacional de Mamíferos, Universidad Nacional Autónoma de México. Financial support was provided by Professional Development Grants, Department of Integrative Biology and the M.L. Bean Life Science Museum, Brigham Young University (to DSR), the Honors Office and Office of Undergraduate Research, Brigham Young University (to CCF), the Ontario Graduate Student Scholarship program (to JRM), and the Royal Ontario Museum Foundation (to MDE). E. Arellano and F.X. González-Cózatl provided comments on an earlier version of this paper.

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