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Molecular systematics of Middle American harvest mice *Reithrodontomys* (Muridae), estimated from mitochondrial cytochrome *b* gene sequences

Elizabeth Arellano [a], Francisco X. González-Cozátl [a], Duke S. Rogers [b,*]

[a] *Centro de Educación Ambiental e Investigación Sierra de Huautla, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Champila, Cuernavaca, Morelos, C.P. 62210, Mexico*
[b] *Department of Integrative Biology and M. L. Bean Life Science Museum, Brigham Young University, Provo, UT 84602, USA*

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Abstract

We estimated phylogenetic relationships among 16 species of harvest mice using sequences from the mitochondrial cytochrome *b* (cyt *b*) gene. Gene phylogenies constructed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) optimality criteria were largely congruent and arranged taxa into two groups corresponding to the two recognized subgenera (*Aporodon* and *Reithrodontomys*). All analyses also recovered *R. mexicanus* and *R. microdon* as polyphyletic, although greater resolution was obtained using ML and BI approaches. Within *R. mexicanus*, three clades were identified with high nodal support (MP and ML bootstrap, Bremer decay and Bayesian posterior probabilities). One represented a subspecies of *R. mexicanus* from Costa Rica (*R. m. cherrii*) and a second was distributed in the Sierra Madre Oriental of Mexico. The third *R. mexicanus* clade consisted of mice from southern Mexico southward to South America. Polyphyly between the two moieties of *R. microdon* corresponded to the Isthmus of Tehuantepec in southern Mexico. Populations of *R. microdon microdon* to the east of the isthmus (Chiapas, Mexico) grouped with *R. tenuirostris*, whereas samples of *R. m. albilabris* to the west in Oaxaca, Mexico, formed a clade with *R. bakeri*. Within the subgenus *Reithrodontomys*, all analyses recovered *R. montanus* and *R. raviventris* as sister taxa, a finding consistent with earlier studies based on allozymes and cyt *b* data. There was also strong support (ML and BI criteria) for a clade consisting of ((*R. megalotis*, *R. zacatecae*) (*R. sumichrasti*)). In addition, cyt*b* gene phylogenies (MP, ML, and BI) recovered *R. fulvescens* and *R. hirsutus* (ML and BI) as basal taxa within the subgenus *Reithrodontomys*. Constraint analyses demonstrated that tree topologies treating the two subgenera (*Aporodon* and *Reithrodontomys*) as monophyletic (ML criterion) was significantly better ($p > 0.036$) and supported polyphyly of *R. mexicanus* (both ML and MP criteria – $p > 0.013$) and *R. microdon* (MP criterion only for certain topologies; $p > 0.02$). Although several species-level taxa were identified based on multiple, independent data sets, we recommended a conservative approach which will involve thorough analyses of museum specimens including material from type localities together with additional sampling and data from multiple, nuclear gene markers.

Keywords: Reithrodontomys; Aporodon; Middle America; Monophyly; Phylogenetic relationships; Polyphyly; Cytochrome *b*; Candidate species

1. Introduction

Harvest mice belonging to the genus *Reithrodontomys* (Family Muridae, Subfamily Sigmodontinae – Musser and Carleton, 1993) consist of 21 species divided almost evenly between two subgenera, *Reithrodontomys* and *Aporodon* (see Bradley et al., 2004b and Arellano et al., 2003 for recent summaries). These mice are relatively small with long tails and are distinguished from other peromyscine rodents (sensu Bradley et al., 2004a; McKenna and Bell, 1997; Musser and Carleton, 1993) by

* Corresponding author. Fax: +801 422 0090.
E-mail address: Duke_Rogers@byu.edu (D.S. Rogers).

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possession of grooved or sulcate, upper incisors (Le Conte, 1853). Members of the subgenus *Reithrodontomys* differ from species assigned to the subgenus *Aporodon* in characteristics of the molar teeth (Hooper, 1952; Howell, 1914) and other morphological characters summarized by Carleton (1980), allozyme data (Arellano et al., 2003; Nelson et al., 1984), DNA satellite sequences (Hamilton et al., 1990), and DNA sequences (Arellano, 1999; Bell et al., 2001; Bradley et al., 2004b).

Although at least one species of harvest mice can be found in most habitats in North and Central America as well as in extreme northwestern South America (Eisenberg, 1989; Hall, 1981; Hooper, 1952;), biodiversity in this group peaks in Mesoamerica. For example, 12 species of *Reithrodontomys* (*R. bakeri*, *R. chrysopsis*, *R. fulvescens*, *R. gracilis*, *R. hirsutus*, *R. megalotis*, *R. mexicanus*, *R. microdon*, *R. spectabilis*, *R. sumichrasti*, *R. tenuirostris*, and *R. zacatecae*) occur in southern Mexico alone (Reid, 1997). Species of Middle American *Reithrodontomys* typically have restricted geographic distributions, with the exceptions of *R. mexicanus,* which occurs from northeastern Mexico south to Ecuador and *R. megalotis*, which is found from southwestern Canada to the southern plateau of Mexico. Aside from *R. fulvescens*, *R. gracilis* and *R. spectabilis*, Middle American *Reithrodontomys* typically occupy moderate to high altitude habitats which form "mountain islands" throughout the region.

Phylogenetic relationships among some members of the subgenus *Aporodon* recently were evaluated by Arellano et al. (2003) using allozmyes. In addition, Bradley et al. (2004b) described a new species of *Aporodon* (*R. bakeri*) using in major part, cytochrome *b* (cyt *b*) sequence data provided by our laboratories. In this report we present additional cyt *b* data for several species that were not included in previous molecular systematic treatments (Bell et al., 2001; Bradley et al., 2004b; Nelson et al., 1984). Therefore, the primary objective of this study was to estimate relationships among Middle American species (members of the subgenus *Aporodon*) using the mitochondrial cyt *b* gene based on a larger taxonomic and geographic sampling than was available previously. Secondarily, inclusion of several additional taxa assigned to the subgenus *Reithrodontomys* allowed us to make inferences with regard to relationships in that lineage as well.

2. Materials and methods

2.1. Specimens

Sixty specimens were included (Appendix A; see Fig. 1 for geographic locations) representing seven species of harvest mice in the subgenus *Aporodon* (*Reithrodontomys bakeri*, *R. creper*, *R. gracilis*, *R. mexicanus*, *R. microdon*, *R. spectabilis*, and *R. tenuirostris*), nine species in the subgenus *Reithrodontomys* (*R. chrysopsis*, *R. fulvescens*, *R. hirsutus*, *R. humulis*, *R. megalotis*, *R. montanus*, *R. raviventris*, *R. sumichrasti*, and *R. zacatecae*), plus two outgroup taxa (*Peromyscus leucopus* and *P. maniculatus*: Watrous and Wheeler, 1981).

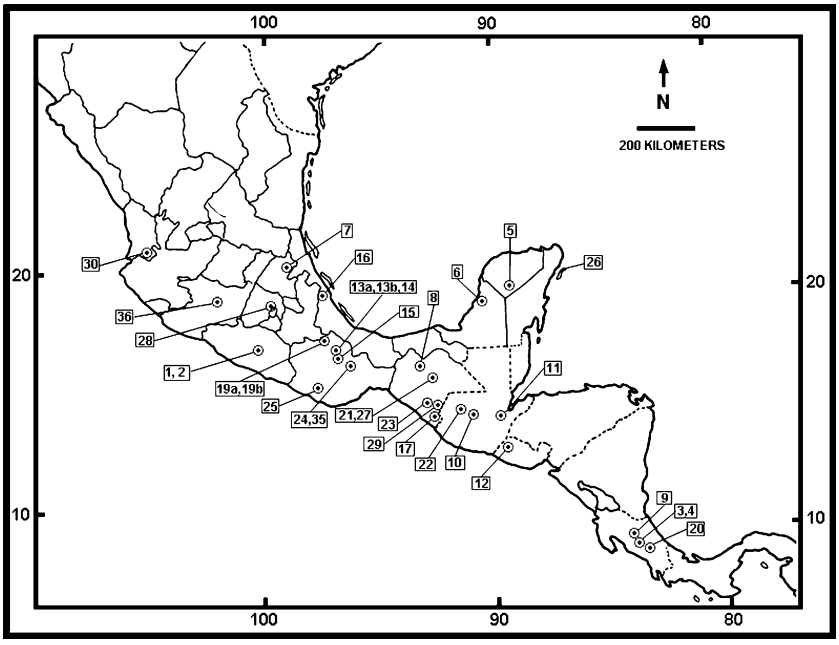

Fig. 1. Map of Mexico and Central America illustrating the geographic location for samples of Mexican and Central American *Reithrodontomys* included in this study. Numbers on this map are the same as those proceeding general geographic designations in Figs. 2 and 3. Complete locality information is provided in Appendix A.

2.2. Data collection

Total genomic DNA was extracted from liver tissue either frozen or preserved in 95% ethanol following Arellano (1999) or using the Qiagen DNeasy™ Tissue Kit (Cat. No. 69504). Four microliters of DNA extraction product were electophoresed on 1.75–2.0% agarose gels stained with ethidium bromide to qualitatively estimate amount of genomic DNA. If DNA bands were relatively bright, then samples were diluted prior to polymerase chain reaction (PCR) amplification. The following primers were used to amplify the entire mitochondrial cyt *b* gene: L14724 (5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′) with H15915 (5′-AAC TGC AGT CAT CTC CGG TTTA CAA GA-3′) (Irwin et al., 1991) or MVZ-05-M (5′-CTT GAT ATG AAA AAC CAT CGT TG-3′) with MVZ-14-M (5′-CTT GAT ATG AAA AAC CAT CGT TG-3′) (modified from Smith and Patton, 1993). Light strand internal primers used included 700L (5′-CCC AGC ACA TAT TAA ACC AGA AT-3′) (Peppers and Bradley, 2000), F1 (5′-TGA GGA CAR ATA TCH TTY TGR GG) (Whiting et al., 2003), L15162 (5′TCG YCT YCC ATG AGG RCA TAT ATC-3′) (Irwin et al., 1991), MVZ 45 (5′-GTH ATA GCH ACA GCA TTY ATA GG-3′) (Smith and Patton, 1993), MVZ 17 (5′-ACC TCC TAG GAG AYC CAG AHA AYT-3′) (Smith and Patton, 1999), MVZ 41 (5′-CAC CCT TAY TAY ACM AYY AAA GA-3′), and MVZ 47 (5′-AGA AAT WAY HCC GCA AYC-3′) (Smith personal communications), whereas heavy strand internal primers used were CBH3 (5′-GGC AAA TAG GAA RTA TCA TTC-3′) (Palumbi, 1996), H15149 (5′-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3′ (Irwin et al., 1991), MVZ 04 (5′-CCT CAR AAK GAT ATT TGB CCT C-3′), and MVZ 16 (5′-TAG GAA RTA TCA YTC TGG TTT RAT) (Smith and Patton, 1993).

The cyt *b* gene was amplified using PCR in a master mix containing 1.0 μl template DNA (approximate concentration estimated on a 2% agarose gel), 4 μl dNTPs (1.25 mM), 2 μl 10× *Taq* buffer, 0.5 μl of each primer (100 μM), 3 μl $MgCl_2$ (25 mM), 14 μl distilled water, and 0.25 μl *Taq* polymerase (5 u/μl; Promega Corp., Madison, WI). 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 50 °C, and 1 min at 72 °C), plus 5 min at 72 °C. Some samples were more difficult to amplify. In these instances, annealing temperature was reduced to 45 °C and number of cycles increased to 40. Four microliters of double-stranded PCR amplified product was assayed by electrophoresis on a 2% agarose gel. The remaining product (ca. 21 μl) was purified either by the QIAquick PCR purification protocol (QIAGEN, Chatsworth, CA), the Gene-Clean purification method (Bio 101, La Jolla, CA) or by using a Millipore Multiscreen™ PCR 96-Well Filtration System (Cat. No. MANU03050). Sequencing was performed 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 a solution of Sephadex 50G (3g/50 ml H_2O) or by using Millipore Multiscreen™ Filter Plates for High Throughput Separations (Cat. No. MAHVN4510). Cytochrome *b* sequences were determined using either a Perkin–Elmer ABI Prism 377 automated sequencer or an ABI 3100 automated sequencer housed in the DNA Sequencing Center at Brigham Young University. To verify the accuracy of our data, we included negative controls in every reaction, complementary strands of each DNA fragment were sequenced and sequences were edited manually using the original chromatograph data in the program Sequencher versions 3.1.1 and 4.1.1 (Gene Codes, 2000). All sequences have been deposited on the GenBank database (see Appendix A for accession numbers).

2.3. Phylogenetic analyses

Gene phylogenies were estimated using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) optimality criteria. Genetic distance values, MP and ML analyses were performed using PAUP* 4.0b10 (Swofford, 2002). MP analyses were conducted with equal character weighting and 10,000 random addition sequences with tree-bisection reconnection (TBR) branch swapping. For MP trees, branch support for nodes was assessed using non-parametric bootstrapping (Felsenstein, 1985) with 1000 bootstrap replicates of 100 random sequence additions. Bootstrap values >70% were considered well supported (Hillis and Bull, 1993). Because resampling methods in general are sensitive to problems of character independence and distribution (Page and Holmes, 1998), we also calculated Bremer support values (Bremer, 1994) for each node in the tree using TREEROT (Sorenson, 1999) and considered Bremer decay values >5 as indicating strong nodal support.

Under the ML criterion, the model of evolution most appropriate for our data was selected using MODELTEST v3.6 (Posada and Crandall, 1998). The general time reversible model with invariable sites and rate heterogeneity (GTR+Γ+I) was selected as the best-fit model of nucleotide substitution ($\pi_A = 0.319$, $\pi_C = 0.299$, $\pi_G = 0.106$, and $\pi_T = 0.275$; $r_{CT} = 5.539$, $r_{CG} = 27.000$, $r_{AT} = 5.772$, $r_{AG} = 1.114$, and $r_{AC} = 65.879$; $I = 0.56$; $\alpha = 1.412$). This model was then used for ML searches consisting of 100 random addition sequences with TBR branch swapping. Nodal support was determined using 180 bootstrap replicates of 10 random addition sequences additions each with TBR branch swapping. All ML analyses were conducted on an IBM SP2 supercomputer to reduce computation time.

Bayesian inference was conducted using MrBayes 3.0b4 software. In this methodology, a posterior probability of a phylogeny is estimated by sampling trees from the overall distribution of posterior probabilities using Metropolis-coupled Markov Chain Monte Carlo (MCMC) to sample phylogenies according to their posterior probabilities (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). We did not define *a priori* a model of evolution. Instead, a likelihood ratio test was performed to compare likelihood scores for each of 56 evolutionary models as evaluated by MODELTEST v3.6 (Posada and Crandall, 1998). We partitioned our cyt *b* data into three classes (codon positions 1, 2 and 3 – Ronquist and Huelsenbeck, 2003). Separate series of BI analyses were run incorporating these models of evolution. In one, codon positions were treated as unlinked. In a second, the first and third codon positions were designated as linked. Both linked and unlinked analyses were conducted four times yielding a total of eight BI analyses (Nylander et al., 2004). Each analysis was started from a different, randomly chosen tree and four simultaneous incrementally heated chains were run for 5×10^6 generations, with sampling every 1000th interval. To ensure the Markov Chain had become stable, ln-likelihood values for sampling points were plotted against generation time. All sample points prior to reaching stationarity (conservatively, the first 150 of 5001 trees) were discarded as "burn in". Posterior probabilities for individual clades obtained from independent analyses were compared for congruence (Huelsenbeck and Imennov, 2002; Huelsenbeck et al., 2002; Nylander et al., 2004).

Although uncorrected *p* distances require no model assumptions, these measures commonly are used to report divergence among populations/taxa. We calculated this estimate for comparative purposes using PAUP* 4.0b10 (Swofford, 2002).

2.4. Hypothesis testing

Alternative phylogenetic hypotheses were tested with both maximum parsimony and likelihood-based 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 and Hasegawa test (K–H; Kishino and Hasegawa, 1989) for trees generated using the maximum parsimony criterion and the Shimodaira and Hasegawa test (S–H; Shimodaira and Hasegawa, 1999) with restricted likelihood as implemented in PAUP* 4.0b10 (Swofford, 2002). Goldman et al. (2000), Buckley (2002), and Strimmer and Ranbaut (2002) noted that the S–H test may be biased in that the number of trees included in the confidence set tends to be large as the number of trees to be compared increases, which results in a conservative test. However, as Shimodaira (2002) concluded and others have recognized, the S–H test is appropriate when the number of candidate trees is not very large. Ten thousand replicates were performed using the S–H topology test by resampling the partial likelihoods for each site (RELL model). Multiple ML hypotheses were maximized under the GTR+Γ+I model in PAUP* 4.0b10 and the resulting best-scored hypothesis for each case was compared to the single ML tree (see below).

3. Results

3.1. mtDNA sequence variation

The mitochondrial cyt *b* gene (1143 bp) was sequenced and aligned for a total of 60 individuals representing 18 taxa (*Mus* positions 14139-1282, GenBank Accession No. J01420; Bibb et al., 1981). Of this total, 52 sequences were generated in our laboratory and 46 are reported for the first time (see Appendix A for a list of taxa, locality and voucher specimen information and GenBank Accession numbers). Of 1143 characters, 444 were variable across all samples; 380 of these were potentially informative phylogenetically. Exclusion of outgroup taxa (*Peromyscus leucopus* and *P. maniculatus*) reduced the number of variable and phylogenetically informative characters to 434 and 371, respectively. Nucleotide composition was similar to those reported for the majority of mammals (Irwin et al., 1991). In this study, guanines occurred less frequently (12.73%) relative to adenine, cytosine and thymine (30.84 , 27.13 and 29.60%, respectively). Genetic distances (uncorrected *p* distances) among members of the subgenus *Aporodon* ranged from 1.27 to 13.25%, whereas the range among species belonging to the subgenus *Reithrodontomys* was 8.48–15.39% (Table 1).

3.2. MP analysis

Parsimony analysis with equal character weighting resulted in 393 most parsimonious trees with 2009 steps (CI = 0.31, RI = 0.73). The 50% majority rule consensus tree (Fig. 2) indicated that members of the subgenera *Aporodon* (clades I – V) and *Reithrodontomys* (clade VI) were monophyletic but with relatively weak boostrap support. However, Bremer decay values were 10 and 5, respectively, in support for monophyly of the two subgenera.

Clades I and III and group D of clade II correspond to mice originally identified as *R. mexicanus* and each of these lineages is strongly supported (Fig. 2). Within clade I there is moderate bootstrap support for grouping harvest mice from Mexico (samples 8, 13b, 16, 17 and 19b) and stronger support for kinship among samples from

Table 1
Ranges of pair-wise uncorrected distances, in percent, for 16 species in the genus *Reithrodontomys*

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 *R. bakeri*	(0.0–4.0)																		
2 *R. creper*	12.1–13.9	(0.3–0.5)																	
3 *R. gracilis*	12.4–14.5	12.3–12.8	(1.5)																
4 *R. mexicanus* (III)	11.9–14.1	13.2–14.3	11.9–12.8	(0.0–2.7)															
5 *R. mexicanus* (I)	11.4–15.0	11.3–13.2	10.8–12.5	11.8–13.5	(0.0–8.1)														
6 *R. mexicanus* (II-D)	9.9–11.5	10.9–11.2	12.5–12.8	12.2–12.9	11.1–12.7	(0.44)													
7 *R. microdon* (II-A)	7.8–10.6	11.1–12.0	12.0–12.5	11.7–13.5	10.9–12.7	9.1–10.8	(0.2–3.8)												
8 *R. microdon*(II-B)	9.3–11.3	11.9–12.5	11.8	12.2–13.0	11.1–12.5	10.2–10.5	8.9–10.0	(0.5)											
9 *R. spectabilis*	12.2–14.3	12.2–12.5	1.2–1.3	11.8–12.4	10.8–12.2	12.4–12.7	11.8–12.5	11.9	—										
10 *R. temairostris*	9.1–10.9	11.8–12.2	12.5	12.6–13.0	12.2–13.0	10.1–10.2	8.1–8.3	9.8	12.5	—									
11 *R. chrysopsis*	16.2–16.7	14.3–14.5	16.6–16.8	15.3–15.7	14.2–16.0	14.9–15.1	14.4–15.3	14.9–15.4	16.7	16.6	—								
12 *R. fulvescens*	14.6–15.4	11.9–12.2	13.9–14.4	13.7–14.4	12.8–14.7	13.0–13.1	13.1–13.7	13.6–14.0	14.0	13.1	14.2	—							
13 *R. hirsutus*	13.9–15.2	14.1–14.4	15.1–15.3	13.3–13.8	14.3–15.6	13.7–13.9	12.5–13.3	14.3–14.5	15.0	13.9	15.4	12.2	—						
14 *R. humulis*	15.1–15.8	14.5–15.0	15.3–15.5	13.6–14.2	13.9–15.3	14.0–14.1	13.5–14.7	13.9–14.0	15.4	15.0	13.6	13.4	13.5	—					
15 *R. megalotis*	14.9–16.0	15.4–15.5	16.0	13.9-14.4	14.2–15.4	13.7–13.9	13.2–14.4	13.7–13.9	15.9	14.7	13.0	13.1	9.2	11.7	—				
16 *R. montanus*	16.2–17.3	15.5–15.8	16.0–16.2	15.8–16.2	15.0–16.5	14.9	15.2–16.4	15.2	16.1	15.3	15.4	13.9	14.1	13.9	14.1	—			
17 *R. raviventris*	15.5–17.4	14.6–14.9	14.8-14.9	15.1–15.4	15.0–16.1	15.5–15.6	14.7–15.9	15.4	15.1	14.6	13.8	13.2	13.2	13.3	13.6	12.7	—		
18 *R. sumichrasti*	14.7–15.9	13.7–13.9	15.8	14.4–14.7	13.5–14.6	13.4	12.9–13.5	14.5–14.6	15.8	15.3	12.6	12.3	12.0	12.9	8.4	14.2	12.6	—	
19 *R. zacatecae*	14.9–16.0	15.6–15.9	15.1–15.3	14.1–14.8	14.0–15.3	14.4–14.5	14.6–15.7	13.4	15.0	16.1	14.7	12.5	12.5	12.7	8.5	13.9	13.9	9.36	—

Numbers in parentheses are within species values, dashes indicate a single specimen examined. Roman numerals and letters in parentheses following species names refer to clades as depicted in Figs. 2 and 3.

Mexico and Central America (samples 10, 12 and 20) relative to sample 18 from Colombia (Fig. 1). Overall, nodal support for clade II is weak. This group is comprised of five lineages and kinship among them is unresolved, however, each internal node is supported strongly (100% bootstrap and Bremer decay values ranging from 12 to 31). Harvest mice confined to the Sierra Madre Oriental of Mexico form clade III and this node has strong support. Samples of *R. creper* form a lineage (clade IV) that has strong bootstrap and Bremer support. *R. gracilis* is paraphyletic relative to *R. spectabilis,* but internal support for this arrangement is weak (clade V). Clade VI contains species belonging to the subgenus *Reithrodontomys.* Support for the ((*R. megalotis* – *R. zacatacae*) *R. sumichrasti*)) clade is weak. The relationships of *R. chrysopsis*, *R. hirsutus* and *R. humulis* are unresolved with the 50% majority rule criterion, but a clade comprised of *R. montanus* – *R. ravivientris* is supported by a Bremer decay value of 5.

3.3. ML and BI analyses

Modeltest (Posada and Crandall, 1998) for the ML analyses indicated that GTR+Γ+I was the most appropriate model of nucleotide substitution for our data. We also partitioned our data by codon position and performed Modeltest for each position prior to BI analyses. The HKY+Γ+I model of evolution was selected for the second codon position and identical evolutionary models best fit the partitioned data for the first and third codon positions (GTR+Γ+I). Phylogenetic estimates based on ML, linked BI analyses (four replicates) as well as unlinked BI analyses (four replicates) all converged on essentially identical tree topologies. Nodal support varied only slightly (values available upon request from DSR) among the four linked and unlinked BI replicates. Nodal differences among these analyses were limited to relationships at the tips of trees (among individuals from within the same population) with only two exceptions. Both the ML (not shown) and linked BI analyses fully resolved relationships among clades I–V (Fig. 3). However, the BI estimates based on the unlinked model failed to resolve relationships among clades II and V relative to III and IV (not shown). The second discrepancy among analyses involved individuals that comprise clade III. Maximum parsimony, ML and the linked BI analyses divided this clade into two well supported lineages. However, the BI unlinked analyses did not recover two groups.

All ML and BI analyses indicated strong nodal support for monophyly of the subgenus *Aporodon* (clades I–V) relative to clade VI (subgenus *Reithrodontomys*) and the five *Aporodon* clades identified in the MP analysis also were recovered using ML and BI approaches – each with 100% posterior probability and strong bootstrap support (Fig. 3). Relationships among members of clade

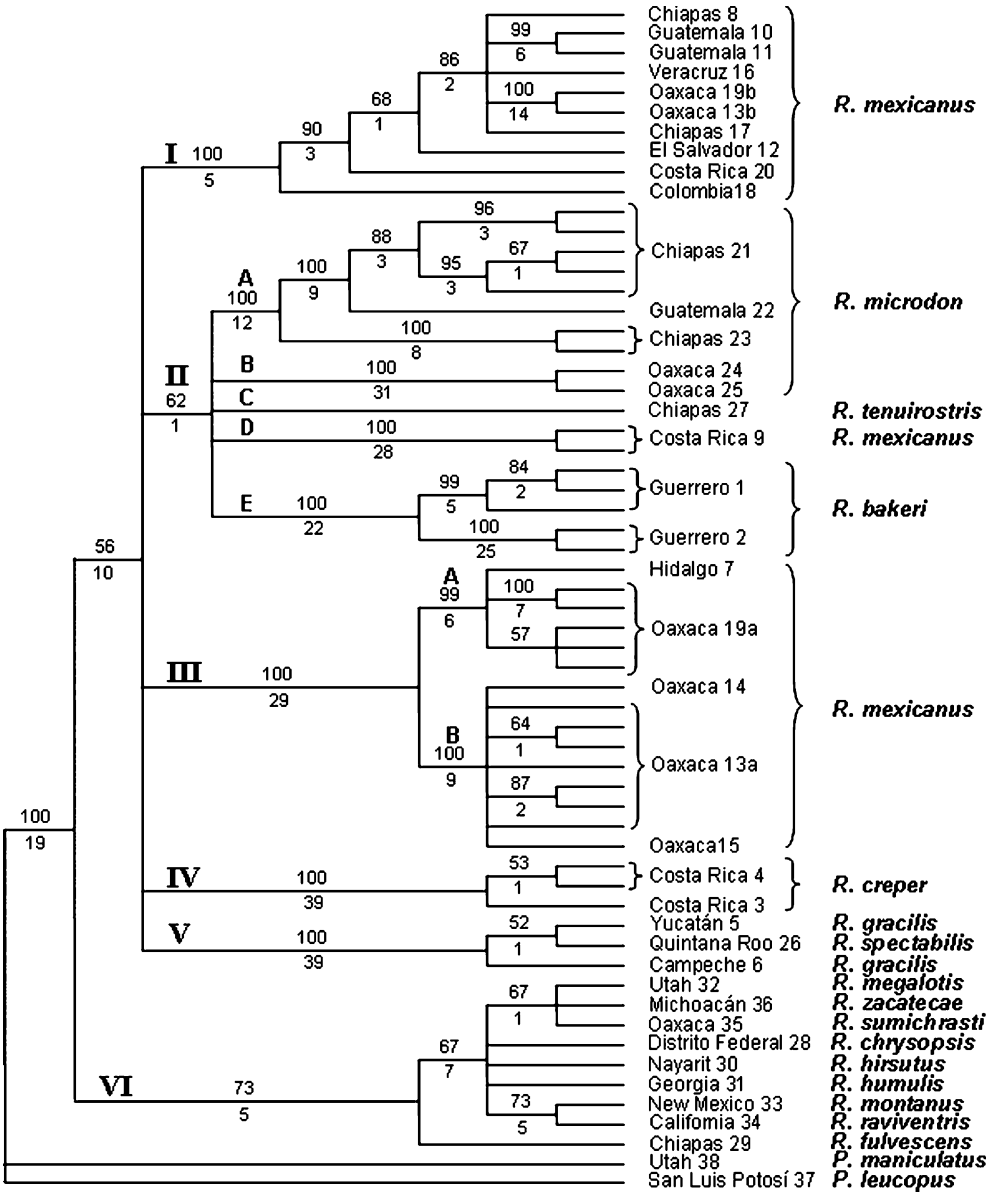

Fig. 2. MP consensus tree (50% majority rule) of 393 equally parsimonious trees (2009 steps; CI = 0.31; RI = 0.73) derived from analysis of cyt *b* sequence data for the genus *Reithrodontomys* (*Peromyscus leucopus* and *P. maniculatus* are outgroup taxa). Numbers to the left indicate sampling localities (Appendix A). Nodal support is represented by bootstrap values derived from 10,000 pseudoreplicates (above nodes) and Bremer support values (below nodes).

II (groups A–E) also were fully resolved by BI and ML analyses (Fig. 3). Within this clade, samples of *R. microdon* are polyphyletic. Samples 21, 22, and 23 of *R. m. microdon* form a lineage whose sister group is *R. tenuirostris*, but nodal support for this arrangement is not particularly strong. *R. m. albilabris* (samples 24 and 25) are most closely related to *R. bakeri*, but nodal support is relatively weak (Fig. 3). Compared to the MP analysis, both ML and BI approaches yielded additional resolution among representatives of the subgenus *Reithrodontomys* (Clade VI). *R. fulvescens* is arranged as the basal taxon and *R. hirsutus* is positioned as the sister group to the remaining taxa. Nodal support for a clade comprised of *R. megalotis*, *R. sumichrasti* and *R. zacatacae* is high, however, the sister group relationship between *R. megalotis* and *R. zacatecae* is only weakly supported. *R. raviventris* and *R. montanus* form a group with strong nodal support. In turn, these two taxa form the sister group to *R. humulis*, although support for this clade is weak. The relationships among these two lineages relative to *R. chrysopsis* are unresolved.

3.4. Hypotheses tested

We tested for monophyly of the two subgenera of harvest mice, *Reithrodontomys mexicanus* and *R. microdon*

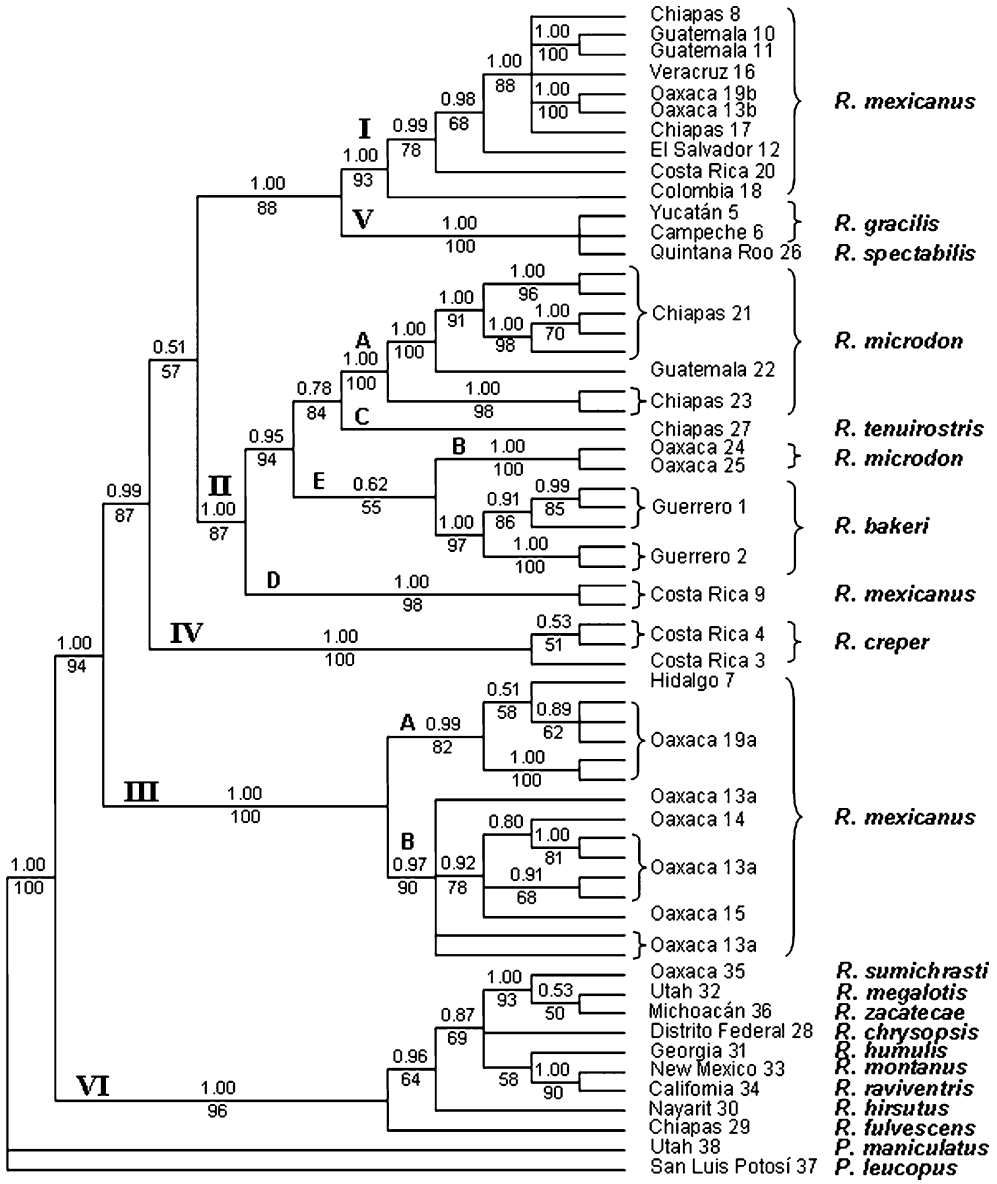

Fig. 3. Bayesian phylogenetic hypothesis (50% majority rule) for the genus *Reithrodontomys* (*Peromyscus leucopus* and *P. maniculatus* are outgroup taxa) derived from analysis of cyt *b* sequence data partitioned by codon position using the HKY+Γ+I model of evolution for the second codon position and the GTR+Γ+I model for the first and third codon positions. Numbers to the left indicate sampling localities (Appendix A). Nodal support is represented by posterior probabilities (above nodes), whereas values below nodes are ML bootstrap support based on 180 replicates. Nodes for which no bootstrap values are given were not present in the ML tree (not shown).

using both MP and ML optimality criteria (Table 2). Constrained trees differed significantly from optimal tree topologies for monophyly of the subgenus *Aporodon* and *Reithrodontomys* using the S–H test (Shimodaira and Hasegawa, 1999). Because *R. mexicanus* was recovered as polyphyletic (clades labeled as I, II–D and III in Figs. 2 and 3), we held these three clades as monophyletic in our constraint analyses but varied relationships among these clades with respect to one another. All permutations of the K–H tests (Kishino and Hasegawa, 1989) and S–H tests for monophyly of *R. mexicanus* resulted in significant differences between optimal and constrained trees. Monophyly of *R. microdon* was supported only by the K–H constraint test which placed *R. bakeri* as the sister group to *R. microdon*.

4. Discussion

4.1. Relationships among members of the subgenus *Aporodon*

Maximum parsimony, ML, and BI analyses produced very similar estimates of tree topologies; the analyses differed in that ML and BI yielded greater resolution

Table 2
Kishino and Hasegawa (1989) and Shimodaira and Hasegawa (1999) test results

Hypothesis	K–H test		S–H test	
	ΔSteps	*p*-Value	ΔLikelihood	*p*-Value
Monophyly of *Aporodon* and *Reithrodontomys*	+6	>0.343	+11.724	>0.036
Monophyly of *R. mexicanus*				
((I,III)(II-D))	+35	>0.001	+25.496	>0.013
((I,II-D)(III))	+41	>0.001	+29.974	>0.004
((II-D,III)(I))	+36	>0.001	+30.244	>0.004
((I)(III)(II-D))	+50	>0.001	+30.697	>0.003
Monophyly of *R. microdon*				
(((II-A,B)(II-C))(II-E)))	+8	>0.157	+5.321	>0.105
(((II-A,B)(II-E))(II-C)))	+12	>0.018	+5.321	>0.105
((II-A,B)(II-C)(II-E))	+16	>0.002	+14.946	>0.051

The optimal maximum parsimony tree had a length of 2009 steps, whereas the optimal maximum likelihood tree had a log likelihood = −9787.049. Δsteps and Δlikelihood are difference between optimal and constrained phylogenetic hypotheses. Multiple tests for monophyly of *R. mexicanus* and *R. microdon* were conducted; tree topologies are indicated in parentheses, whereas Roman numerals and letters refer to clades as depicted in Fig. 1

within and among clades. The most striking finding was that *R. mexicanus*, as presently defined, is polyphyletic and consists of three highly differentiated clades (p-values among these clades range from 12.12 to 12.86%). The first lineage is comprised of harvest mice from the Sierra Madre Oriental of Mexico (clade III, Figs. 2 and 3) and the second consists of samples spanning the range of the "classic" *R. mexicanus*, which extends from Mexico south into northern South America (clade I). The third lineage formerly regarded as *R. mexicanus* (group D in clade II) is more closely related to *R. microdon*, *R. tenuirostris* and *R. bakeri*. Recovering polyphyly within *R. mexicanus* is consistent with findings reported by Arellano et al. (2003), who examined relationships among species of *Aporodon* using allozymes as well as those of Bradley et al. (2004b) based on cyt *b*. Both studies concluded that harvest mice previously regarded as *R. mexicanus* from the Sierra Madre Oriental of Mexico actually comprise a lineage that is basal to all other *Aporodon* taxa, although sampling was limited to a single individual in both papers. Our study includes additional individuals and localities and documents that harvest mice belonging to clade III occur in sympatry with *R. mexicanus* sensu stricto (clade I) at two localities in Oaxaca (La Esperanza and Puerto de la Soledad – Fig. 1). Arellano et al. (2003) hypothesized that *R. mexicanus cherrii* from Costa Rica likely represented a species-level taxon whose affinities lie with the *R. tenuirostris* species group (sensu Hooper, 1952) and our data support that conclusion.

Our cyt *b* data also demonstrate that *R. microdon* is polyphyletic. These results are consistent with those of Arellano et al. (2003) who found that *R. microdon* was paraphyletic relative to *R. tenuirostris* (*R. bakeri* was not included in their analysis). These two lineages of *R. microdon* differ by 6.47% and represent allopatric populations separated by the Isthmus of Tehuantepec in southern Mexico. The isthmus likely represents a low elevation barrier to dispersal for other highland rodent species whose ranges span the isthmus. Sullivan et al. (2000) compared phylogeographic concordance between cyt *b* gene phylogenies for two co-distributed rodent species (*Peromyscus aztecus* and *R. sumichrasti* in Mesoamerica. Populations of both species south of the Isthmus of Tehuantepec formed the deepest clade within each species. Further, levels of genic divergence documented by Sullivan et al. (2000) are consistent with the view that populations of *P. aztecus* and *R. sumichrasti* on either side of the Isthmus of Tehuantepec likely represent species level taxa. The pattern of sequence divergence exhibited by *R. microdon* is essentially identical to that recovered by Sullivan et al. (2000).

The data presented by Arellano et al. (2003) are equivocal with regard to placement of *R. creper*. In an analysis using *Peromyscus maniculatus* as the outgroup taxon, *R. creper* shows affinities with *R. mexicanus*, *R. spectabilis* and *R. gracilis*. In a second analysis using several members of the subgenus *Reithrodontomys* as outgroup taxa, *R. creper* shows kinship with other members of the *R. microdon* species group (sensu Hooper, 1952; Arellano et al., 2003). Our cyt *b* data also are equivocal in that the phylogenetic affinities of *R. creper* are unresolved (MP analysis) or this taxon forms a lineage that is basal to all members of the subgenus *Aporodon* with the exception of *R. mexicanus* from the Sierra Madre Oriental of Mexico.

Maximum parsimony, ML and BI analyses all demonstrated that *R. gracilis* and *R. spectabilis* were closely related and were not divergent genetically from each other (Table 1). Arellano et al. (2003) also demonstrated kinship between *R. gracilis* from Yucatan, Mexico and the geographically adjacent *R. spectabilis* from Isla Cozumel, (Figs. 1 and 2), relative to *R. gracilis* from further west in the state of Campeche, Mexico. This relationship was based on frequency differences or the presence of autapomorphic alleles among these taxa for eight genetic loci; the fixed difference involved the *ADH* locus (allele "d" was present in *R. spectabilis* whereas

allele "b" was found in both populations of *R. gracilis*). Regardless of the phyletic relationships among these taxa, it is clear that *R. spectabilis* represents a relatively recent derivative of *R. gracilis*.

4.2. Relationships among members of the subgenus *Reithrodontomys*

Although our primary objective was to elucidate relationships among *Aporodon* taxa, our cyt *b* data add to previous work done using allozymes (Arellano et al., 2003; Nelson et al., 1984) and cyt *b* data (Bell et al., 2001) by the inclusion of two additional species – *R. hirsutus* and *R. chrysopsis*. Neither MP nor ML analyses shed light on the phylogenetic positions of these two taxa. Bayesian inference, however, supported a hypothesis that *R. hirsutus* is basal to a clade comprised of all taxa with the exception of *R. fulvescens*. Our analysis of relationships among the remaining members of the subgenus *Reithrodontomys* are essentially congruent with those of Bell et al. (2001).

4.3. Species-level phylogenetics

We made decisions regarding species boundaries by employing the phylogenetic species concept (Cracraft, 1983; Nixon and Wheeler, 1990), which defines a species as the smallest group of organisms delimited by a unique combination of character states, within which there is a pattern of ancestry and descent. Because strict application of this species concept can result in recognition of temporarily isolated demes as species, we also applied the tree-based species delimitation methodology as outlined by Wiens and Penkrot (2002). Accordingly, delimiting species requires concordance among two or more independent data sets and can involve both non-tree and tree-based methods (Marshall and Sites, 2003).

The available data are equivocal with regard to the systematic status of *R. spectabilis*. This taxon was described on the basis of its overall larger size (Jones and Lawler, 1965) and represents the only harvest mouse found on Isla Cozumel, located to the east of the Yucatan, Mexico. Allozyme data (Arellano et al., 2003) documented that *R. spectabilis* possessed a single apomorphic character, but based on all characters, *R. gracilis* was paraphyletic relative to *R. spectabilis*. Cytochrome *b* data presented herein (depending on the optimality criterion used) either do not support the monophyly of *R. gracilis* (MP, ML and BI unlinked analyses) or present these lineages (two sequences representing *R. gracilis* and *R. spectabilis*) as an unresolved trichotomy (BI linked analyses). Although *R. spectabilis* is morphologically distinctive, allozyme and cyt *b* sequence data indicate that this lineage has not yet achieved reciprocal monophyly with *R. gracilis*. Therefore, we conclude that *R. spectabilis* is a relatively recent derivative of *R. gracilis* and given the morphological novelty it represents, poses an interesting evolutionary question regarding the evolution of large size due to island effect (sensu Foster, 1964).

We consider the lineage represented by clade III in this study to represent candidate species A. This lineage has six fixed allozyme difference (Arellano et al., 2003), possesses a different chromosomal complement (Urbina-Sánchez et al., submitted), differs substantially in cyt *b* sequences from other *Aporodon* taxa and individuals representing this clade and those of *R. mexicanus* (sensu stricto) are sympatric at two locations in Oaxaca, Mexico (Puerto de la Soledad and La Esperanza). Moreover, trees that force monophyly of this clade with other lineages of *R. mexicanus* differ significantly from our optimal MP and ML trees. According to Hall and Dalquest (1963), two morphological forms of *R. mexicanus* occur in Veracruz, Mexico. One is larger overall and can be distinguished by several skull characteristics. Whether or not the two morphological types identified by Hall and Dalquest (1963) represent candidate species A and *R. mexicanus* sensu stricto is not known. Decisions regarding assignment of an available name (junior synonym) or a new specific epithet to this candidate species cannot be made without a comprehensive analysis of morphological variation that involves comparison of specimens with "known" genetic data to museum voucher specimens, including specimens representing junior synonyms (*R. costaricensis jalapae*, *R. goldmani* [Merriam, 1901] and *R. mexicanus scansor* [Hooper, 1950]) for which genetic data are lacking.

These data, together with that of Arellano et al. (2003), demonstrate that *R. microdon* is not monophyletic. Rather, *R. m. albilabris* and *R. m. microdon* show kinship with *R. bakeri* and *R. tenuirostris*, respectively. *R. m. albilabris* is distinguished from *R. m. microdon* by two fixed alleles (Arellano et al., 2003) and results of the constraint analysis on our cyt *b* data indicate that forcing monophyly of *R. m. albilabris* and *R. m. microdon* results in significantly longer trees (ML criterion). Therefore, we propose that *R. m. microdon* from Chiapas, Mexico and Guatemala be considered as *R. microdon* (sensu stricto) and *R. m. albilabris* from Oaxaca, Mexico, should be regarded as candidate species B.

Our cyt *b* data demonstrate that *R. mexicanus cherrii* forms a clade related to taxa from Mexico and Guatemala (*R. microdon* and *R. tenuirostris*) rather than to populations of *R. mexicanus* from Costa Rica. Patterns of mtDNA variation fit the cladistic haplotype aggregation method of Brower (1999) and the genealogical exclusivity method presented by Baum and Shaw (1995). This taxon also is distinct based on nuclear markers. Arellano et al. (2003) discovered two fixed allozyme differences and four autapomorphic alleles that distinguished this population from all other samples of *Reithrodontomys*. Therefore, we consider *R. m. cherrii* to be distinct on the basis of genealogical exclusivity

(Baum and Shaw, 1995). Finally, our constraint tests failed to support tree topologies that grouped *R. m. cherrii* with other clades identified as *R. mexicanus*.

Hooper (1952, p. 153) remarked that *R. mexicanus* from Costa Rica (subspecies *cherrii*) is one of the "largest and brightest colored of the subspecies of *R. mexicanus*." Arellano et al. (2003) documented that samples of *R. mexicanus* used in their study were indistinguishable, morphologically, from *R. mexicanus cherrii* and that in turn, this taxon differed morphologically from other species of harvest mice in the subgenus *Aporodon* currently recognized in Costa Rica (*R. brevirostris* and *R. rodriguezi*). Based on these criteria, we propose that *R. m. cherrii* (*Reithrodontomys* sp. sensu Arellano et al., 2003) from Costa Rica should be recognized as a separate species. Accordingly, the appropriate name available for this distinctive Costa Rican harvest mouse is *Reithrodontomys cherrii* Allen. This newly recognized species (synonymy provided below) fits, as best as we can determine, the morphological and distributional parameters given under its original description (Allen, 1891).

Reithrodontomys cherrii (Allen, 1891, p. 211)*Type Locality*—San Jose, [Costa Rica]. *Synonyms.— costaricensis* Allen, 1895:139; type locality "La Carpintera, 5000 ft., Costa Rica".

Characteristics and Distribution.—Subsequent redefinition by Osgood (1907) restricted its geographic distribution to a small area south and east of San José, Costa Rica. However, we anticipate that the range of this form actually encompasses areas of central and southern Costa Rica at elevations from ca. 1000 to 2500 m and may include the range of what is now known as *R. m. garichensis*.

Remarks.—For a summary of the taxonomic history and designation of type material, see Hooper (1952, p.153) . Based on mitochondrial cyt *b* sequence data, this species belongs in the *R. tenuirostris* species group (Hooper, 1952).

4.4. Interspecific phylogenetics

Recently, Arellano et al. (2003) summarized the alpha taxonomy of the genus *Reithrodontomys* with regard species descriptions subsequent to Hooper's (1952) compendium. Hooper (1952) recognized two species groups within the subgenus *Aporodon*; *R. mexicanus* and *R tenuirostris*. More recently, Bradley et al. (2004b) described *R. bakeri*, a species owing kinship to the *R. tenuirostris* species group. Currently, the *R. mexicanus* species group is comprised of *R. brevirostris*, *R. darienensis*, *R. gracilis*, *R. mexicanus*, *R. paradoxus* and *R. spectabilis* (Arellano et al., 2003), whereas the *R. tenuirostris* species group is formed by *R. bakeri*, *R. creper*, *R. microdon*, *R. rodriguezi* and *R. tenuirostris*. Of these, *R. brevirostris*, *R. darienensis*, *R. paradoxus* and *R. rodriguezi* were unavailable for study. Our findings, together with those of Arellano et al. (2003), are not consistent with the recognition of two lineages within the subgenus *Aporodon*. Instead, we favor recognition of four. The first lineage corresponds to candidate species A (clade III). The second is formed by clades I and V and is equivalent with Hooper's (1952) *R. mexicanus* group (herein known to include *R. gracilis*, *R. spectabilis*, and *R. mexicanus*, sensu stricto, but may include *R. brevirostris*, *R. darienensis* and *R. paradoxus* as well). The third group we recognize (clade II) also is equivalent to Hooper's (1952) *R. tenuirostris* group (*R. bakeri*, *R. cherrii*, *R. microdon* [a composite taxon] and *R. tenuirostris*). Finally, we recognize a fourth lineage comprised of *R. creper* (clade IV).

4.5. Conclusions and prospectus

With regard to species recognition, we adopted the null hypothesis that lineages were conspecific unless strong evidence indicated otherwise. As a result, we took a conservative approach in making taxonomic recommendations (identifying candidate species in most cases) because we felt that committing a type I error (rejecting a true Ho) would be more problematic than making a type II error (failure to reject a false Ho). Type I errors will inevitably introduce more junior synonyms into the literature and ultimately, will reduce the credibility of systematic treatments at a time when papers dealing with topics that impact conservation strategies or biodiversity estimates are critically needed. The candidate species identified in this paper highlight the need for additional data (morphological, nuclear DNA sequences) that may be brought to bear on issues relevant to species-level status of these entities. Additional sampling of populations currently assigned to *R. microdon* and *R. mexicanus* will help shed light on relationships within this complex. Likewise, additional sampling and sequence data are needed to resolve the systematic status of *R. chrysopsis R. hirsutus,* and *R. spectabilis*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.07.021

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