Original Study

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Mitochondrial DNA and other lines of evidence clarify species diversity in the *Peromyscus truei* species group (Cricetidae: Neotominae)

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Abstract: Deer mice (genus *Peromyscus*) are among the commonest small mammals in the Nearctic zoogeographic region. Nevertheless, systematic relationships are only partially settled and numerous taxonomic questions await resolution. For instance, researchers have found that some members of the *Peromyscus truei* species group contain high levels of genetic divergence that could indicate the presence of cryptic species. We analyzed the systematics and phylogenetic relationships of the *P. truei* group using new and previously published mitochondrial cytochrome *b* sequences. Our analyses verify several earlier conclusions, but we also detected new clades that deserve recognition. Considering their mitochondrial distinctiveness, allopatric ranges, and previously reported molecular, biochemical, chromosomal, morphological, and ecological differences, we elevate three previously described taxa to species. We support the recognition of two subgroupings. The first comprises *P. gratus*, *P. truei*, and possibly *P.* cf. *martirensis* and *P.* cf. *zapotecae*. The second contains to *P. amplus*, *P. attwateri*, *P. collinus*, *P. difficilis*, *P. felipensis*, *P. laceianus*, *P. nasutus*, *P. ochraventer*, and *P. pectoralis*. Placement of *P. bullatus* will likely remain unknown until genetic data are available. Further research could improve our understanding of the evolutionary history of *Peromyscus*, but in some cases taxonomic issues must be resolved first.

Keywords: cytochrome *b*; molecular phylogeny; *Peromyscus difficilis*; *Peromyscus pectoralis*.

1 Introduction

The 66 currently recognized species of deermice (*Peromyscus*) represent a dominant element of North American small mammal faunas (Pardiñas et al. 2017). Despite considerable effort to resolve their taxonomy, the true number of species and their phylogenetic relationships remain unclear (Platt et al. 2015; Sullivan et al. 2017). Taxonomic revisions (Carleton 1989; Osgood 1909) divided deermice into several species groups, including the large-eared mice in the *P. truei* species group, which inhabit much of Mexico and the United States (Pardiñas et al. 2017). Originally this species group included *P. bullatus*, *P. difficilis*, *P. nasutus*, *P. polius*, and *P. truei* (Osgood 1909), but several taxa were added or removed in later decades (mainly moving species between the *P. truei*, *P. boylii*, and *P. aztecus* species groups) (Durish et al. 2004; Sullivan et al. 1991). Based on morphological, karyotypic and electrophoretic investigations Carleton (1989) defined this species group as comprising *P. bullatus*, *P. difficilis*, *P. gratus*, *P. nasutus*, and *P. truei*; subsequent analyses added *P. attwateri* (DeWalt et al. 1993; Janecek 1990), and other authors suggested *P. pectoralis*, *P. laceianus* (Durish et al. 2004), and *P. ochraventer* (Bradley et al. 2007) may belong in the *P. truei* species group. It was also shown that *P. difficilis*, *P. nasutus*, *P. gratus*, *P. truei*, and *P. pectoralis* possessed high levels of intraspecific mitochondrial divergence, and future tests for the existence of undescribed taxa were recommended (Bradley et al. 2015; Durish et al. 2004). Even though these genetic studies were based only on cytochrome *b* (cyt *b*), their analysis deserves attention because

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mitochondrial diversity is often the first clue that cryptic species are present (e.g., Arellano et al. 2005; Edwards and Bradley 2002; Hernández-Canchola and León-Paniagua 2021; Rogers et al. 2007; Vallejo and González-Cózatl 2012).

Several members of the *P. truei* species group also contain multiple subspecies. Firstly, the saxicoline deermouse, *P. gratus*, includes four subspecies: *P. g. gratus* (from Jalisco to Tlaxcala), *P. g. erasmus* (only from type locality in Durango), *P. g. gentilis* (from New Mexico to Guanajuato), and *P. g. zapotecae* (from Tlaxcala to Oaxaca) (Pardiñas et al. 2017). In addition, based on cyt *b* sequences, it was reported that *P. g. zapotecae* may represent an independent species (genetic distances = 4.5%) (Durish et al. 2004). The sister taxon of *P. gratus* is the pinyon deermouse, *P. truei*, which includes 11 subspecies, nine of them found in the southwestern United States and Baja California Peninsula (*P. t. chlorus*, *P. t. dyselius*, *P. t. gilberti*, *P. t. lagunae*, *P. t. martirensis*, *P. t. montipinoris*, *P. t. nevadensis*, *P. t. preblei*, *P. t. sequoiensis*), and two in the south-central United States (*P. t. comanche*, *P. t. truei*) (Rogers et al. 2019). Durish et al. (2004) also reported a high mitochondrial divergence between these southwestern U.S./Baja and the south-central *P. truei* populations (4.5%), but the lack of variation in intron 7 of the fibrinogen beta chain suggested that they do not deserve specific status (Rogers et al. 2019).

The southern rock deermouse, *P. difficilis*, includes five subspecies: *P. d. difficilis* from the Sierra Madre Occidental into Guanajuato; *P. d. amplus* from the north and east Valley of Mexico; *P. d. felipensis*, with a fragmented range in the mountains surrounding the Valley of Mexico, and northeast of the city of Oaxaca; *P. d. petricola* from northern Sierra Madre Oriental; and *P. d. saxicola* from Querétaro and Hidalgo (Fernández et al. 2010; Pardiñas et al. 2017). Two studies found high levels of mitochondrial divergence and suggested that the northern populations of *P. d. difficilis* and *P. d. petricola* could represent an independent species (Durish et al. 2004; Fernández 2011). The sister species of *P. difficilis* is the northern rock deermouse, *P. nasutus*, which includes three subspecies: *P. n. nasutus* from Utah to Coahuila; *P. n. griseus* is known only from areas near the type locality in New Mexico; and *P. n. penicillatus* from along the border between Texas and Coahuila (Pardiñas et al. 2017). There is a lack of clear morphological differentiation between *P. difficilis* and *P. nasutus*, so their geographic boundaries are not clear (Durish et al. 2004; Robbins and Baker 1981).

Finally, the northern white-ankled deermouse, *P. laceianus* (south-center Oklahoma, south-east New Mexico, and Texas), was originally described as a subspecies, but based on high levels of genetic divergence with cyt *b* (5.9%) and morphological differences from *P. pectoralis*, it was elevated to a species (Bradley et al. 2015; Durish et al. 2004). The southern white-ankled deermouse, *P. pectoralis*, still includes three subspecies: *P. p. pectoralis* from Chihuahua to Jalisco, and from Guanajuato to Hidalgo; *P. p. collinus* from Tamaulipas to Hidalgo; and *P. p. zimmermani* from Coahuila to Hidalgo (Bradley et al. 2015). High levels of genetic divergence also suggested that *P. p. collinus* could represent an independent species, but a phylogeny constructed with 16 skin clips from *P. p. collinus* (average size of sequenced cyt *b* = 417 base pairs, range from 363 to 430 base pairs) did not supported its specific status (Bradley et al. 2015).

These studies have improved our understanding of the systematics and phylogenetic relationships in some members of the *P. truei* species group (Bradley et al. 2015; Carleton 1989; Durish et al. 2004; Fernández 2011; Osgood 1909; Rogers et al. 2019). Herein, we focus on taxa that possess high levels of intraspecific genetic diversity that still deserve attention. To assess their phylogenetic relationships, and to analyze if mitochondrial DNA supports past morphological and molecular hypotheses, we obtained complete cyt *b* sequences from new localities and subspecies from most members of the *P. truei* species group (the micro endemic *P. bullatus* is the only missing species). Fresh tissue samples from *P. d. felipensis* (from both Valley of Mexico and Oaxaca), and *P. p. collinus* were available for the first time, and we also increased the sampling in *P. difficilis*, *P. gratus*, *P. nasutus*, *P. pectoralis*, and *P. truei* (Figure 1).

2 Materials and methods

2.1 Molecular protocols

We used morphological traits defined by Álvarez-Castañeda et al. (2015) to verify the identity of analyzed samples. All samples came from specimens hosted in scientific collections: Universidad Nacional Autónoma de México, Facultad de Ciencias, Mexico City, Mexico (MZFC); Louisiana State University, Museum of Natural Science, Baton Rouge, USA (LSUMZ); Instituto Politécnico Nacional, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional unidad Durango, Durango, Mexico (CRD); University of New Mexico, Museum of Southwestern Biology, Albuquerque, USA (MSB). We sequenced individuals of *P. attwateri* ($n = 1$), *P. difficilis amplus* ($n = 4$), *P. d. saxicola* ($n = 2$), *P. d. felipensis* ($n = 3$), *P. gratus gentilis* ($n = 1$), *P. g. gratus* ($n = 1$), *P. g. zapotecae* ($n = 1$), *P. nasutus* ($n = 1$), *P. ochraventer* ($n = 3$), *P. pectoralis collinus* ($n = 4$), *P. p. pectoralis* ($n = 2$), *P. p. zimmermani* ($n = 2$), *P. truei nevadensis* ($n = 1$), *P t. truei* ($n = 1$), in addition to *Onychomys arenicola* ($n = 1$), *Habromys ixtlani* ($n = 1$), *P. aztecus* (*P. aztecus* species group; $n = 1$), and *Peromyscus schmidlyi* (*P. boylii* species group; $n = 1$) that were used as outgroups (Bradley et al. 2007; Durish et al. 2004). We followed the molecular protocols previously

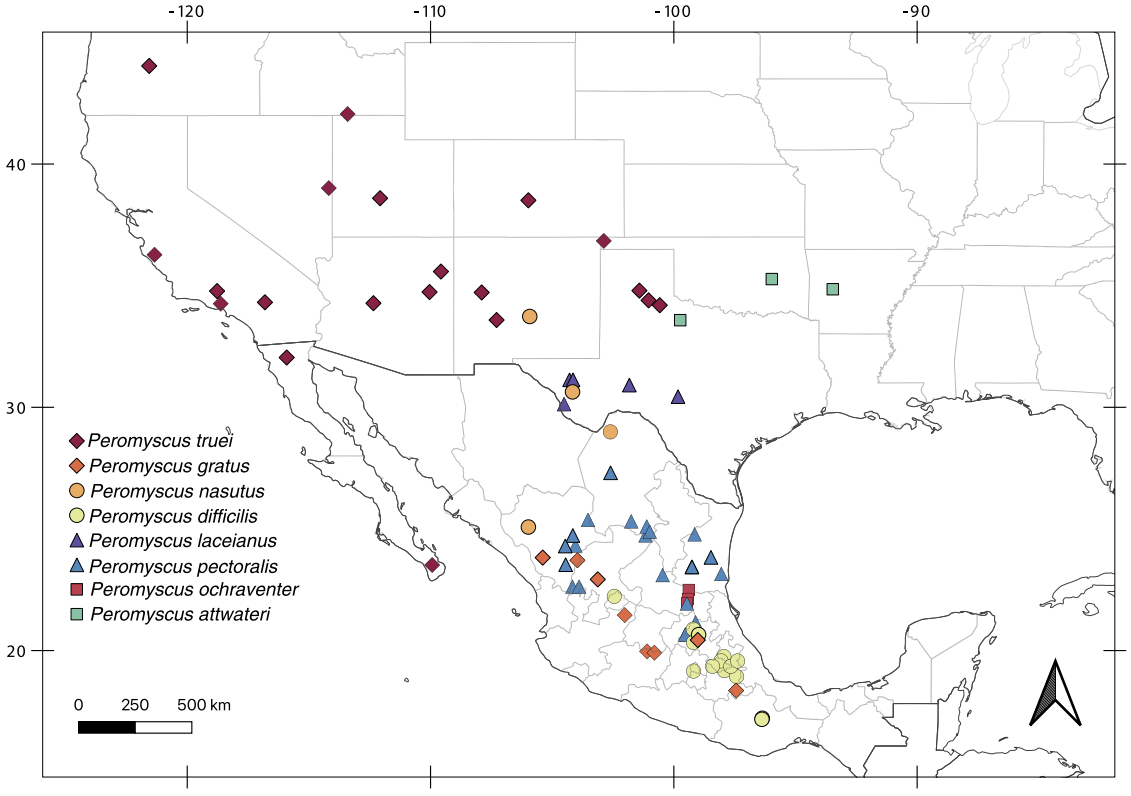

Figure 1: Map of Mexico and the United States showing the localities of *Peromyscus truei* species group samples analyzed in this work.

reported in Hernández-Canchola et al. (2021) to amplify and sequence cyt *b*. DNA sequences were edited and aligned using MEGA X (Kumar et al. 2018) and FINCHTV 1.4 (Patterson et al. 2004), and all sequences were visually inspected and aligned. We aligned our new data with selected sequences available in Genbank (Bradley et al. 2015; Durish et al. 2004; Rogers et al. 2019). Our final alignment included sequences generated from skin clips from two specimens (TTU38387 and TCWC29004), but the rest of the sequences were derived from tissue samples (Supplementary Alignment S1).

2.2 Phylogenetic relationships

We conducted maximum likelihood analyses (ML) and Bayesian inferences (BI) to construct the *P. truei* species group's phylogenetic relationships. In PARTITIONFINDER 2 (Lanfear et al. 2016), we selected the best substitution model and partition scheme (maximally divided by codon position) through an exhaustive search among all available models in MRBAYES 3.2 (Ronquist et al. 2012), using the Bayesian Information Criterion (BIC). We used this result for both ML and BI. In IQ-TREE 1.6.12 (Nguyen et al. 2015), we estimated the ML gene tree, with branch support estimated by 10,000 replicates of ultrafast bootstrap (Hoang et al. 2018). In MRBAYES 3.2 we used three hot chains and one cold chain in two independent runs of 10 million generations, sampling data every 1000 iterations. We checked for convergence of MCMC results by examining whether trace plots had leveled and ensuring that effective sample sizes >200 in TRACER 1.7 (Rambaut et al. 2018). The final topology was obtained using a majority-rule consensus tree after a burn-in of 25%.

Once we identified which of our samples were related to *P. p. collinus*, we removed the sequences TTU38387 and TCWC29004 from subsequent analyses because they represent short and highly divergent sequences obtained from skin-clips (Bradley et al. 2015).

2.3 Genetic distances

To examine genetic variation within the *P. truei* species group clades, we calculated intra-clade genetic distances in MEGA X (Kumar et al. 2018). Once we detected lineages with values ≤1.5% (there are no sister species in Neotominae rodents with lower genetic distances; Baker and Bradley 2006), we calculated genetic distances between these clades, and we drew a heat map using the ggplot2 R package (Wickham 2011). In both cases (intra and inter clades), we used the pairwise deletion option and the Kimura 2-parameter model (Kimura 1980) with the goal of continuity and comparability with previous works (Baker and Bradley 2006; Bradley et al. 2015; Durish et al. 2004; Rogers et al. 2019).

2.4 Haplotype networks and genetic diversity

To observe the genealogical relationships between sister species and to visualize their levels of intraspecific and interspecific differentiation, we constructed haplotype networks in POPART 1.7 (Leigh and

Bryant 2015) using the TCS algorithm (Clement et al. 2002). Finally, to describe their genetic diversity, in DnaSP 5.10 (Librado and Rozas 2009) we calculated the following genetic measurements: number of segregating sites (S), number of haplotypes (h), haplotype diversity (*Hd*), and nucleotide diversity (π).

3 Results

We sequenced 1143 base pairs of cyt *b* in 31 individuals (GenBank accession numbers OL631899-OL631929; Supplementary Table S1). With these and previously published sequences, we analyzed an alignment of 88 individuals that had 423 variable sites, 337 of which were parsimony informative.

3.1 Phylogenetic relationships and genetic distances

The best evolutionary model scheme was SYM+Γ, HKY + I, and GTR + I+Γ applied to the first, second, and third codon positions, respectively. Topologies from ML and BI were similar, but we observed minor differences between them (Figure 2). In the BI estimate the *P. truei* species group was monophyletic but this grouping was not statistically supported, whereas in the ML result it formed a polytomy with *P. aztecus* and *P. schmidlyi*. Within the *P. truei* species group we detected 18 clades with intraspecific genetic distances ≤1.32% (Figure 3), and most of the relationships between them were the same in both topologies.

In the BI tree we inferred three clades in *P. gratus* that correspond to the subspecies *P. g. gentilis* (*P. gratus* I), *P. g. gratus* (*P. gratus* II), and *P. g. zapotecae* (*P. gratus* III), but their relationships are not resolved (Figure 2). In the ML estimate, *P. g. gratus* and *P. g. zapotecae* were monophyletic, but *P. g. gentilis* was paraphyletic. The genetic distances between *P. g. gentilis* and *P. g. gratus* was 1.74%, whereas between *P. g. gentilis* and *P. g. zapotecae* was 4.48%, and between *P. g. gratus* and *P. g. zapotecae* was 4.18%. In *P. truei* we observed two clades that correspond to the south-central US populations (*P. truei* I: *P. t.*

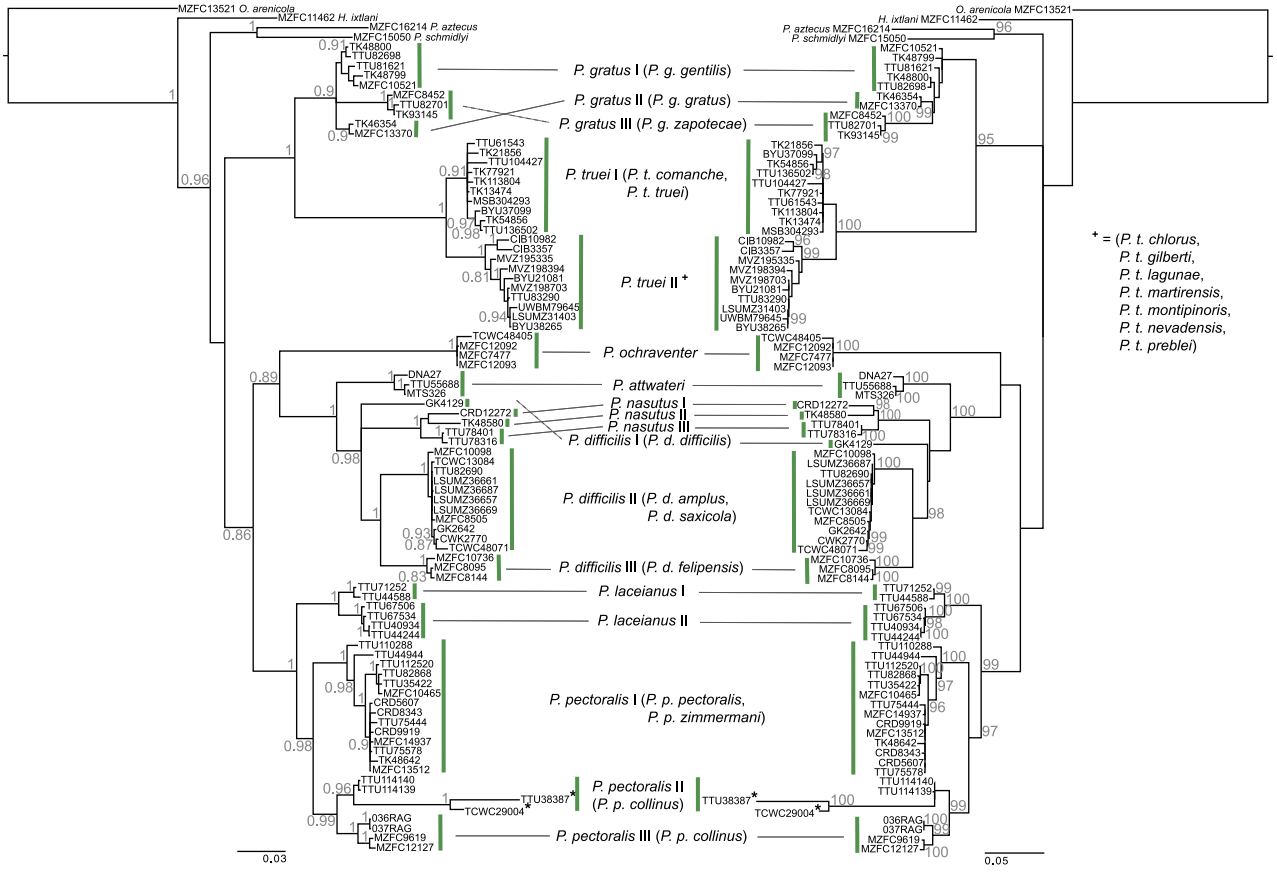

Figure 2: Phylogenetic relationships of members in the *Peromyscus truei* species group based on the mitochondrial cyt *b*. At the left the majority-rule consensus tree obtained from Bayesian analysis, and at the right the maximum-likelihood tree. Support values are shown as posterior probabilities and ultrafast bootstrap, respectively; values < 0.8/94 are not shown. Green bars indicate the 18 clades with intraspecific genetic distances ≤1.5, and the asterisk show short sequences obtained from skin-clips. Tip labels show the catalog number of each analyzed specimen.

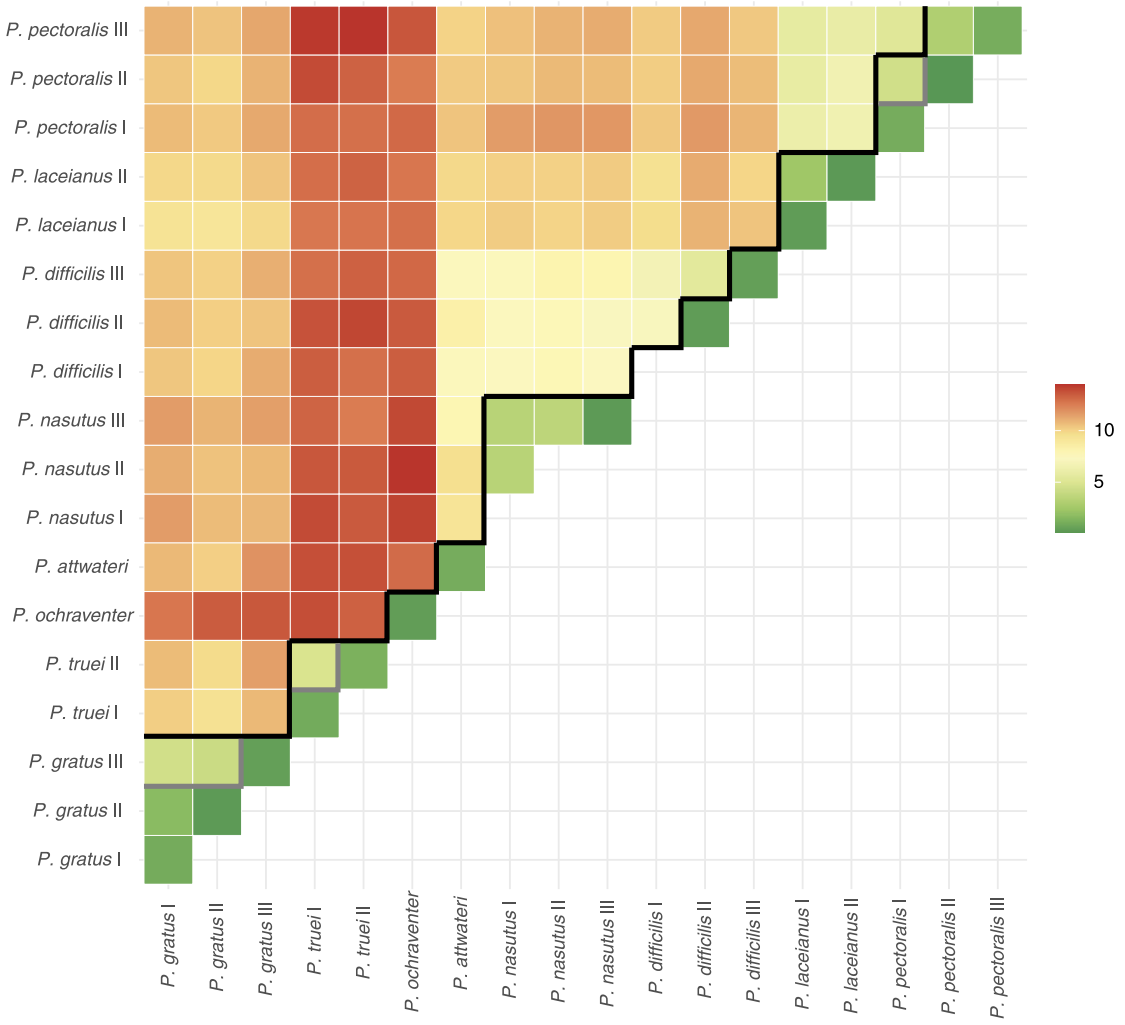

Figure 3: Heat map showing genetic distances (K80) as % between the 18 clades with intraspecific genetic distances ≤1.5 in the *Peromyscus truei* species group. Genetic distances >4% are shown above the gray line, and values >5% above the black line. Clade labels on the *x*- and *y*-axes match those from Figure 2.

comanche, and *P. t. truei*), and the southwestern US and Baja Californian populations (*P. truei* II: *P. t. chlorus*, *P. t. gilberti*, *P. t. lagunae*, *P. t. martirensis*, *P. t. montipinoris*, *P. t. nevadensis*, and *P. t. preblei*), which have a genetic distance of 4.87%. The sister relationship between *P. gratus* and *P. truei* was strongly supported by both ML and BI.

P. ochraventer and *P. attwateri* were related to *P. nasutus* and *P. difficilis* in both ML and BI, but the placement of *P. ochraventer* as sister to the other species received only modest statistical support (Figure 2). Within *P. nasutus* we detected three clades, *P. nasutus* I from Coahuila, Mexico; *P. nasutus* II from Durango, Mexico; and *P. nasutus* III with samples from the United States (New Mexico and Texas). The genetic distance between the Coahuilense and the Duranguense samples was 3.46%, between the Coahuilense and samples from the US was 3.46%, and between the Duranguense and the samples from the US was 3.62%. In *P. difficilis* we detected three clades that align with previously recognized subspecies: *P. d. difficilis* (*P. difficilis* I), *P. d. amplus* + *P. d. saxicola* (*P. difficilis* II), and *P. d. felipensis* (*P. difficilis* III). The sister relationship between *P. d. amplus* + *P. d. saxicola* and *P. d. felipensis* was strongly supported by ML and BI, but the phylogenetic position of *P. d. difficilis* remains enigmatic. In the ML topology *P. d. difficilis* was sister to all other *P. difficilis*, but this relationship was not supported; however, in the BI *P. d. difficilis* appears in a polytomy that includes *P. difficilis* II + *P. difficilis* III, and *P. nasutus*. The genetic distance between *P. d. difficilis* and all other subspecies of *P. difficilis* were > 6.69%, and between *P. d.*

amplus + *P. d. saxicola* and *P. d. felipensis* was 5.50%. The relationship between the *P. nasutus* and *P. difficilis* populations was supported only in the BI topology.

Within *P. laceianus* we detected two clades separated by a genetic distance of 2.44%, one located in central Texas (*P. laceianus* I), and the other one in southwestern Texas (*P. laceianus* II). Within *P. pectoralis* we observed three clades: the first one includes two samples previously classified as "*P. pectoralis* undetermined subspecies" (Bradley et al. 2015) from central and northern Tamaulipas (TTU 44,944 and TTU 110,288) in addition to the subspecies *P. p. pectoralis* and *P. p. zimmermani* (*P. pectoralis* I), the second clade includes two samples previously classified as "*P. pectoralis* undetermined subspecies" (Bradley et al. 2015) from southern Tamaulipas (TTU 114,139, TTU 114,140), and the skin clips samples from southern Tamaulipas and San Luis Potosí (*P. pectoralis* II, or *P. p. collinus* north), whereas the third clade includes the southern samples of *P. p. collinus* (*P. pectoralis* III). The genetic distances between *P. p. pectoralis* + *P. p. zimmermani* and *P. p. collinus* north was 4.46%, between *P. p. pectoralis* + *P. p. zimmermani* and *P. p. collinus* south was 5.28%, and between *P. p. collinus* north and south was 3.12%. The sister relationship between *P. laceianus* and *P. pectoralis* was strongly supported by both ML and BI topologies.

3.2 Haplotype networks and genetic diversity

Haplotype networks support the phylogenetic relationships observed in the ML and BI topologies. We detected divergent haplotype groups within *P. gratus*, *P. truei*, *P. nasutus*, *P. difficilis*, *P. laceianus*, and *P. pectoralis*, especially between clades with genetic distances >4% (Figure 4). For example, in the case of the sister species *P. gratus* and *P. truei*, the more similar haplotypes between *P. gratus* I and *P. gratus* II are differentiated by 9 mutational steps (genetic distance [g. d.] = 1.74%), but the more similar haplotypes between *P. gratus* III and all other *P. gratus* are separated by at least 22 mutational steps (g. d. > 4%); and the two groups in *P. truei* are differentiated by at least 28 mutations (g. d. = 4.87%). In the sister species *P. nasutus* and *P. difficilis*, more than 36 mutation steps were inferred between the three groups in *P. nasutus* (g. d. from 3.46 to 3.62%), but more than 70 mutational steps were detected between *P. difficilis* I and all other *P. difficilis* (g. d. > 6.69%), and more than 50 mutations between *P. difficilis* II and III (g. d. = 5.5%). Finally, in the sister species *P. laceianus* and *P. pectoralis*, 7 mutational steps were found between the most similar haplotypes of *P. laceianus* I and II (g. d. = 2.44%), but 15 mutations were observed between *P. pectoralis* I and III (g. d. = 5.28%), 10 mutations between both *P. pectoralis* I and II (g. d. = 4.46%) and II and III (g. d. = 3.12%). All genetic diversity indices (Table 1) corroborated the observations made with haplotype networks: the haplotype diversity was high, but within each grouping haplotypes were similar in general (low values of nucleotide diversity and segregating sites).

4 Discussion

After Carleton (1989) recognized *P. bullatus*, *P. difficilis*, *P. gratus*, *P. nasutus*, and *P. truei* as members of the *P. truei* species group, *P. attwateri* was soon added (DeWalt et al. 1993; Janecek 1990), but it was not the same for other species. Durish et al. (2004) suggested *P. pectoralis* and *P. laceianus* also belong in the *P. truei* species group, but this hypothesis was not subsequently supported, so they were not classified in any species group (Bradley et al. 2007). Even though *P. ochraventer* was considered as closely related to *P. attwateri*, *P. difficilis*, and *P. nasutus*, it was also classified as *incertae sedis* (Bradley et al. 2007). Sample sizes were limited to a maximum of two *P. pectoralis*, one *P. laceianus* and one *P. ochraventer* in these studies, but here we analyzed more specimens from more localities and that helped us determine that *P. laceianus*, *P. ochraventer*, and *P. pectoralis* belong in the *P. truei* species group.

Although mitochondrial DNA sequences are but one source of character data, mitochondrial genetic divergences are often consistent with species boundaries, hence their wide adoption as barcodes in mammals (Baker and Bradley 2006; Greenbaum et al. 2019; Hernández-Canchola and León-Paniagua 2021; Molinari et al. 2017; Shpirer et al. 2021). On the one hand, we detected high haplotypic diversity despite the similarity of haplotypes, and these parameter values are within the range of interspecific cyt *b* variation reported in other *Peromyscus* species (Hernández-Canchola et al. in press; León-Tapia et al. 2021; Pérez-Consuegra and Vázquez-Domínguez 2015). On the other hand, between sister *Peromyscus* species, genetic distances from 3.2% to 3.9% have been reported in the species groups *P. boylii* (Bradley et al. 2017), *Peromyscus maniculatus* (Bradley et al. 2019), *Peromyscus mexicanus* (Álvarez-Castañeda et al. 2019; Lorenzo et al. 2016), and in the subgenus *Haplomylomys* (Cornejo-Latorre et al. 2017). But a more conservative approach where interspecific distance values are close to 5% is more common (Baker and Bradley 2006; Bradley and Baker 2001). We detected high genetic distances between *P. truei* I and *P. truei* II; *P. gratus*

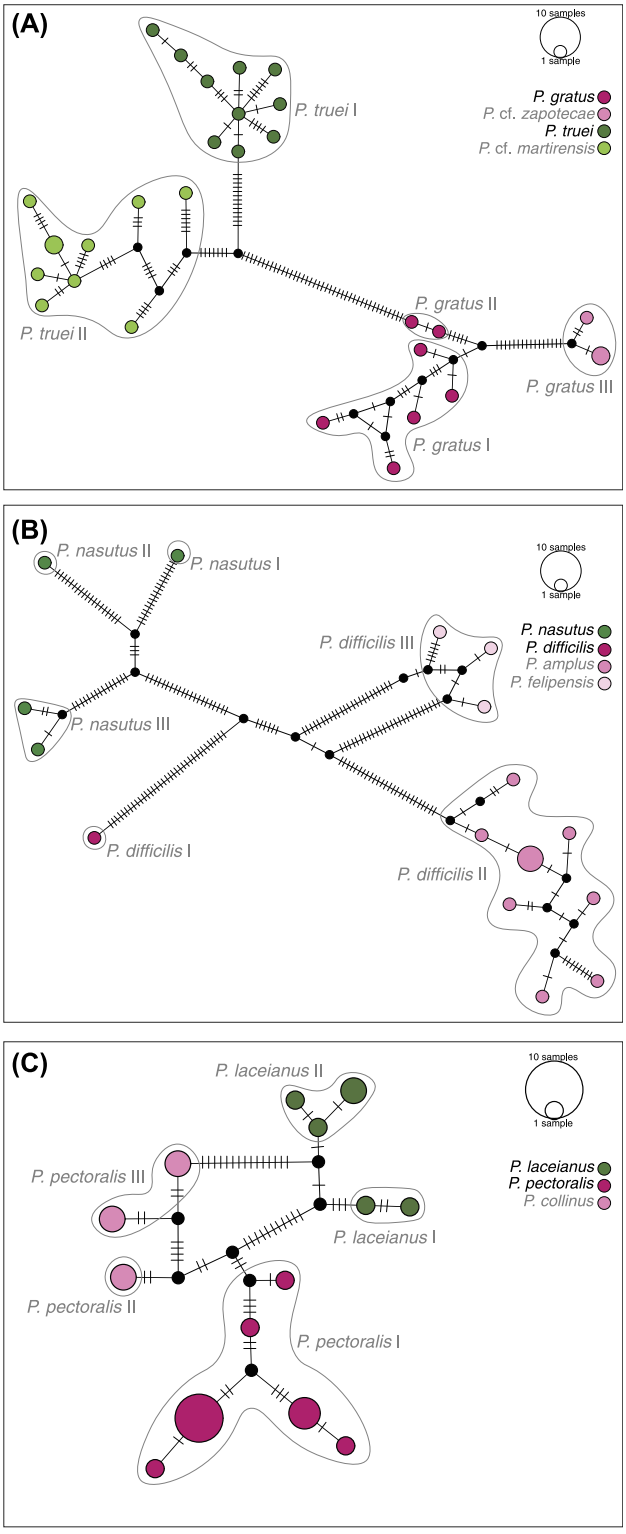

Figure 4: Haplotype networks based on the mitochondrial cyt *b* of sister species in the *Peromyscus truei* species group: (A) *P. gratus* (pink) + *P. truei* (green); (B) *P. nasutus* (green) + *P. difficilis* (pink); and (C) *P. laceianus* (green) + *P. pectoralis* (pink). In each case, lighter colors represent possible unrecognized taxa based on their high genetic divergence (see Figure 5 and discussion). The grey outlines show the 18 clades with intraspecific genetic distances ≤1.5, the circle size is proportional to the frequency of haplotypes, short perpendicular lines show mutational steps, and black dots represent missing haplotypes.

I + II and *P. gratus* III; *P. difficilis* I, *P. difficilis* II, and *P. difficilis* III; and between *P. pectoralis* I and *P. pectoralis* II + III. However, additional character types are needed to confidently determine their taxonomic status (Baker and Bradley 2006; Greenbaum et al. 2019), and to avoid under- or over-estimating the number of species (Dávalos and Russell 2014). Consistent with our results, previous studies in the *P. truei* species group showed morphological, allozymic, and/or karyological variation between the mitochondrial clades we detected (see below).

Within *P. gratus* we detected a genetic distance of 4.39% between the specimens of *P. g. gentilis* + *P. g. gratus* and *P. g. zapotecae* (Figure 3), two groups externally and cranially discernible (Hooper 1957). In addition, *P. g. zapotecae* has different X and Y chromosomal morphologies and G chromosomic band patterns than the other two subspecies (Martínez-Vázquez et al. 2020). On the other hand, it was reported that *P. g. gentilis* and *P. g. gratus* were monophyletic and sister to *P. g. zapotecae* in a mitochondrial gene tree (Durish et al. 2004), but we did not infer this relationship. A better sampling could improve our understanding about the phylogenetic relations and taxonomic status of *P.* cf. *gratus* (*P. gratus* I + II) and *P.* cf. *zapotecae* (*P. gratus* III). The clades *P. truei* I (*P. t. comanche*, *P. t. truei*) and *P. truei* II (*P. t. chlorus*, *P. t. dyselius*, *P. t. gilberti*, *P. t. lagunae*, *P. t. martirensis*, *P. t. montipinoris*, *P. t. nevadensis*, *P. t. preblei*, *P. t. sequoiensis*) are separated by a genetic distance of 4.87%, similar to values previously reported (Durish et al. 2004; Rogers et al. 2019). The close relationship between *P. t. comanche* and *P. t. truei* is backed with morphologic (Schmidly 1973) and karyologic (Modi and Lee 1984) data, but also the clades *P. truei* I and *P. truei* II are clearly morphologically discernible, except in the contact area, which hints at the possibility of secondary contact and hybridization (Rogers et al. 2019). Additionally, G-banded karyotypes in *P. t. preblei* (*P. truei* II) are similar to those from *P. t. comanche* and *P. t. truei* (*P. truei* I), except for the short arms in chromosomes 6 and X (Modi and Lee 1984). However, these two clades were not given specific recognition because the lack of variation in one nuclear locus (Rogers et al. 2019). Analyses of multiple nuclear loci are needed to clearly test the status of *P. truei* I (*P.* cf. *truei*) and *P. truei* II (*P.* cf. *martirensis*).

In *P. difficilis*, we detected three highly divergent clades (genetic distances from 5.5 to 7.09%): *P. d. difficilis* (*P. difficilis* I), *P. d. amplus* + *P. d. saxicola* (*P. difficilis* II) and

Table 1: Genetic diversity summary statistics for some members of the *Peromyscus truei* species group.

	n	*S*	*h*	Hd	SD (Hd)	π	SD (π)
P. truei	20	66	19	0.995	0.018	0.029	0.002
P. truei I	10	19	10	1	0.045	0.007	0.001
P. truei II	10	34	9	0.978	0.054	0.012	0.003
P. gratus	10	50	9	0.978	0.054	0.024	0.003
P. gratus I + II	7	26	7	1	0.076	0.013	0.002
P. gratus III	3	10	3	1	0.272	0.006	0.002
P. nasutus	4	59	4	1	0.177	0.029	0.007
P. difficilis	15	116	12	0.943	0.054	0.028	0.007
P. difficilis I	1						
P. difficilis II	11	21	8	0.891	0.092	0.005	0.001
P. difficilis III	3	10	3	1	0.272	0.006	0.002
P. laceianus	6	33	5	0.933	0.122	0.014	0.004
P. pectoralis	20	25	9	0.858	0.061	0.021	0.003
P. pectoralis I	14	13	7	0.802	0.094	0.010	0.002
P. pectoralis II + III	6	47	5	0.933	0.122	0.021	0.004

We include results for clades with genetic distances >4% in the mitochondrial cyt *b*. *n*, sample size; *S*, number of segregating sites; *h*, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; SD, standard deviation.

P. d. felipensis (*P. difficilis* III). The findings of Durish et al. (2004) with cyt *b* were supported in a dissertation based on two mitochondrial and two nuclear loci: two highly divergent clades were proposed as different species, *P. d. difficilis* + *P. d. petricola* as sister to *P. d. saxicola* + *P. d. amplus* + *P. d. felipensis* (Fernández 2011). However, we analyzed *P. d. felipensis* from both Oaxaca and Valley of Mexico and found that these animals represent a third divergent clade (Figure 2). Morphological studies pointed out that *P. d. felipensis* has the largest skull and the darkest coloration of all subspecies within *P. difficilis* (Hoffmeister and de la Torre 1961), possibly because it has been collected in rocky areas within coniferous forest or humid oak-pine forests, whereas all other subspecies have been mainly collected in rocky dry or semi-arid environments (Fernández et al. 2010). Protein electrophoresis analyses also showed that a *P. d. felipensis* from Morelos, Mexico was the least similar to other subspecies (Janecek 1990). Chromosomal fundamental numbers (FN) have served as important characters in cryptic *Peromyscus* species (Avise et al. 1979; Zimmerman et al. 1975), and the three *P. difficilis* clades also differ in these traits: reported values in *P. d. amplus* are FN = 66 (*P. difficilis* II), in *P. d. petricola* FN = 56 (*P. difficilis* I?), and in *P. d. felipensis* FN = 76 (*P. difficilis* III) (Arellano-Meneses et al. 2000; Müdespacher-Ziehl et al. 2005; Zimmerman et al. 1975). Based on morphology, coloration, allopatric ranges, ecology, allozymes, chromosomes, and mitochondrial and nuclear data, we recommend these three clades be recognized as the species *P. difficilis* (Allen 1891), *P. felipensis* Merriam 1898, and *P. amplus* Osgood 1904 (Figure 5).

Chromosomal differences and molecular data were crucial to validate "*P. difficilis*" (*P. amplus*, *P. difficilis*, *P. felipensis*) and *P. nasutus* as independent species (Avise et al. 1979; Durish et al. 2004; Tiemann-Boege et al. 2000; Zimmerman et al. 1975), but their morphological and allozymic similarity (Hoffmeister and de la Torre 1961; Janecek 1990) have obscured our understanding. Firstly, populations of "*P. difficilis*" from northern Sierra Madre Occidental possess cranial characteristics that approach those of *P. nasutus* (Hoffmeister and de la Torre 1961; Osgood 1909), and from the central Sierra Madre Occidental researchers have reported "*P. difficilis*" with chromosomal rearrangements and haplotypes like *P. nasutus* (Durish et al. 2004; Robbins and Baker 1981), so we agree with the previous hypothesis that suggested that *P. nasutus* ranges along the Sierra Madre Occidental into northwestern Mexico (Figure 5) (Durish et al. 2004; Fernández 2011). Secondly, it was reported that *P. amplus amplus* and *P. a. saxicola* were sister to *P. nasutus* and all of them were sister to *P. difficilis*, but these relationships were not supported (Durish et al. 2004). We constructed two phylogenetic trees and obtained an unsupported topology that placed to *P. nasutus* as sister to *P. amplus*, *P. difficilis*, and *P. felipensis* (ML), and a supported polytomy between *P. nasutus* and *P. amplus*, *P. difficilis*, and *P. felipensis* (BI), so their mitochondrial relationships are not clear yet. Thirdly, we found three *P. nasutus* clades with genetic distances of ca. 3.5%, similar to values reported in other sister *Peromyscus* species (see above). Until additional forms of character data are available, we consider this diversity to be phylogeographic structure. Although the

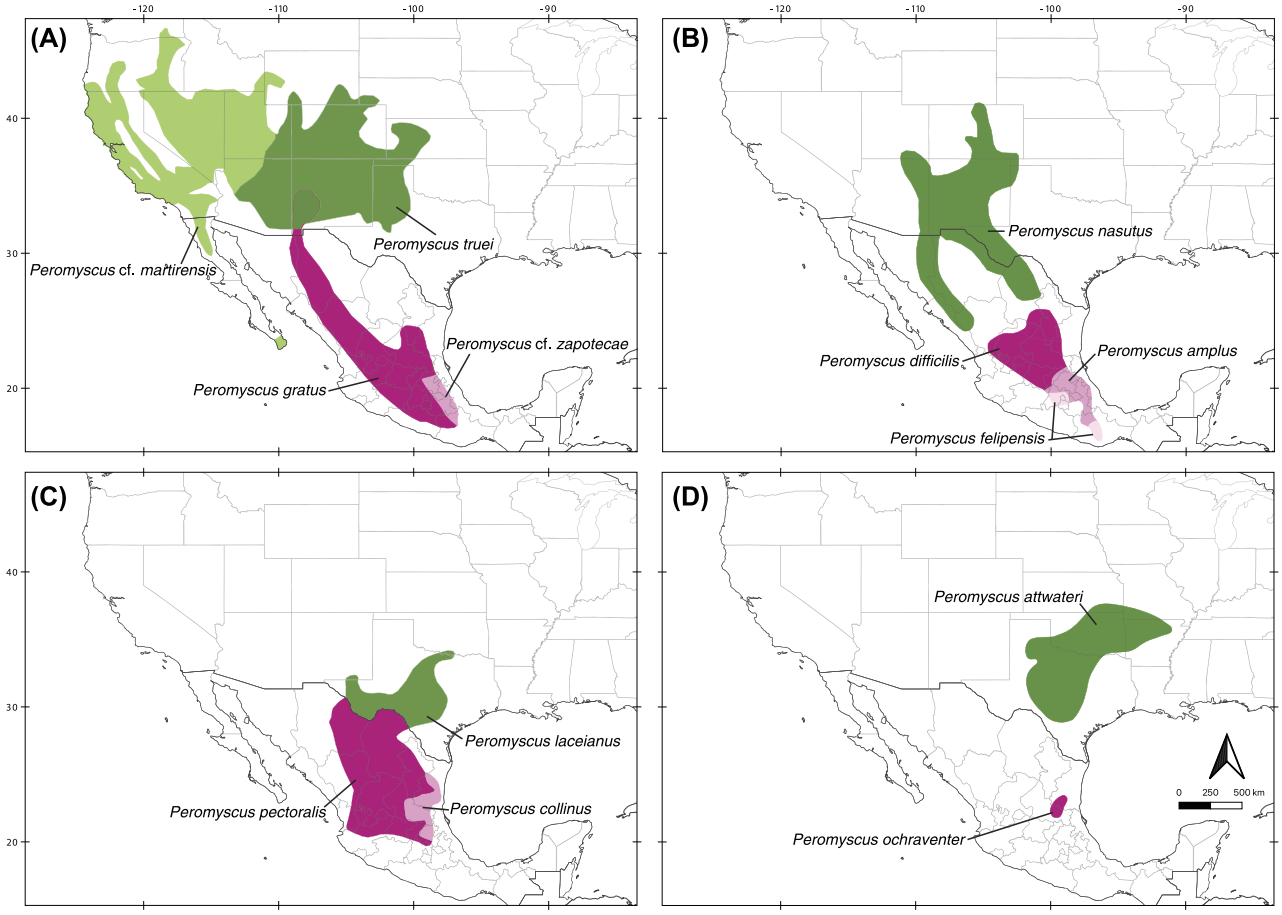

Figure 5: Geographic ranges of members of the *Peromyscus truei* species group showing taxonomic changes proposed in this work: (A) *P. gratus* (pink) and *P. truei* (green); (B) *P. nasutus* (green) and *P. difficilis* (pink); (C) *P. laceianus* (green) and *P. pectoralis* (pink); (D) *P. attwateri* (green) and *P. ochraventer* (pink). Lighter colors represent possible unrecognized taxa: we suggest recognizing the species *P. amplus*, *P. collinus*, and *P. felipensis* because their high mitochondrial divergence and its consistency with multiple lines of evidence previously reported; however, the specific status of the highly divergent *P.* cf. *martirensis* and *P.* cf. *zapotecae* should be tested with additional data. Maps modified from the IUCN.

Coahuilan *P. nasutus* is geographically closer to samples from the United States, it was genetically related to the Duranguense specimen. The addition of more specimens and loci could test if the Bravo River (between the US and Mexican samples) and the Chihuahuan desert province (between the Coahuilense and the Duranguense samples) are biogeographic barriers in these mice.

In a revision of *P. pectoralis*, four individuals from Tamaulipas, Mexico were more closely related to *P. pectoralis* than to *P. laceianus*, but based on morphological and molecular data they could not be classified in any subspecies (Bradley et al. 2015). These results warrant some caution because the genetic data from *P. p. collinus* were short sequences of cyt *b* amplified from skin clips. Here, for the first time we analyzed long sequences from fresh tissue samples of *P. p collinus*, and we estimated relationships that verified the identification of our *P. p. collinus*, and placed unassignable specimens from central and northern Tamaulipas in *P. pectoralis* I and from southern Tamaulipas in *P. pectoralis* II. The high genetic distances (~5%) detected between the clades *P. pectoralis* I (*P. p. pectoralis*, and *P. p. zimmermani*) and *P. pectoralis* II + III (*P. p. collinus*), their allopatric ranges, morphological variation (Bradley et al. 2015; Schmidly 1972), and allozyme variation (Kilpatrick and Zimmerman 1976), support specific recognition as *P. pectoralis* Osgood 1904 and *Peromyscus collinus* Hooper 1952, respectively (Figure 5). We also detected some phylogeographical structure, so more samples could be used to test whether the Pecos River between clades *P. laceianus* I and II, and some geographic features in the Sierra Madre Oriental between clades *P. pectoralis* II and III, could be promoting differentiation.

With our increased sampling, we verified earlier findings about mitochondrially divergent populations in the *P. truei* species group (Bradley et al. 2015; Durish et al. 2004; Fernández 2011; Rogers et al. 2019), but we also detected new clades that may warrant specific recognition. However, species delimitation using only mitochondrial sequences can be biased by female philopatry, a common trait in many mammal species (Dávalos and Russell 2014). Besides, mtDNA is just one non-recombining locus with reduced effective population size; as such, it properties can limit its utility in systematic studies (Rubinoff et al. 2006). Nevertheless, mitochondrial sequences are often useful in taxa where other character data are available, to confirm earlier hypotheses and generate new ones (Rubinoff et al. 2006). That is why we supported our mitochondrial and geographic conclusions by examining other evidence from previous studies of nuclear loci, allozymes, chromosomes, morphology, and ecological data. Based on these different lines of evidence, two groups exist within the *P. truei* species group (Tiemann-Boege et al. 2000): the first includes *P. gratus*, *P. truei*, and possibly *P.* cf. *zapotecae* and *P.* cf. *martirensis*, respectively. The second contains *P. amplus*, *P. attwateri*, *P. collinus*, *P. difficilis*, *P. felipensis*, *P. laceianus*, *P. nasutus*, *P. ochraventer*, and *P. pectoralis* (Figure 5), but placement of *P. bullatus* will likely remain unknown until genetic data are available. Further systematic analyses of the *P. truei* species group should be conducted to verify our taxonomic conclusions, but also phylogeographical analyses are desirable to analyze whether the Sierra Madre Oriental promoted the differentiation of multiple endemic clades (*P. amplus*, *P. collinus*, *P. ochraventer*, and *P.* cf. *zapotecae*), as has been reported in other *Peromyscus* species (Bradley et al. 2017; Cruz-Gómez et al. 2021; Hernández-Canchola et al. in press). Further research will be crucial to understand the number of *Peromyscus* species, their phylogenetic relationships, and the processes that generated their high diversity in the Nearctic region.

4.1 Taxonomy

Based on our results and those from Hoffmeister and de la Torre (1961), Schmidly (1972), Zimmerman et al. (1975), Kilpatrick and Zimmerman (1976), Janecek (1990), Arellano-Meneses et al. (2000), Durish et al. (2004), Müdespacher-Ziehl et al. (2005), Fernández (2010, 2011), and Bradley et al. (2015) we suggest recognizing next subspecies as valid species:

4.1.1 *Peromyscus amplus* Osgood 1904

P. amplus Osgood 1904

P. difficilis amplus Osgood 1909

P. difficilis saxicola Hoffmeister and de la Torre 1959

Holotype: USNM 70,158, adult female collected 12 November 1894, skin and skull.

Type locality: "Coixtlahuaca, Oaxaca, Mexico".

Characters and comparison: Compared with *P. felipensis*, *P. amplus* is paler with long and soft pelage and it has a higher and more inflated braincase (Osgood 1904). Compared with *P. difficilis*, *P. amplus* is larger and has dull and reddish pelage (Fernández et al. 2010; Hoffmeister and de la Torre 1961). These morphological differences between allopatric populations are supported by mitochondrial and nuclear sequences (Durish et al. 2004; Fernández 2011; this study), and specific chromosomal fundamental numbers (FN = 66 in *P. amplus*) (Arellano-Meneses et al. 2000).

Remarks: For detailed descriptions of morphological differences between *P. amplus* and its related species, see Hoffmeister and de la Torre (1961) and Fernández et al. (2010).

4.1.2 *Peromyscus felipensis* Merriam 1898

P. felipensis Merriam 1898

P. difficilis felipensis Osgood 1909

Holotype: USNM 68,409, adult male collected 22 August 1894, skin and skull.

Type locality: "Cerro San Felipe, Oaxaca, Mexico (alt. 10,200 ft)".

Characters and comparison: Compared with *P. difficilis*, *P. felipensis* is larger and darker; has smaller ears and coarser pelage; and has a larger skull and heavier rostrum (Merriam 1898). Compared with *P. amplus*, *P. felipensis* has a larger skull and is much darker (Hoffmeister and de la Torre 1961; Fernández et al. 2010). These morphological differences between allopatric populations are consistent with results from analyses of protein electrophoresis (Janecek 1990), mitochondrial and nuclear sequences (Durish et al. 2004; Fernández 2011; this study), and specific chromosomal fundamental numbers (FN = 76 in *P. felipensis*) (Müdespacher-Ziehl et al. 2005).

Remarks: For detailed descriptions of morphological differences between *P. amplus* and its related species, see Hoffmeister and de la Torre (1961) and Fernández et al. (2010). *P. felipensis* inhabits coniferous and humid oak-pine forests, whereas its sister species has been collected mainly in semi-arid environments (Fernández et al. 2010).

4.1.3 *Peromyscus collinus* Hooper 1952

P. pectoralis collinus Hooper 1952

Holotype: UMMZ 61,310, adult male collected 11 July 1930, skin and skull.

Type locality: "México, Tamaulipas, Sierra San Carlos, 12 miles northwest of San Carlos, San José, 2000 feet elevation".

Characters and comparison: Compared with *P. pectoralis*, *P. collinus* has larger external measurements; larger cranium; broader rostrum, maxillary bone, and interorbital region; larger palatine foramen, molar toothrow, and auditory bullae; darker and reddish upper parts, and darker tarsi. Compared with *P. laceianus*, *P. collinus* has larger total length, tail, hind foot, and ear; braincase narrower and longer; larger auditory bullae; darker and reddish upper parts; and darker tarsi (Hooper 1952; Schmidlyi 1972). These morphological differences between allopatric populations are consistent with mitochondrial data (this study), and allozyme variation (Kilpatrick and Zimmerman 1976).

Remarks: For detailed descriptions of morphological differences between *P. collinus* and its related species, see Schmidlyi (1972) and Bradley et al. (2015).

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