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ABSTRACT

DNA sequence data obtained from the 96 year old holotype of *Marmosa elegans coquimbensis* Tate, 1931, support the hypothesis that this nominal taxon is a synonym or subspecies of *Thylamys elegans* (Waterhouse, 1839) and is not conspecific with *T. pallidior* (Thomas, 1902).

INTRODUCTION

The nominal taxon *Marmosa elegans coquimbensis* was named by Tate (1931) based on a single specimen in the Field Museum of Natural History (FMNH) collected on 20 June 1923 by Colin C. Sanborn at or near Paiguano (30°02′ S, 70°27′ W, ca. 1600 m elev.) in the Coquimbo region of Chile. Tate's trinomial usage was maintained by most subsequent researchers (e.g., Cabrera, 1958) until *elegans* Waterhouse, 1839, and other species of fat-tailed mouse opossums were transferred to the genus *Thylamys* Gray, 1843, by Gardner and Creighton (1989). Most subsequent authors have treated *coquimbensis* as a subspecies (Palma, 1997) or a junior synonym (Creighton and Gardner, 2008) of *T. elegans*, but we, together with S. Jansa (Giarla et al., 2010), transferred *coquimbensis* from the synonymy of *T. elegans* to that of its sister species *T. pallidior* (Thomas, 1902) based on morphological traits of the holotype (FMNH 22302). If valid, our reidentification of FMNH 22302 as *T.*

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pallidior would have been the first record of that species from the Coquimbo region. Recently, however, Boric-Bargetto et al. (2016) analyzed cytochrome *b* sequence data from several specimens of *Thylamys* collected near Paiguano and recovered them as members of a northern haplogroup of *T. elegans*. Although the authors admitted the possibility that *T. elegans* and *T. pallidior* might be sympatric near Paiguano, they concluded that the haplogroup in question should be called *T. e. coquimbensis*. In order to test their conclusions, we extracted and sequenced DNA from the 96 year old holotype of *coquimbensis* for phylogenetic analysis.

Materials and Methods

A single claw was clipped from the dried study skin of FMNH 22302. To avoid contamination from exogenous DNA, all pre-PCR laboratory procedures were performed in a biological safety cabinet with UV sterilization in a lab where mammalian DNA is never amplified and in which contaminating mammalian PCR products are unlikely to be present. Moreover, no other didelphid DNA samples had ever been present in the building where the work was conducted prior to the experiment. The sample was soaked in ethanol overnight and then rinsed with water three times. The same wash procedure was performed the next day. On the third day, the sample was allowed to air-dry before DNA extraction was performed with a modified DNeasy Blood and Tissue Kit (Qiagen) protocol. The sample was added to a mixture of 40 μl proteinase K, 160 μl Buffer ATL, and 30 μl 1 M dithiothreitol and incubated at 56° C in a shaking mixer for 24 hours. The digested sample was then lysed by adding 200 μl Buffer AL, mixing, and incubating at 70° C for 10 minutes. DNA was precipitated using 200 μl cold ethanol and incubated at 4° C for one hour. The sample was added to a QIAquick PCR Purification Kit (Qiagen) spin column. This column was chosen over the standard DNeasy spin columns because it is designed to preferentially bind fragments of DNA less than 10 kb. The subsequent wash and elution steps followed the usual DNeasy tissue extraction protocol.

Nested pairs of primers were designed to span 174–359 bp pieces of the mitochondrial cytochrome *b* (CYTB) gene in the degraded sample. To ensure that primers would amplify both a relative of *Thylamys pallidior* and *T. elegans*, primers were designed using a consensus sequence of two *T. pallidior* CYTB sequences (GenBank accession numbers HM583398 and HM583387) and one *T. elegans* whole mitochondrial genome sequence (NC005825). Primers were designed in Geneious R9 (Biomatters, Inc.) using the Primer3 algorithm (Untergasser et al. 2012), and only regions that exhibited sequence conservation between the two reference species were used (table 1). Seven sets of PCR reactions were conducted using seven primer pairs. Each reaction contained 13 μl of GoTaq Green Master Mix (Promega), 9 μl of water, 1 μl of each 10 μ M primer solution, and 1 μl of undiluted sample DNA. A negative control reaction, where no DNA was added, was conducted for each separate PCR mixture. The reaction mixture was PCR amplified using an initial 2 min. melting phase at 95° C; then 40 cycles of 30 s at 95°, 30 s at 55°, and 30 s at 72°; then 5 min at 72°. PCR product size was verified on a 1% agarose gel. PCR products were purified using ExoSAP-It (Thermo Fisher) and sent to GENEWIZ (South Plainfield, NJ) for Sanger sequencing in both directions.

For each PCR amplicon, chromatograms were examined, trimmed, and assembled in Geneious. To determine whether individual amplicons might be derived from contaminant DNA, each was subjected to a standard nucleotide BLAST search (Altschul et al., 1990) against Gen-

TABLE 1. Pairs of primers used to sequence overlapping pieces of cytochrome *b* and associated information about PCR amplicons.

Amplicon number	Forward Primer	Reverse Primer	Amplicon Length	GenBank Accession of Best BLAST Hit
1	Coqui-CYTB-32F: TGGCATGAAAAACCATTGTTGT	Coqui-CYTB-331R: TCCATTGGCGTGAATGTTTCG	300	KP994523.1
2	Coqui-CYTB-222F: TCCTAGCCATACATTACACATCAGA	Coqui-CYTB-511R: AATTACTGTAGCTCCTCAGAAGG	290	KP994527.1
3	Coqui-CYTB-445F: AGCTACTGCATTTGTAGGTTATGT	Coqui-CYTB-739R: GGGGTGGAAAGGAATTTTGTCTG	295	KP994526.1
4	Coqui-CYTB-457F: TCCTTCTGAGGGGCTACAGT	Coqui-CYTB-815R: GGGGTGAAGTTATCGGGGTC	359	KP994526.1
5	Coqui-CYTB-717F: CAGACAAAATTCCTTTCCACCCC	Coqui-CYTB-1016R: AGCTTCGTTGGTTTGATGTATGA	300	KP994522.1
6	Coqui-CYTB-901F: TGCCTACGCAATTCTACGATCT	Coqui-CYTB-1191R: TCAAGTATGCCTGCTAAGGGT	291	KP99452651
7	Coqui-CYTB-1079F: ACCTGAATTGGAGGACAACC	Coqui-CYTB-1293R: TGTCCAAGGAAGGAGTTTTCC	215	AJ508401.2

Bank's nonredundant nucleotide database. After ruling out contamination, FMNH 22302 CYTB amplicons were assembled to a reference *Thylamys pallidior* sequence and trimmed to include only the CYTB coding region. A representative set of 72 *Thylamys* CYTB sequences and one sequence from the outgroup *Lestodelphys halli* were downloaded from GenBank (appendix 1). This set included sequences from all the *Thylamys* species recognized by Giarla et al. (2010), broad geographic sampling within the Elegans Group, and all the sequences that Boric-Bargetto et al. (2016) identified as *T. elegans coquimbensis*. These sequence data were aligned in Geneious using MUSCLE (Edgar 2004). The alignment was partitioned by codon position, and different partitioning schemes and nucleotide substitution models were tested in PartitionFinder2 (Lanfear et al. 2016). A Bayesian phylogeny was inferred in MrBayes 3.2.6 (Ronquist et al., 2012) using the best-fitting partitioning scheme and substitution models. Two simultaneous Markov chain Monte Carlo (MCMC) runs were initiated, each for 10 million generations and with four chains. Convergence of the MCMC runs was assessed in Tracer 1.7.1 (Rambaut et al., 2018) to ensure that each parameter estimate had an effective sample size greater than 200.

RESULTS

A complete CYTB sequence from FMNH 22302 (GenBank accession MK907779) was assembled from seven individually amplified and overlapping PCR amplicons derived from high-quality Sanger reads. When subjected to individual BLAST searches, each amplicon matched a *Thylamys elegans* sequence in the nonredundant nucleotide database (table 1), ruling out contamination from nondidelphids. Once assembled, the CYTB amplicons overlapped each other by 84.5 bp on average (range: 20–218 bp), with no conflicting bases. After alignment, PartitionFinder2 identified a best-fitting partitioning scheme and associated nucleotide substation models in which each codon posi-
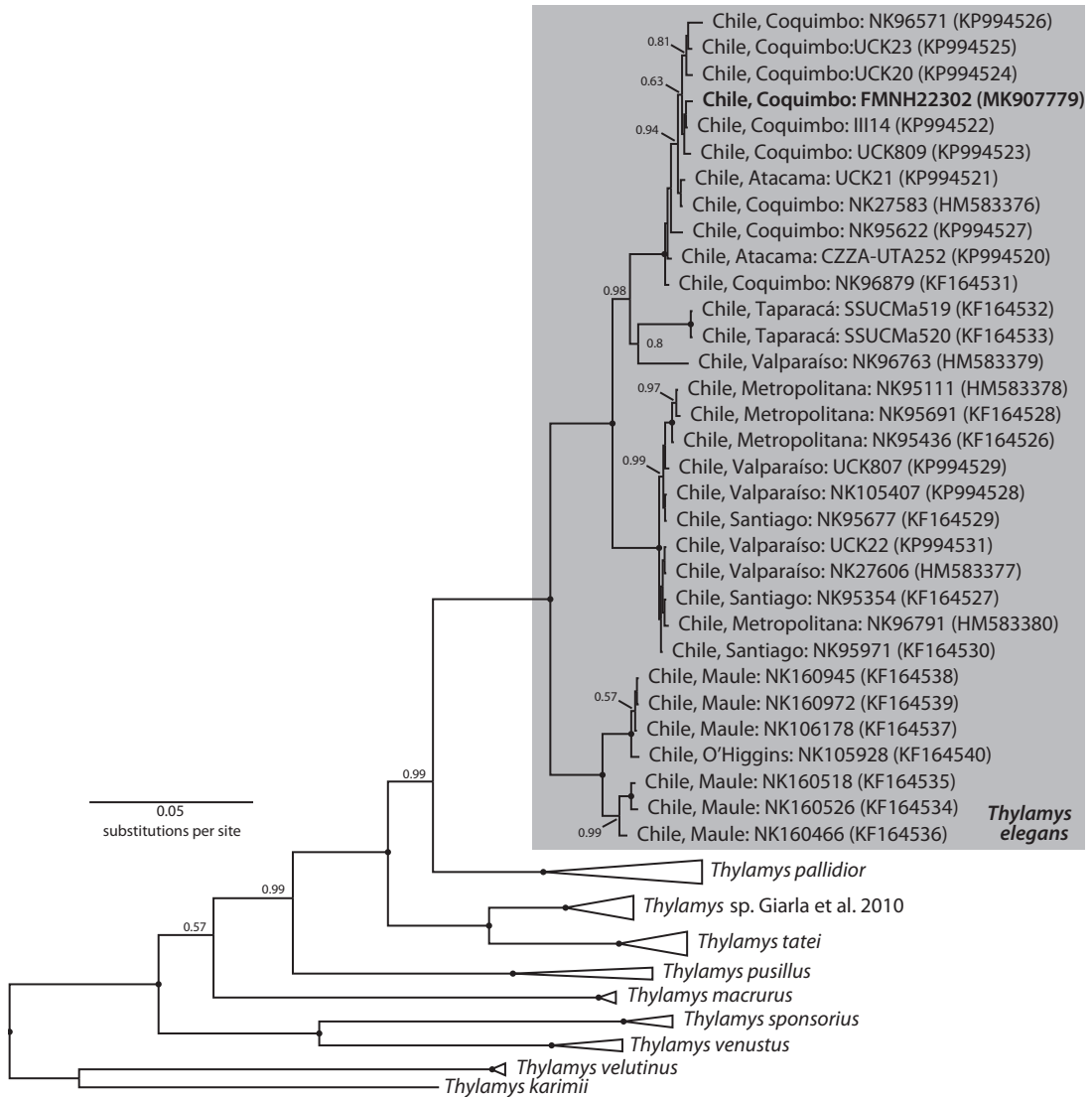
FIG. 1. Bayesian phylogenetic tree of *Thylamys* cytochrome *b* sequences. Numbers at nodes indicate posterior probabilities (PP). Filled circles at nodes denote PPs equal to 1.0. Unmarked nodes received PPs less than 0.5. Within *T. elegans*, tips are labeled with country, region, specimen identifier, and, in parentheses, a GenBank accession number. The holotype of *Marmosa elegans coquimbensis* Tate, 1931, is in boldface type. For other species, tips of the phylogeny are collapsed and the outgroup is not shown. See appendix 1 for a full list of sequences included in the phylogeny. A full tree file corresponding to this topology is available on TreeBase (doi: http://purl.org/phylo/treebase/phylows/study/TB2:S25505).

tion was allowed its own substitution model (position 1: GTR+G; position 2: GTR+I; position 3: GTR+I+G). The resulting phylogeny unambiguously recovered the holotype of *coquimbensis* as nested within *T. elegans* as that species is currently recognized (fig. 1).

DISCUSSION

Our results unequivocally support Boric-Bargetto et al.'s (2016) conclusion that *coquimbensis* is more closely related to *Thylamys elegans* than to *T. pallidior*, although the gene tree we recovered does not address the issue as to whether *coquimbensis* should be treated as a subspecies or as a synonym of the former species. Indeed, the trinomial classification of what is currently considered *T. elegans* is complicated by several divergent but unnamed mtDNA lineages, one consisting of two sequences from Tarapacá (SSUCMa519, SSUCMa520; the "Loa lineage" of Boric-Bargetto et al., 2016), another of a single sequence from Valparaíso (NK96763), and a third of seven sequences from Maule and O'Higgins (NK105928, NK106178, NK160466, NK160518, NK160526, NK160945, NK160972). Of these, only the "Loa" haplogroup from Tarapacá was analyzed by Boric-Bargetto et al. (2016), although the Maule/O'Higgins and Tarapacá sequences had previously been analyzed by Palma et al. (2014).

Our results also raise questions about phenotypic character variation within *Thylamys elegans*. The type of *coquimbensis* (FMNH 22302) is—as previously reported by us (Giarla et al., 2010: 45)—phenotypically very similar to *T. pallidior*, notably in pelage coloration (much paler dorsally than typical *T. elegans*, and with almost entirely self-white ventral fur), hind-foot length, and bullar inflation. Apparently, the Paiguano topotypes examined by Boric-Bargetto et al. (2016) are also pale-furred, but other specimens that they refer to *T. e. coquimbensis* (e.g., NK 27583, one of a series of specimens that we examined at the Museum of Southwestern Biology in Albuquerque, NM) are much darker, so this trait is evidently not diagnostic of the haplogroup that they associate with this trinomen.

The other traits of FMNH 22302 that led us to synonymize *coquimbensis* with *Thylamys pallidior* (smaller feet, more inflated bullae) were not mentioned by Boric-Bargetto et al. (2016), who emphasized the diagnostic value of nasal morphology, in which their Paiguano specimens were said to resemble *T. elegans* rather than *T. pallidior*. We had previously evaluated this character (which was also mentioned by Solari, 2003) as a diagnostic criterion for our revision (Giarla et al., 2010), but found too much intraspecific variation for it to be useful, and we invite readers to inspect Boric-Bargetto et al.'s (2016: fig. 2) illustration to decide this issue for themselves.

In summary, the mtDNA sequence data at hand clearly support Boric-Bargetto et al.'s (2016) decision that the nominal taxon *coquimbensis* should be associated with *Thylamys elegans* rather than with *T. pallidior*, but there remain unresolved issues of trinomial nomenclature and geographic variation that should be addressed in any future revision of these taxa.

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APPENDIX 1

Cytochrome *b* Sequences Downloaded from Genbank and Used in the
Phylogenetic Analysis

Species	Museum Code	Specimen Identifier	Genbank Accession Number
Lestodelphys halli		PNG1399	KF164579
Thylamys elegans		CZZA-UTA252	KP994520
Thylamys elegans		III14	KP994522
Thylamys elegans	MSB207075	NK105407	KP994528
Thylamys elegans	MSB153832	NK105928	KF164540
Thylamys elegans	MSB 207538	NK106178	KF164537
Thylamys elegans		NK160466	KF164536
Thylamys elegans	MSB 230411	NK160518	KF164535
Thylamys elegans	MSB 230254	NK160526	KF164534
Thylamys elegans		NK160945	KF164538
Thylamys elegans		NK160972	KF164539
Thylamys elegans	MSB 87097	NK27583	HM583376
Thylamys elegans	MSB 87098	NK27606	HM583377
Thylamys elegans	MSB 133104	NK95111	HM583378
Thylamys elegans	MSB 209719	NK95354	KF164527
Thylamys elegans	MSB 209755	NK95436	KF164526
Thylamys elegans	MSB 209206	NK95622	KP994527
Thylamys elegans	MSB 209225	NK95677	KF164529
Thylamys elegans	MSB 209239	NK95691	KF164528
Thylamys elegans	MSB 133100	NK95971	KF164530
Thylamys elegans	MSB 210062	NK96571	KP994526
Thylamys elegans	MSB 133095	NK96763	HM583379
Thylamys elegans	MSB 133097	NK96791	HM583380
Thylamys elegans	MSB 210242	NK96879	KF164531
Thylamys elegans		SSUCMa519	KF164532
Thylamys elegans		SSUCMa520	KF164533
Thylamys elegans		UCK20	KP994524
Thylamys elegans		UCK21	KP994521
Thylamys elegans		UCK22	KP994531
Thylamys elegans		UCK23	KP994525
Thylamys elegans		UCK807	KP994529
Thylamys elegans		UCK809	KP994523
Thylamys karimii	MZUSP 32094	APC1561	HM583381
Thylamys macrurus	MZUSP 32094	APC932	HM583382
Thylamys macrurus	MSB 70700	NK27536	HM583383
Thylamys pallidior	MLP 24.X.01.3	AC47	HM583392

APPENDIX 1 Continued

Species	Museum Code	Specimen Identifier	Genbank Accession Number
Thylamys pallidior		DUS29	KF164522
Thylamys pallidior		EP434	KF164517
Thylamys pallidior		EP476	KF164515
Thylamys pallidior		HZP2691	KF164519
Thylamys pallidior		HZP3069	KF164511
Thylamys pallidior		HZP3079	KF164510
Thylamys pallidior		HZP3083	KF164509
Thylamys pallidior		JPJ1292	KF164524
Thylamys pallidior	MVZ115634	MVZ115634	HM583388
Thylamys pallidior	MVZ143696	MVZ143696	HM583389
Thylamys pallidior	MVZ173937	MVZ173937	HM583391
Thylamys pallidior	FMNH 162495	NBH7697	HM583384
Thylamys pallidior	MSB 57003	NK14721	HM583385
Thylamys pallidior	MSB 209881	NK96045	KF164513
Thylamys pallidior	MSB 210395	NK96067	KF164512
Thylamys pallidior	MSB 133108	NK96072	HM583387
Thylamys pallidior	OMNH 34908	OCGR7279	HM583406
Thylamys pallidior	OMNH 34911	OCGR7390	HM583408
Thylamys pallidior		PNG1055	KF164525
Thylamys pallidior		RRG34	KF164518
Thylamys pallidior		UCK481	KF164514
Thylamys pallidior		UP793	KF164521
Thylamys pallidior		VII485	KP994534
Thylamys pusillus	AMNH 260025	NK12539	HM583414
Thylamys pusillus	OMNH 23483	OCGR1525	HM583369
Thylamys sp. Giarla *et al.* 2010	MVZ116614	MVZ116614	HM583420
Thylamys sp. Giarla *et al.* 2010	MVZ137896	MVZ137896	HM583423
Thylamys sp. Giarla *et al.* 2010		HZP30	KF164554
Thylamys sponsorius	MSB 67015	NK23763	HM583441
Thylamys sponsorius	OMNH 34534	OCGR7432	HM583434
Thylamys tatei		MUSM23121	KF164555
Thylamys tatei		MUSM23253	KF164556
Thylamys tatei	MVZ135504	MVZ135504	HM583449
Thylamys velutinus	OMNH22284	OMNH22284	HM583450
Thylamys velutinus	OMNH37216	OMNH37216	HM583451
Thylamys venustus	MSB 63268	NK21664	HM583491
Thylamys venustus	AMNH 275428	NK22844	HM583468