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A first species-wide phylogenetic analysis of small mammals from Costa Rica using mitochondrial cytochrome *b*

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Abstract

Costa Rica is within the Mesoamerican biodiversity hotspot and has about 53 native species of small mammals. This high diversity, along with recent records of new species and indications of cryptic genetic diversity, suggest that application of the DNA barcoding approach would be worthwhile. Here we used 131 tissue samples of small mammals from multiple localities in Costa Rica and sequenced the complete mitochondrial cytochrome *b* (1140 bp). These samples represented 17 recognized species and two taxa of uncertain status. The new sequence data were supplemented with previously published data from INSDC. Our phylogenetic analyses are consistent with and extend upon recent revisions in *Heteromys*, *Peromyscus* and *Reithrodontomys* and suggest possible new cryptic forms within what are currently named *Melanomys chrysomelas*, *Nyctomys sumichrasti* and *Proechimys semispinosus.* The previously named "*Heteromys* sp" is indeed likely a new species requiring a full taxonomic description. Moreover, we found new localities for previously described species substantiating recent taxonomic surveys and field guides for the small mammals of Costa Rica. To confirm the presence of cryptic species and major genetic forms in *Heteromys*, *Peromyscus*, *Reithrodontomys*, *Melanomys*, *Nyctomys* and *Proechimys* there needs to be greater sampling, additional genetic markers, morphometrics and other studies. *Scotinomys* also shows interesting phylogenetic subdivision, requiring further investigation.

Keywords DNA barcoding · Phylogenetics · Taxonomy · Mitochodrial DNA · Conservation genetics

Introduction

Even for a well-studied group like mammals, there are regions of the world where there is incomplete knowledge of the range of species and the major genetic forms within them. Addressing situations like this, the DNA barcoding

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approach developed by Hebert et al. (2003) exploits phylogenetics of simple mitochondrial markers to uncover previously undescribed diversity. For mammals, cytochrome *b* (cyt*b*) is the mitochondrial marker of choice (Tobe et al. 2009). Here we used a barcoding approach with cyt*b* to learn more about mammal diversity in a small but particular country, Costa Rica.

The need to reveal previously undescribed diversity is particularly important in areas already known to be rich in species – which typically are in understudied tropical regions at high risk of habitat destruction and species loss (Bradshaw et al. 2009) including the tropical biodiversity hotspots inferred by Myers et al. (2000). The Mesoamerican hotspot that includes Costa Rica and Panamá, plus nearby islands, represent one of these high diversity regions. It is one of the most physically and biologically complex areas on the planet, with among the highest levels of biodiversity per km^2 worldwide (Reid and Miller 1989; Bagley and Johnson 2014) and yet only covering ~0.09% (127,050 km^2) of global land area (Bagley and Johnson 2014). A considerable proportion of this region (36%) is a tropical forest biome, incorporating vegetation zones ranging from lowland wet and dry forests to mangrove estuaries, rolling savannahs and grasslands, and once-pristine montane habitats (Marshall 2007). The mainland forms a long (~1170 km), narrow isthmus that is ~240 km wide across Costa Rica but only 65 km wide at the Panama Canal basin. The Talamanca Cordillera mountain range reaches its highest peak at 3820 m in Chirripó Hill (Costa Rica), creating sky-islands of isolated montane habitat (Bagley and Johnson 2014). This mountainous area has the largest forest cover in the country, with the greatest number of life zones and the most extensive national park (~404,500 ha; La Amistad International Park) (Rodríguez-Herrera et al. 2014). Costa Rica has a diversity of mammals comparable to much larger countries (such as Mexico and USA) reflecting both its geographical position within the tropics and a location on a dynamic dispersal route between two continents (Cody et al. 2010; Wilson et al. 2014).

When considering mammalian diversity with an eye to conservation, small mammals (i.e. species less than 500 g: https://www.neonscience.org/data-collection/small-mammals) are particularly understudied and undervalued (Fisher 2011). This is unfortunate given their merit as study systems of ecological and evolutionary significance and conservation value (e.g. Rodríguez-Estival and Smits 2016; Pardini et al. 2005; Jumeau et al. 2017; Sullivan et al. 2012). Studies of their phylogeny (e.g. Jaarola et al. 2004; Jansa and Weksler 2004; Piaggio et al. 2013) and phylogeography (e.g. Jaarola and Searle 2002; Tougard et al. 2013; Barbosa et al. 2017), highlight the importance of molecular tools for understanding their evolutionary history and conservation. There are indications that cryptic diversity is common in small mammals (e.g. Lecompte et al. 2005; Paupério et al. 2012; Demos et al. 2014; Rivera et al. 2018).

Following Reid (2009), Reid and Gómez Zamora (2022) and Ramírez-Fernández et al. (2023), we consider 53 native species of small mammals in Costa Rica, from three orders: Didelphimorphia, Eulipotyphla and Rodentia (Table 1). Small mammals are therefore not a monophyletic group but they do constitute an ecological guild (Simberloff and Dayan 1991). In this paper we are considering the small mammals of Costa Rica that spend sufficient time on the ground to be caught in live traps there (the live traps being intended for individuals up to 500 g). We exclude the single carnivoran under 500 g in Costa Rica (*Neogale frenata*) on the grounds that a single species of a major group had little value for a

Table 1 Numbers of species of small mammals per genus in Costa Rica following Rodríguez-Herrera et al. (2014) and Ramírez-Fernández et al. (2023). See text for further explanation

Order	Family	Genera	Number of species
Didelphimorphia	Didelphidae	*Caluromys*	1
		Marmosa	4
		Metachirus	1
		Philander	1
		Didelphis	2
Eulipotyphla	Soricidae	*Cryptotis*	5
Rodentia	Heteromyidae	*Heteromys*	4
	Cricetidae	*Handleyomys*	1
		Icthyomys	1
		Melanomys	1
		Nephelomys	1
		Nyctomys	1
		Oecomys	1
		Oligoryzomys	2
		Oryzomys	1
		Ototylomys	1
		Peromyscus	2
		Reithrodontomys	9
		Rheomys	2
		Scotinomys	2
		Sigmodon	1
		Sigmodontomys	1
		Tanyuromys	1
		Transandinomys	2
		Tylomys	1
		Zygodontomys	1
	Echimyidae	*Diplomys*	1
		Hoplomys	1
		Proechimys	1
		Total	53

phylogenetic study such as ours. We include *Didelphis*, even though adults are larger than 500 g, because small (presumably immature) individuals are part of the guild that we consider. Also, their inclusion provides phylogenetic completeness for the Didelphidae. Among the small mammals under investigation, rodents are more diverse than other groups, with the 39 species distributed among three families and 23 genera (Table 1). The derivation of the Costa Rican fauna from both North and South America (Cody et al. 2010) can be seen in the small mammals we are considering (Table 1), with the didelphids and the echimyids showing ancestry from South America and all the other taxa deriving from North America.

Furthermore, there are six species of endemic small mammals within the political boundaries of Costa Rica, five rodents (*Heteromys oresterus*, *Heteromys nubicolens*, *Reithrodontomys cherrii*, *Reithrodontomys musseri*, *Reithrodontomys rodriguezi*) and one soricid (*Cryptotis monteverdensis*). This number increases to 15 species when considering the geographical region of the highlands of the Talamanca Mountain range in Costa Rica and the Chiriquí zone in the west of Panamá. In the extreme north of the country, four other species are only shared with Nicaragua: *Marmosa nicaraguae, Peromyscus nicaraguae*, *Reithrodontomys brevirostris* and *Reithrodontomys paradoxus*, increasing the total number of endemic species to 19. Of these, 14 are distributed in high altitudinal areas (above 1500 m) and mainly in the Talamanca Mountain range (Rodríguez-Herrera et al. 2014).

Our understanding of the diversity of small mammals of Central America is based on a mixture of traditional taxonomy and molecular phylogenetic studies, particularly using cyt*b*. The objective of this paper is to build on that knowledge, with an intensive study of the molecular phylogenetics of small mammals of Costa Rica. Given their high diversity and substantial endemism, that is particularly needed. With some exceptions (e.g. the study of *Heteromys* by Rogers and Vance 2005; Rogers and González 2010), Costa Rica has generally not been a specific target for the study of small mammals, and the existing knowledge derives mainly from broader studies of Central America. Therefore, we have conducted a major effort to collect tissue samples of small mammals from localities throughout Costa Rica (Fig. 1) to obtain complete cyt*b* sequences. These sequence data were subjected to a species-wide phylogenetic analysis, following the DNA barcoding approach (Hebert et al. 2003). Our rationale was to establish whether the genetic subdivisions that we find in this phylogenetic analysis mirror the current taxonomy, or whether there are undescribed genetic lineages (new major genetic forms within species or new species).

Fig. 1 Relief map of the provinces of Costa Rica and sampling localities within them (Table S1). LAIP: La Amistad International Park (LAIP-EA, LAIP-PT, LAIP-VS); SRNP: Santa Rosa National Park; CERS: Cuatro Esquinas Ranger Station; AFRS: Aguas Frías Ranger Station; BCNP: Braulio Carrillo National Park; MANP: Manuel Antonio National Park. Map adapted from: https://es.m.wikipedia.org/wiki/Archivo:Costa_Rica_relief_location_map.jpg

One rodent genus stands out as particularly species-rich: *Reithrodontomys* (Table 1). We subjected that genus to a separate analysis, to establish whether cyt*b* could confirm already described species boundaries and to investigate if there is even greater species level diversity in Costa Rica and surrounding areas.

Materials and methods

Sampling and DNA extraction

A total of 131 individuals (Table S1), representative of the small mammals of Costa Rica, were sampled from 10 localities using live traps in 2017 (Fig. 1). We obtained samples from 17 recognized species and two taxa of uncertain status (indicated as 'sp') (Table S1). Individuals were identified using Reid (2009) and by reference to the scientific literature available at the time of fieldwork. Tissue samples were taken under anaesthetic with an ear punch and the animals were subsequently released. DNA extraction was performed using the ExtractMe Genomic DNA 96-Well kit (DNA GDAŃSK).

Amplification and sequencing

The complete cytochrome *b* gene (cyt*b*, 1143 bp) was amplified in all samples. Each 10 µL polymerase chain reaction (PCR) included 5 µL of Qiagen© PCR Multiplex Kit Master Mix (Qiagen, Hilden, Germany), 0.4 µL of each primer (10 µM) and 1 µL of genomic DNA. Details of primers are given in Table S2. A standard protocol was followed for thermal cycling: 15 min at 95 °C, 45 s at 95 °C, 45 s at the annealing temperature, and 1 min at 72 °C, plus 5 min at 60 °C for a total of 35 cycles. The combination of primer pair and annealing temperature differ between genera (Table S3).

The product obtained by PCR was purified using the ExoSAP-IT® PCR clean-up Kit (GE Healthcare, Piscataway, NJ, USA) and sequences were generated with the amplification primers. Cycle sequencing reactions were carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) or by Genewiz Inc. (USA). Forward and reverse sequences were obtained on a 3130XL automated sequencer (Applied Biosystems, USA) and were assembled and edited in Geneious version 8.1.9.

Data analysis

The sequences we generated were analysed together with 129 cyt*b* sequences from INSDC (International Nucleotide Sequence Database Collaboration) incorporating all the taxa represented by our new sequences (although there were no INSDC sequences classified as '*Reithrodontomys* sp'), plus an additional 24 species of small mammals (Table S4). This dataset was used for our 'all-species analysis'. For the '*Reithrodontomys* specific analysis', there were 20 additional INSDC sequences included, with three extra species represented (Table S5). In essence, the all-species analysis included all species of small mammal from Costa Rica where INSDC sequences were available, while for the *Reithrodontomys* specific analysis we included all species of *Reithrodontomys* from Central America represented by INSDC sequences.

To make the phylogenetic tree for the all-species analysis more manageable, we reduced the number of our own *Reithrodontomys* and *Scotinomys* sequences to no more than five per species or major genetic lineage, maximising the geographic spread (these particularly well-represented genera are considered fully in separate analyses: *Reithrodontomys* in this work and *Scotinomys* in González et al. in prep.). For every species in the all-species analysis, we had no more than five INSDC sequences, preferentially selecting sequences from Costa Rica or Central America. Platypus (*Ornithorhynchus anatinus*) and echidna (*Tachyglossus aculeatus*) were included as outgroup species.

For the *Reithrodontomys* analysis, we incorporated all sequences that we generated for this genus. INSDC sequences were treated in a similar way as for the all-species analysis, except that all species of *Reithrodontomys* with a distribution that includes at least one of the seven Central American countries were included. Once again, for every species of *Reithrodontomys* considered, up to five INSDC sequences were included according to availability. *Peromyscus nudipes* and *Microtus agrestis* were added as outgroup sequences.

The cytochrome *b* sequences were aligned in both the all-species and *Reithrodontomys* datasets with Clustal Omega v1.2.2 (Madeira et al. 2022) under default parameters. Alignments were examined by eye and trimmed to 1140 bp with the subseq function in SeqKit v2.4.0 (Shen et al. 2016) to remove trailing gaps.

Maximum likelihood trees were inferred using IQ-TREE 2 v2.2.0 (Minh et al. 2020) using default search parameters. Our alignment was partitioned by codon position (1st + 2nd and 3rd) with independent rates (Chernomor et al. 2016) and the best-fit substitution model for each was determined using ModelFinder (Kalyaanamoorthy et al. 2017). Branch support was assessed using ultrafast bootstrap approximation (Hoang et al. 2018) and the SH-like approximate likelihood ratio test, each with 1000 replicates.

In addition, Bayesian inference of phylogenies was performed with MrBayes v3.2.7 (Ronquist et al. 2012), using rjMCMC to sample across a GTR + I + gamma substitution model. We ran four independent chains for 10 million generations each, sampling every 1000 generations and with a burn-in of 25%. However, given that Bayesian inference

revealed the same branching patterns as maximum likelihood both within species and among closely related species, we only report the maximum likelihood results here.

Results

All-species analysis

Of the 17 recognized species that we sampled in this study, the following provided the first complete cyt*b* sequences from Costa Rica: *Didelphis marsupialis*, *Nyctomys sumichrasti*, *Oryzomys couesi*, *Peromyscus nudipes*, *Philander melanurus, Proechimys semispinosus*, *Scotinomys teguina*, *Sigmodon hirsutus*. In those instances where complete cyt*b* sequences were already available for Costa Rica, our data often provided new locations within the country.

Combining our new data with previously published sequences in INSDC, we generated a cyt*b* phylogeny using IQ-TREE (Fig. S1). The general features of the phylogeny were as expected, with placentals and marsupials each forming monophyletic groups sister to each other, although the support for the placental grouping is weak. Within the placentals, the Eulipotyphla and the three families of rodents (Cricetidae, Echimyidae, Heteromyidae: Table 1) all form well-supported monophyletic groups. The relative positioning of these four groups is unresolved, so that Rodentia do not form a monophyletic group. This may be a consequence of long-branch attraction (Bergsten 2005).

Nearly all the 41 named species analysed form monophyletic groups within the tree. Nevertheless, some are paraphyletic, or have an otherwise unclear relationship, namely: *Peromyscus nudipes/nicaraguae*, *Scotinomys xerampelinus* and *Transandinomys talamancae*. *Heteromys* sp shows features that sets it apart from named species, forming its own clade. Interpreting the results with *Reithrodontomys* sp is more complex. Sequences classified as *Reithrodontomys* sp mostly occupy a large independent clade. However, there is one sequence that is positioned separately with the *Reithrodontomys brevirostris* sequence as its closest relative.

The following list considers each species in turn, with reference to location data in Tables S1 and S4 and Fig. 1, and referring to the phylogenetic structure in Fig. S1 (with highlighted branches in Fig. 2):

Caluromys derbianus (Fig. 2D): There are no new sequences and no phylogenetic structure with the sequences from Costa Rica, Panamá and Ecuador.

Cryptotis (3 species) (Fig. 2C): These three species are represented by four sequences, clustered together. We contributed a new sequence of *C. nigrescens* from BCNP in the province of Heredia, while the previous sequence was also from Costa Rica but from a different province (Puntarenas). They cluster together with long branch lengths.

Didelphis marsupialis (Fig. 2D): Our three sequences from Costa Rica, from close to the border with Panamá, add to previous sequences from Panamá, Brazil and Mexico. There is little structure but our sequences cluster most closely with those from Panamá while those from Mexico are most distinctive.

Didelphis virginiana (Fig. 2D): There are no new sequences, and minor differentiation between the sequences from Mexico and the United States.

Diplomys labilis (Fig. 2C): There are no new sequences, just one existing sequence from Panamá.

Handleyomys alfaroi (Fig. 2A): Our new sequence from Costa Rica (LAIP-PT, Puntarenas) clusters with another sequence from Puntarenas and a sequence from Panamá. The sequences from Honduras, Guatemala and Nicaragua are marginally separate, but there is very little discernible subdivision.

Heteromys desmarestianus (Fig. 2C): Our six sequences from coastal central Costa Rica (MANP, Puntarenas) cluster closely with a previous sequence from Puntarenas and a sequence from Cartago (inland central Costa Rica). A sequence from another more northern Costa Rican province (Alajuela) is more distantly related. More distantly related still are two sequences from Honduras (one was ours), which cluster together, and a sequence from Mexico. Thus, there is some subdivision in *H. desmarestianus* over a wide geographic area.

Heteromys nubicolens (Fig. 2C): There are no new sequences, and the two sequences previously obtained (from Guanacaste and Puntarenas in Costa Rica) are very similar.

Heteromys oresterus (Fig. 2C): There are no new sequences, and the five sequences previously obtained (from San José and Cartago in central inland Costa Rica) are very similar.

Heteromys salvini (Fig. 2C): Our two sequences from SRNP (Guanacaste) in Costa Rica group very closely with previous sequences from Guanacaste and Puntarenas and a sequence from Honduras.

Heteromys sp (Fig. 2C): This provisional designation by Rogers and González (2010) was based on a distinct lineage located in Costa Rica (found in Alajuela and Limón). We added another sequence from Limón (AFRS). All sequences are very closely related. On the basis of all the cyt*b* data available for this form and in the context of our whole phylogenetic tree, *Heteromys* sp most likely represents a separate species, having a similar level of distinctiveness as other recognized species (both considering *Heteromys* and other genera in our phylogenetic tree).

Hoplomys gymnurus (Fig. 2C): There are no new sequences, just one existing sequence from Panamá.

Fig. 2 Highlighted branches of the cytochrome *b* phylogeny for small mammals in Costa Rica (see Fig. S1 for the complete phylogeny). Maximum likelihood tree (IQ-TREE). Branch support: ultrafast bootstrap approximation/SH-like approximate likelihood ratio test results. Taxa and sequences shown in blue were collected in this study. **A**: Branch including the cricetid rodent genera *Handleyomys*, *Melanomys*, *Nephelomys*, *Nyctomys*, *Oecomys*, *Oligoryzomys*, *Oryzomys*, *Ototylomys*, *Rheomys*, *Sigmodon*, *Sigmodontomys*, *Transandinomys*, *Tylomys*, *Zygodontomys*; **B**: Branch including the cricetid rodent genus *Peromyscus*; **C**: Branch including rodent (echimyid and heteromyid) and eulipotyphlan genera; **D**: Branch including the marsupial genera

Marmosa alstoni (Fig. 2D): There are no new sequences, just two identical existing sequences from Panamá.

Marmosa mexicana (Fig. 2D): There are no new sequences, and most of the five sequences previously obtained from Guatemala and Mexico are very similar to each other. One sequence from Guatemala (HM106344) stands out as distinctive.

Melanomys chrysomelas (Fig. 2A): The four previous sequences from Nicaragua and Costa Rica (Heredia) are closely related to each other, and one of our four new sequences is closely related to those (SM3690). However, the other three new sequences from the same locality (MANP, Puntarenas) form a somewhat distinct lineage.

Metachirus nudicaudatus (Fig. 2D): There are no new sequences, and the five sequences from four countries (Ecuador, Guyana, Panamá, Peru) are distinctive from each other – the sequence from Guyana particularly so. The two sequences from Panamá do form a clade though.

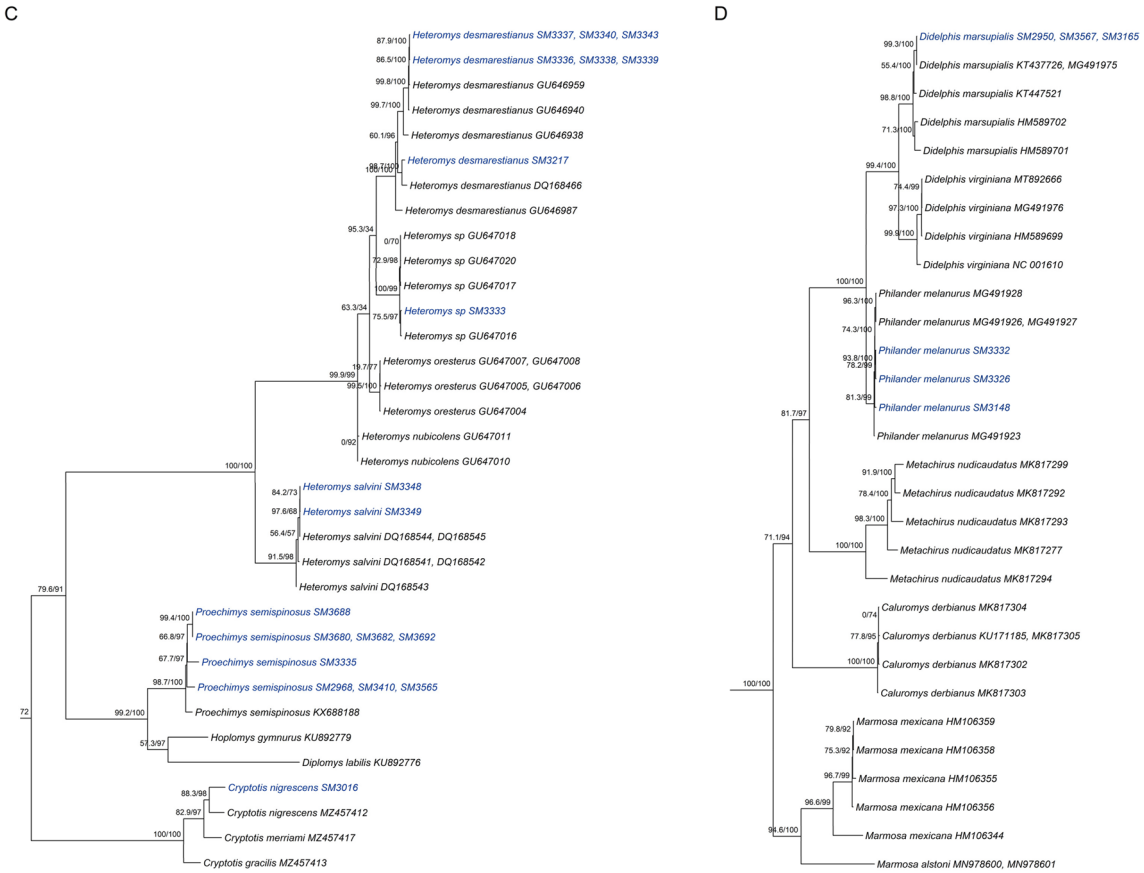

Fig. 2 (continued)

Nephelomys devius (Fig. 2A): All three sequences are from Costa Rica and our sequence (from LAIP-VS, Limón) and previous sequences from Puntarenas and Cartago are extremely similar.

Nyctomys sumichrasti (Fig. 2A): The five existing sequences from three different countries (El Salvador, Guatamala, Mexico) are generally distinctive from each other except the two from El Salvador that are very similar. The two sequences from Mexico are particularly divergent. Our two new sequences from Costa Rica, although both coming from the same northern inland locality (S. Verde, Heredia) are different from each other and from the other sequences.

Oecomys trinitatis (Fig. 2A): There are no new sequences, just one existing sequence from Peru.

Oligoryzomys costaricensis (Fig. 2A): There are no new sequences, just two identical existing sequences from Panamá.

Oryzomys couesi (Fig. 2A): Our new sequence from Costa Rica (LAIP-PT, Puntarenas) is very similar to existing sequences from Guatemala, Honduras and Nicaragua.

Ototylomys phyllotis (Fig. 2A): There are no new sequences, just one existing sequence from Honduras.

Peromyscus nicaraguae/nudipes (Fig. 2B): We contributed 18 new sequences from Costa Rica (BCNP, LAIP) and one from Honduras (SM3205). They were all labelled *P. nudipes* but in fact they fall into one of two distinct clusters of tightly related individuals. One of these clusters, which includes our sequence from Honduras and all our sequences from BCNP in Heredia (and one from LAIP-PT, Puntarenas), also includes five previous sequences from Costa Rica (Puntarenas), Honduras and Nicaragua. Four of these previous sequences have been named *P. nicaraguae*, and it appears likely that the sequences in this cluster should all be classified as *P. nicaraguae*. The other clade with ten of our sequences from LAIP-VS (Limón) and a previous sequence from Panamá are likely appropriately named *P. nudipes*.

Philander melanurus (Fig. 2D): Our new sequences from Costa Rica (LAIP-EA in Puntarenas, CERS and AFRS in Limón) cluster very closely with previous sequences from Colombia and Panamá.

Proechimys semispinosus (Fig. 2C): Our new sequences from Costa Rica (MANP and Sansi in Puntarenas and S. Verde in Heredia) cluster with a sequence from Colombia. We have contributed eight new sequences from Costa

Rica and there appears to be substantial variation among those.

Reithrodontomys: The genus-specific analysis is given below.

Rheomys raptor (Fig. 2A): There are no new sequences, just one existing sequence from Costa Rica.

Scotinomys teguina/xerampelinus: The combination of our new sequences from Costa Rica and existing sequences from Honduras (*S. teguina*) and Costa Rica (*S. xerampelinus*) indicate greater genetic subdivision than just the two named species. More detailed analysis is provided in González et al. (in prep.).

Sigmodon hirsutus (Fig. 2A): Our new sequences from Costa Rica (SRNP, Guanacaste) cluster very closely with previous sequences from Honduras, Mexico and Nicaragua.

Sigmodontomys alfari (Fig. 2A): There are no new sequences, and minor differentiation between the existing sequences from Panamá and Ecuador.

Transandinomys bolivaris/talamancae (Fig. 2A): There are no new sequences and these two species are represented by five existing sequences. The three *T. talamancae* sequences from Panamá cluster together. However, the *Transandinomys* from Ecuador form two further branches with the disposition making *T. talamancae* paraphyletic.

Zygodontomys brevicauda (Fig. 2A): There are no new sequences, and the five sequences from three countries (Bolivia, Panamá, Venezuela) show a degree of genetic subdivision, though it does not relate in a simple way to geography (e.g. the three sequences from Venezuela are not each other's closest relatives).

Reithrodontomys analysis

The sequences that we contributed to the phylogeny of the *Reithrodontomys* genus were *Reithrodontomys* sp, *R. creper* and *R. sumichrasti* (Fig. 3). Our sequences of *R. creper* and *R. sumichrasti* are very similar to INSDC sequences of the same species and confirm the well-supported monophyly of those two species within the tree. For *R. creper*, the existing sequences are from the central northern interior of Costa Rica (Cartago, Heredia). Our new sequences came both from this region (BCNP, Heredia) but also at the interior border with Panamá (LAIP-VS, Limón). All 24 sequences are very similar to each other. The previously obtained sequences of *R. sumichrasti* come from a broader geographic area (Costa Rica, Guatemala, Honduras, Nicaragua, Panamá) and our two new sequences are identical to the existing sequence from Costa Rica, being from the same general region in the central interior of the country (our sequences: BCNP, in the south of Heredia; the earlier sequence: Cartago).

The five sequences from *R. fulvescens* are all from INSDC and form a distinct monophyletic grouping at the base of the *Reithrodontomys* clade. All the sequences are from Mexico and there is considerably more structure within the lineage than in *R. creper* and *R. sumichrasti*.

The situation elsewhere within the *Reithrodontomys* lineage is more complex and our *Reithrodontomys* sp sequences are on three separate branches. One of those sequences found in three individuals (SM3143, 3149, 3150) from LAIP-EA and LAIP-PT (Puntarenas) is closely related to a published *R. brevirostris* sequence from Cartago, suggesting that those particular *Reithrodontomys* sp are *R. brevirostris.* This is supported by the inclusion of a shorter published *R. brevirostris* sequence from Alajuela in the same clade (Fig. S2). The addition of another short published sequence of *R. dariensis* suggests that the *R. brevirostris* lineage is in a larger clade consisting of *R. dariensis*, *R. gracilis* and *R. mexicanus* lineages as well (Fig. S2). The apparent misplacement of one *R. mexicanus* sequence (AF108708) clustering with *R. dariensis* is not surprising because "*R. mexicanus*" has been a standard "catch-all" designation for *Reithrodontomys* in Central America until a recent taxonomic re-evaluation (Gardner and Carleton 2009).

A similar phenomenon may explain the clade containing two short sequences of *R. cherrii* together with *R. mexicanus* sequences all from the same province in Costa Rica (San José) (Fig. S2). That apparent *R. cherrii* lineage is grouped with *R. tenuirostris* and *R. microdon* (showing paraphyly) (Fig. S2). The sequences from these two species are from Mexico and Guatemala.

The final two lineages consisting solely of *Reithrodontomys* sp in the main analysis are notable for the lack of genetic subdivision despite large numbers of sequences (33 in one lineage, six in the other) (Fig. 3). Again, a short sequence suggests that the larger clade maybe *R. garichensis* (Fig. S2).

Discussion

This study examined genetic diversity in Costa Rican small mammals applying the widely-used DNA barcoding approach (Hebert et al. 2003; Dasmahapatra and Mallet 2006; Jones et al. 2021; Tsoupas et al. 2022). Our application of DNA barcoding involved use of the complete cyt*b* gene to potentially identify new genetic forms or species, following a rich heritage of this molecular marker in phylogeographic studies of small mammals (e.g. Ditchfield 2000; Kotlík et al. 2022). The new complete cyt*b* sequences that we obtained were combined with those from INSDC, giving us 217 sequences for a phylogeny of 41 named species of small mammals (plus two species of uncertain identity). We were then able to follow this up with a focused analysis of a particularly speciose genus of small mammals,

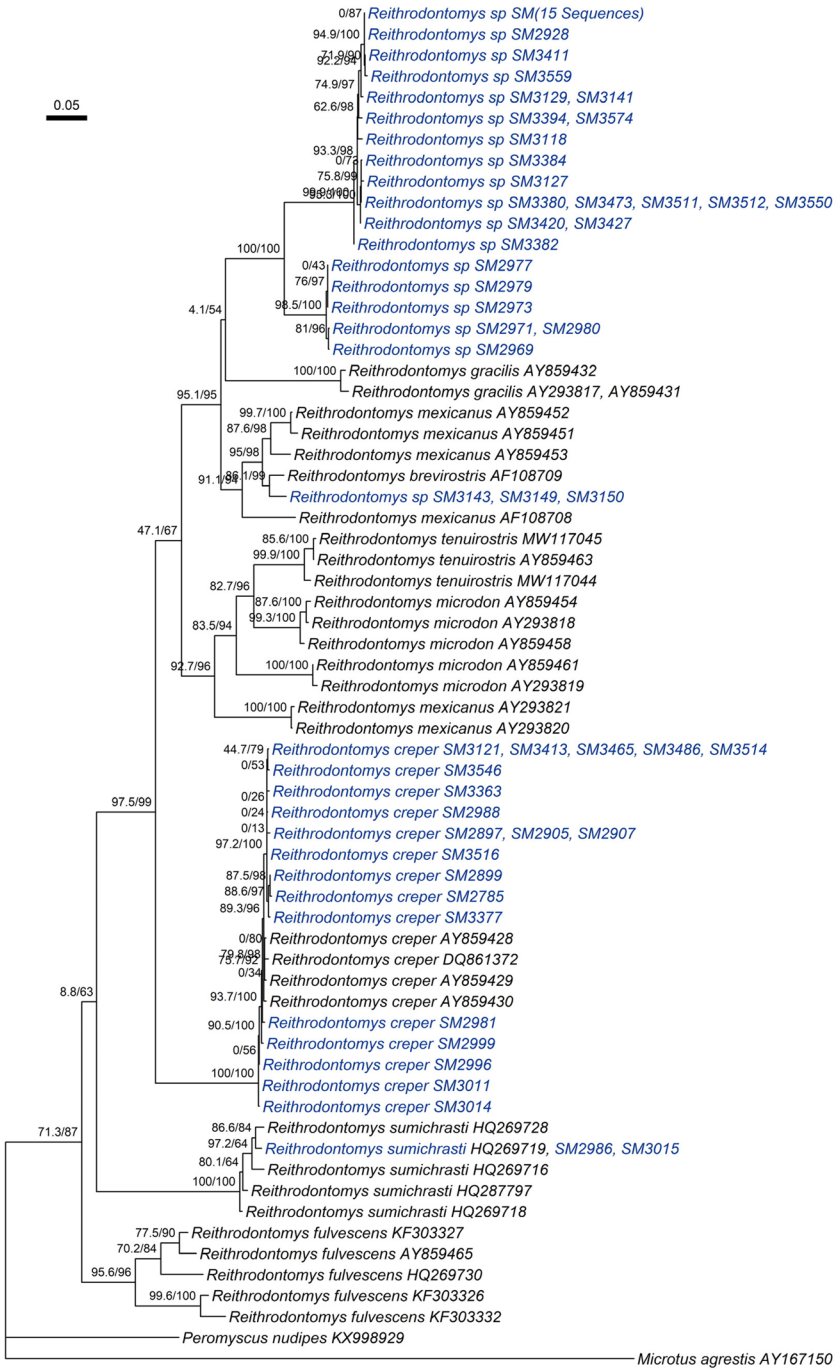

Fig. 3 Cytochrome *b* phylogeny for *Reithrodontomys* in Costa Rica. Maximum likelihood tree (IQ-TREE). Branch support: ultrafast bootstrap approximation/SH-like approximate likelihood ratio test results. Taxa and sequences shown in blue were collected in this study. *SM(15 Sequences)*: *SM2781*, *SM2782*, *SM2903*, *SM3123*, *SM3367*, *SM3412*, *SM3417*, *SM3460*, *SM3462*, *SM3463*, *SM3470*, *SM3471*, *SM3488*, *SM3513*, *SM3535*

Reithrodontomys (adding another 65 sequences for an analysis of eight named species plus a species of uncertain identity). Our work based on Costa Rican samples has been able to build on previous excellent taxonomic studies of small mammals in Central America that have incorporated cyt*b* analysis (e.g. Almendra et al. 2018; Arellano et al. 2005; Bradley et al. 2016; Corley et al. 2011; Gutiérrez et al. 2010; Hanson and Bradley 2008; Hardy et al. 2013; Pérez Consuegra and Vázquez-Domínguez 2015; Rogers and González 2010; Voss et al. 2019).

At the highest taxonomic levels our phylogeny fits reasonably with expectation (separation of the different rodent families, eulipotyphlans, marsupials). But more importantly for our purposes, the phylogeny showed itself to be a valuable tool for species discrimination. Almost all previously described species formed clear monophyletic groups and the clustering of sequences at the within-species level typically mirrored geography, with sequences from geographically close localities typically showing a closer relationship. Our interest was in assessing whether this geography-driven

differentiation was of a sufficient magnitude to indicate presence of major genetic forms or cryptic species. Also of note are those few examples where the sequences of a particular named species do not form a clade or where relationships do not reflect geography – this may indicate previously unexposed taxonomic units. Our approach has been a species-wide barcoding analysis of small mammals and, in this spirit, we will be wide-ranging in our discussion of phylogenetic results of the different species of small mammal that we have examined.

Considering our new sequences, *Didelphis marsupialis*, *Oryzomys couesi*, *Philander melanurus* and *Sigmodon hirsutus* added complete cyt*b* sequences for the first time for Costa Rica, but with no indications of strong genetic differences between specimens in Costa Rica and in other nearby countries. In the case of *O. couesi*, this taxon was differentiated from other related forms using cyt*b* sequences, and its species status defined from this molecular analysis (Hanson et al. 2010). However, we found little further differentiation within the species over the range incorporating Honduras, Guatemala, Nicaragua and Costa Rica.

In the case of *Handleyomys alfaroi*, *Nephelomys devius*, *Heteromys* sp and *Heteromys salvini* our studies added to previous cyt*b* sequences from Costa Rica, but with no new indications of genetic subdivisions in the species. Previous studies on *H. alfaroi* using a range of nuclear and mitochondrial markers suggested cryptic diversity within the species (Almendra et al. 2018), but the differentiation that we found with just cyt*b* was not very substantial, despite using sequences from Costa Rica, Guatemala, Honduras, Nicaragua and Panamá. *Heteromys* sp is particularly interesting because Rogers and González (2010) identified it as a new cryptic species in Costa Rica on the basis of their studies with cyt*b*. Our new sequence and phylogenetic analysis supports that contention, and we recommend a full taxonomic treatment of this form, and a new naming. *Heteromys* sp is clearly distinct from other closely related *Heteromys* found in Costa Rica on the basis of cyt*b* sequence (*H. desmarestianus*, *H. nubicolens* and *H. oresterus*: Fig. 2C). *Heteromys* sp was identified from the cyt*b* analysis of Rogers and González (2010) as one of several cryptic forms within what was known as *H. desmarestianus*. Certainly, considering what is currently classified as *H. desmarestianus* our new sequences reinforce the differentiation between sequences from Costa Rica and those from Honduras and those from Mexico. This differentiation is not of the same magnitude as separates *H. desmarestianus* from *Heteromys* sp, but still noteworthy.

As well as *Heteromys* sp and a Costa Rica lineage of what is currently classified as *H. desmarestianus*, there are other possible examples of cryptic forms in Costa Rica. In addition to the previously described lineage of *Melanomys chrysomelas* from Costa Rica and Nicaragua, to which we added a new sequence, we found a somewhat distinct lineage of three sequences all from the same location (MANP, Puntarenas) (Fig. 2A). Hanson and Bradley (2008) already described *M. chrysomelas* as a cryptic species within a wide-ranging form previously know as *M. caliginosus*. Further subdivision maybe appropriate.

In a similar way as the wide-ranging *M. caliginosus* was subdivided into species with smaller ranges on the basis of cyt*b* data, this may also be appropriate for *Nyctomys sumichrasti*. It ranges from Mexico to Panamá (Corley et al. 2011) and there is considerable differentiation based on cyt*b* sequence (Fig. 2A). Corley et al. (2011) already recognized a need for taxonomic re-evaluation of this species by analysing data from El Salvador, Guatemala, Honduras, Mexico and Nicaragua. We have provided the first complete cyt*b* sequences from Costa Rica and our data reinforce that sentiment. One of the two sequences we provide is distinctly different from other branches considering the set of available sequences for this species.

The particular relevance of using complete cyt*b* sequences to uncover cryptic diversity is illustrated by our analysis of *Peromyscus* (Fig. 2B). At the time of collection we named our specimens as *P. nudipes*. However, Bradley et al. (2016) inferred the presence of *P. nicaraguae* in Costa Rica, so we included the defining cyt*b* sequence and other published Central American *P. nicaraguae* sequences in the phylogenetic tree. Our new sequences fall into two distinct clades in the tree. Judging by the presence of Bradley et al.'s (2016) sequences (both *P. nicaraguae* and *P. nudipes*), one of our "*P. nudipes*" clades should be classified as *P. nudipes* sensu stricto and the other as *P. nicaraguae*.

Another species worth further taxonomic treatment on the basis of our data is *Proechimys semispinosus.* Previously, there were no complete cyt*b* sequences for Costa Rica available and the sequences that we add show differentiation within the country. The *Cryptotis nigrescens* sequence that we obtained was the second for Costa Rica and quite divergent from the first, so again this species is worth further treatment. Our phylogeny reveals other taxa worth further examination based purely on published sequences, particularly *Transandinomys talamancae* (which is paraphyletic in our tree) and both *Metachirus nudicaudatus* and *Marmosa mexicana* which both have single lineages clearly distinctive from the others. The importance of taxonomic revision in *Marmosa* has already been recognised by Voss et al. (2020).

There are two other genera that require particular attention: *Scotinomys*, which is obviously interesting based on the phylogenetic results in Fig. S1 (*S. xerampelinus* is paraphyletic), and *Reithrodontomys*, which is the best represented genus in our study. *Scotinomys* is considered in detail in a separate publication (González et al. in prep.). *Reithrodontomys* is considered below.

Reithrodontomys

Reithrodontomys were included both in the all-species analysis (Fig. S1) and in a separate analysis (Fig. 3). The utility of the DNA barcoding in this instance is in helping to confirm the species identity of living individuals, which is challenging purely based on use of a field guide. Most of the specimens collected were classified by us as *Reithrodontomys* sp because we could not distinguish the species. The range of species of *Reithrodontomys* in Central America is impressively large and based on equally impressive taxonomic effort over many years (Hooper 1952; Arellano et al. 2005, 2023; Gardner and Carleton 2009; Hardy et al. 2013; Martínez-Borrego et al. 2022). The full analysis based on complete cyt*b* sequences allowed us to confirm the identity of *R. creper* and *R. sumichrasti* among our samples. Also, three identical sequences attributed to *Reithrodontomys* sp form a sister lineage to known *R. brevirostris* and are most reasonably attributed to that species. The use of shorter sequences (Fig. S2) suggest that the largest clade of *Reithrodontomys* sp in our trees are in fact *R. garichensis.* These also help convert what we name as "*R. mexicanus*" (following the original descriptions in Smith and Patton (1999), Bradley et al. (2004) and Arellano et al. (2005)) into *R. cherrii* (AY293821, AY293820), *R. dariensis* (AF108708) and *R. mexicanus* sensu stricto (AY839451, AY839452, AY839453). The inclusion of other *Reithrodontomys* species from Central America also allowed us to have a phylogeny of over ten species.

Our study on *Reithrodontomys* builds on several previous taxonomic studies making use of cyt*b* sequences. Our two *R. sumichrasti* sequences were identical to those obtained in a nearby location in Costa Rica by Hardy et al. (2013) which were included in one of the several lineages identified within the species (classified as *R. s. australis*)(Hardy et al. 2013; Arellano et al. 2023). The clade that we found that linked *R. cherrii*, *R. microdon* and *R. tenuirostris* (Fig. S2) has also been demonstrated by Martínez-Borrego et al. (2022) who designate it the "*R. tenuirostris* group". They also define the "*R. mexicanus* group" consisting of *R. brevirostris*, *R. dariensis*, *R. gracilis* and *R. mexicanus*, which we also recovered (Fig. S2) and they identify *R. creper* as an independent lineage (as we do) and another group consisting of *R. fulvescens* and *R. sumichastri* that are the earliest branching lineages in our tree. The only two lineages that have not been described by others are the two largest lineages of *Reithrodontomys* sp in our tree of complete cyt*b* sequences. From where the samples were collected, the most likely species are *R. garichensis*, *R. musseri* and *R. rodriguezi* (Gardner and Carleton 2009; Reid & Gómez Zamora 2022). Based on a single short sequence, the larger of these lineages is probably *R. garichensis*. The other is, at present, unidentified.

One interesting aspect of our phylogeny of *Reithrodontomys* is the variation in branch lengths between lineages (Fig. 3). *R. creper* and our two largest clades of *Reithrodontomys* sp have very short branch lengths indicating very low cyt*b* variation. This could represent biased sampling from a limited geographical area or the possibility of recent bottlenecking and expansion of this species.

Conclusion

Our DNA barcoding approach towards the small mammals of Costa Rica using cyt*b* has newly revealed or supported a number of possible instances of new species or major genetic forms, in *Melanomys chrysomelas*, *Nyctomys sumichrasti, Heteromys*, *Peromyscus* and *Reithrodontomys*. The work on *Scotinomys* is described elsewhere (González et al. in prep.) and our phylogenetic reconstructions based on INSDC sequences of *Marmosa mexicana*, *Metachirus nudicaudatus* and *Transandinomys talamancae* suggest that there may be cryptic species or major genetic forms within those taxa. All the new sequences that we provided came from living small mammal specimens identified with a field guide (Reid 2009). Clearly, this method of field identification coupled with the barcoding molecular analysis means that we have only made a "first pass" at the description of small mammal diversity in Costa Rica. Cyt*b* as a sole discriminator is risky (Galtier et al. 2009; Alves et al. 2008), so further in-depth genetic and morphological studies are needed (DeSalle et al. 2005). DNA barcoding is a quick approach to find new species and major genetic forms – it has been very successful when considering the small mammals of Costa Rica. DNA barcoding should work particularly well for understudied species-rich higher taxa and our results corroborate that.

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Author contribution A Gómez-Lépiz, J Paupério, PC Alves and JB Searle conceived and designed the study. PC Alves funded and formally supervised the study. Specimen and data collection was by S Sampaio, SMC Valdés and A Gómez-Lépiz with support from J Paupério and PC Alves. Analysis was conducted by JJ Hughes and S Sampaio with

support from J Paupério and JB Searle. The first draft of the manuscript was prepared by JB Searle and A Gómez-Lépiz. All authors commented on this and subsequent versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The cytochrome *b* sequences generated in the present study have been deposited into the European Nucleotide Archive, under project PRJEB74466. Details of all data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Competing interests The authors declare no competing interests.

Permits and animal welfare All procedures involving small mammals were approved by CONAGEBIO (National Commission for Biodiversity Management), under the auspices of the Ministry of Environmental of Costa Rica (permit: R-052-2017-OT-CONAGEBIO), and supervised by a veterinary official (National Id: 1074). All equipment for handling of small mammals accorded with internationally accepted standards.

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