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Molecular phylogenetics of mouse opossums: new findings on the phylogeny of *Thylamys* (Didelphimorphia, Didelphidae)

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The mouse opossums of the genus *Thylamys* constitute a group of species mainly adapted to open xeric-like habitats and restricted to the southern portion of South America. We used molecular data (mitochondrial and nuclear sequences) to evaluate the phylogenetic and biogeographical relationships of all currently known living species of the genus, recognizing a new taxon from the middle and high elevations of the Peruvian Andes and evaluating the phylogenetic structuring within *T. pallidior* and *T. elegans*, as well as the validity of *T. sponsorius*, *T. cinderella* and *T. tatei*, and the haplogroups recognized within *T. pusillus*. Our results confirm the monophyly of the genus and that the Caatinga and the Cerrado inhabitants *Thylamys karimii* and *T. velutinus* are the most basal species in the radiation of *Thylamys*. We also calibrated a molecular clock which hypothesized a time of origin of the genus of about 24 My, with most species differentiating in middle and late Miocene and Plio-Pleistocene times of South America.

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Introduction

Mouse opossums are all mouse-sized marsupials inhabitants of the Neotropical region distributed between Mexico and southern Argentina. One of the pionnering works on mouse opossums was Tate's (1933) revision of the genus *Marmosa* (Didelphidae) *sensu lato*, recognizing five species groups based on morphology, later classified as the genera *Marmosa* (*sensu stricto*), *Micoureus*, *Marmosops*, *Gracilinanus* and *Thylamys* (Creighton 1984; Reig *et al.* 1985, 1987; Gardner & Creighton 1989). However, new revisions of the former taxa (Voss *et al.* 2004, 2005; Voss & Jansa 2009) incorporated novelty genera such as *Chacodelphys* and *Cryptonanus* that together with *Gracilinanus*, *Marmosops*, *Lestodelphys* and *Thylamys* comprise the tribe Thylamyini (Voss & Jansa 2009).

Thylamys Gray, 1843 characterizes by its southern or subequatorial distribution in South America, being predominantly a semi-xeric habitat form (Flores *et al.* 2000). *Thylamys* species are of small size (head and body 75–147 mm, tail 65–161 mm; Gardner 2007), lack a marsupium, have nocturnal habits and an arboreal life, and they can be found in the most diverse open-like South American biomes such as Coastal Desert, Puna, Monte Desert, Pampas, Patagonia, Chaco, Caatinga and Cerrado (Palma *et al.* 2002; Gardner 2007; Giarla *et al.* 2010; Formoso *et al.* 2011). However, some species are inhabitants of dense forests such as the temperate forests of the Yungas and the subtropical Atlantic Forests of Paraguay (Flores *et al.* 2007). The tail of most species seasonally becomes thickened (incrassation) with fat deposits particularly in winter seasons (Mann 1978; Gardner 2007). Regarding number of species, recent propositions based on morphology and molecular phylogenetics have recognized between nine and 13 species (see below; Braun *et al.* 2005; Gardner 2005, 2007; Martin 2009; Teta *et al.* 2009; Voss & Jansa 2009; Giarla *et al.* 2010), but new taxa are in need of formal description.

The fossil record of *Thylamys* dates from the Pliocene (Montehermosan; Mones 1980; Reig *et al.* 1985), but recent revisions date back this taxon from the Huayqueran (late Miocene) of Cerro Azul Formation, Argentina (Goin 1997; Goin *et al.* 2000). The origin of *Thylamys* has been ascribed to have occurred via peripheral isolates from ancestral forms in the eastern Andes (Palma *et al.* 2002). Regarding phylogenetic relationships of *Thylamys*, the genus seems to constitute a monophyletic group (Palma *et al.* 2002; Braun *et al.* 2005; Voss & Jansa 2009; Giarla *et al.* 2010), and its sister taxon is the Patagonian mouse opossum *Lestodelphys halli* (*e.g.* Kirsch & Palma 1995). At the beginning of 2000s, few studies considered molecular phylogenetic data to evaluate the origin and relationships of *Thylamys*, and most of them included approximately half of currently recognized species. Palma *et al.* (2002) phylogeny included five species of *Thylamys* based on cytochrome *b* sequences concluding that *T. macrura* from the subtropical forests of Paraguay would be the predecessor from which other species radiated and that the sister taxa *T. elegans* (Coastal Desert) and *T. pallidior* (Puna and Patagonia) would be the most derived forms within the genus. Similar conclusions to the latter study were obtained by Braun *et al.* (2005) using the same molecular marker. In addition, the latter study recovered two subclades within *T. pallidior*, one from the Puna or Altiplano in the Andes which they called '*T. pallidior* north', and the other from the Argentinean Patagonia that Braun *et al.* called '*T. pallidior* south'. According to these authors, these two clades would represent two subspecies of *T. pallidior*. Braun *et al.* (2005) also validated *T. cinderella* from the Yungas of Argentina as different from *T. venustus* of Bolivia and recognized *T. tatei* as a valid species (previously validated by Solari 2002) from Peru, although not to *T. sponsorius* that would be synonymized with *T. cinderella*. Flores *et al.* (2000) stated that *T. sponsorius* could not be distinguished from *cinderella* based on colour pattern, but they can be differentiated based on cranial features. A recent revision of marsupials from the Neotropics considered *T. sponsorius* as a valid species based on information available on morphology (Gardner 2007). More recently, another molecular study that included *T. karimii* from the Brazilian Caatinga and Cerrado recovered the latter as basal to a clade that joined *T. pusillus* (*T. elegans*-*T. pallidior*) (Carvalho *et al.* 2009).

Other more specific revisions have used morphologic and/or molecular data to evaluate the variation observed in some particular taxa. Teta *et al.* (2009) based on morphology and cyt *b* sequences recognized a complex of three taxa within *T. pusillus*: *T. pusillus* sensu stricto restricted to the Bolivian and Paraguayan Chaco, *T. citellus* restricted to the Argentinean provinces of Corrientes and Entre Ríos, and *T. pulchellus* inhabitant of the dry Argentinean Chaco. In addition, Martin (2009) based on cranial and body characters recognized as *T. fenestrae* several specimens from the Pampas and Espinal ecoregions in Argentina, formerly classified as *T. elegans* and *T. pallidior*. Finally, a recent publication by Giarla *et al.* (2010) included most of the currently known species, to the exception of *T. cinderella* (recognized as a full species by Gardner 2007). Giarla *et al.* (2010) study based on mitochondrial DNA sequences and morphology, recognized nine species of *Thylamys* in two subgenera: *Xerodelphys* (new subgenus) for *T. karimi* and *T. velutinus*, and the subgenus *Thylamys* (sensu stricto) for *T. macrurus* and *T. pusillus*, as well as five other species in two monophyletic groups ('groups' based on morphology):

the *elegans* group (*T. elegans*, *T. pallidior* and *T. tatei*) and the *venustus* group (*T. sponsorius* and *T. venustus*).

This study was focused to evaluate the phylogenetic and biogeographical relationships of the genus *Thylamys*, with particular attention to the new classification schema proposed by Giarla *et al.* (2010). In addition, we report the existence of a new species of *Thylamys* from the Andes of Peru, evaluated the phylogenetic structuring within *T. pallidior* and *T. elegans*, as well as the validity of *T. sponsorius*, *T. cinderella* and *T. tatei* and the haplogroups recognized within *T. pusillus*. Finally, we hypothesize a biogeographical scenario for *Thylamys* differentiation and calibrated a molecular clock to propose times of origins for the species of the genus.

Material and methods

Tissues and specimens analysed

Voucher specimens for sequenced individuals were deposited in the Colección de Mamíferos del Centro Nacional Patagónico, Puerto Madryn, Chubut, Argentina (CNP); Colección de Flora y Fauna 'Profesor Patricio Sánchez Reyes' (SSUC), Departamento de Ecología, Pontificia Universidad Católica de Chile, Santiago, Chile; the Museum of Southwestern Biology (MSB), Department of Biology, University of New Mexico, Albuquerque, New Mexico; Programa de Investigaciones de Biodiversidad Argentina (PIDBA), Instituto Miguel Lillo, Universidad Nacional de Tucumán, Tucumán, Argentina; Museo de Historia Natural de la Universidad Nacional de San Agustín de Arequipa (MUSA), Arequipa, Perú; Museo de Historia Natural de la Universidad Nacional Mayor de San Marcos, Lima, Perú (MUSM); Texas Tech University, Natural Science Research Laboratory/Mammals, Lubbock, Texas (TK); Oklahoma Collection of Genomic Resources, Sam Noble Oklahoma Museum of Natural History (OCGR); field catalogs of Fernando Alfaro (FDA), L. Ignacio Ferro (LIF), Randy Gladwell (RRG), Jorge Pablo Jayat (JPJ), Marcela Nabte (MN), R. Eduardo Palma (EP), Ulyses Pardiñas (UP, AC), Enrique Rodríguez-Serrano (ER), Horacio Zeballos Patrón (HZP). Tissues and other data associated with each specimen were cross-referenced directly to each voucher specimen and stored in the collection using a special field catalog number, the NK number used by the SSUC and MSB. A detailed list of the specimens sequenced per locality is given in Table 1. We followed the American Society of Mammalogists guidelines during the collection and handling of all animals used in this work (Sikes *et al.* 2011).

Nucleotide sequence analyses

DNA was extracted from frozen liver samples treated with the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin). The complete cytochrome *b* mitochondrial gene (cyt *b*; 1144 bp) was amplified for 70 individuals representing 52 localities (Fig. 1). In addition, we included 33 cyt *b* sequences from additional 31 localities that were available from the GenBank (see Table 1). We also amplified the nuclear transthyretin intron 1 (TTR; 1058 bp) for 27 of the above 84 individuals from 19 localities. Primers used to amplify the cyt *b* gene were 14724 (L—Irwin *et al.* 1991) and LBE13 (5′ TTG TTG GCT TAC AAG GCC AGT 3′), using the following thermal cycle protocol: initial denaturation for 8 min at 95 °C, followed by 30 cycles of 94 °C (1 min 30 s), 46 °C (45 s) and 72 °C (1 min 30 s). A final extension at 72 °C for 8 min terminated the reaction. Primers to amplify the TTR were the MP1 (forward) y MM2 (reverse) (Duan *et al.* 1995; Aldred *et al.* 1997), and the thermal cycle was performed using the following protocol (modified from Aldred *et al.* 1997): initial denaturation for 3 min at 94 °C, followed by 30 cycles of 94 °C (30 s), 65 °C (30 s) and 72 °C (1 min). The final extension was at 72 °C for 3 min. Double-stranded PCR products were purified with Qiaquick (Qiagen, Valencia, California). Cycle sequencing (Murray 1989) was performed using primers 14724, LBE13 and 15162 (Irwin *et al.* 1991) for the cyt *b* gene and MP1, MM2, MP5 y MM6 (Steiner *et al.* 2005) for the TTR intron, labelled with the Big Dye Terminator kit (Perkin Elmer, Norwalk, Connecticut). Sequencing reactions were analysed on an Applied Biosystems Prism 3100 (Foster City, CA, USA) automated sequencer. Sequences were aligned using the CLUSTAL X program (Thompson *et al.* 1997) and by eye. All sequences have been deposited in GenBank, and accession numbers for both cyt *b* and TTR sequences are given in Table 1.

Phylogenetic analyses

Phylogenetic analyses were conducted using maximum parsimony, maximum likelihood and Bayesian methodology on the cyt *b*, TTR and concatenated cyt *b*-TTR sequences. For parsimony analysis congruence between cyt *b* and TTR, data sets were tested using the partition homogeneity test (Farris *et al.* 1994) implemented in PAUP* 4.0 b10 (Swofford 2002) with 1000 replicates excluding invariant characters (Cunningham 1997). Phylogenetic trees were rooted with the outgroup criterion using the outgroups of *Thylamys*, *Lestodelphys halli* (Kirsch & Palma 1995) and *Marmosops impavidus*. Outgroups for the concatenated cyt *b*-TTR analysis also included *L. halli* and a chimerical sequence assembled from two different specimens (see Table 2 for GenBank accession numbers). For maximum parsimony (MP), we used PAUP* 4.0 b10 (Swofford 2002), treating all characters as unordered with four possible states (A, C, G, T) and using only those characters that were phylogenetically informative. For parsimony, a heuristic search was performed

Table 1 Collecting localities of *Thylamys* spp. Refer to Methods for acronyms of voucher specimens. Geographical coordinates were not available for all collecting sites

Voucher	GenBank access cyt *b*	GenBank Access TTR	Species	Locality	Region/ Province	Latitude	Longitude	Country	Elevation
HZP3083	KF164509		*T. pallidior*	Hacienda Ventilla, Huacullani	Puno	16°35′45.18″S	69°20′05.18″W	Perú	3800
HZP3079	KF164510		*T. pallidior*	Hacienda Ventilla, Huacullani	Puno	16°35′45.18″S	69°20′05.18″W	Perú	3800
HZP3069	KF164511	KF164581	*T. pallidior*	Hacienda Ventilla, Huacullani	Puno	16°35′45.18″S	69°20′05.18″W	Perú	3800
NK96067	KF164512		*T. pallidior*	Enquelga	Arica-Parinacota	19°13′13.8″S	68°44′42.6006″W	Chile	3900
NK96045	KF164513		*T. pallidior*	Enquelga	Arica-Parinacota	19°13′13.8″S	68°44′42.6006″W	Chile	3900
NK23533	HM583386*	KF164580	*T. pallidior*	Serranía Sama	Tarija	21° 26′ 59.99″S	64°52′ 1.2″W	Bolivia	3200
NK14721	HM583385*		*T. pallidior*	68 km (by road) N Comargo	Chuquisaca	20°8′ 59.99″S	65°16′ 58.8″W	Bolivia	3400
UCK481	KF164514		*T. pallidior*	Río Loa Alto	Antofagasta	21°56′49.6″S	68°36′37.3″W	Chile	3053
EP476	KF164515		*T. pallidior*	La Huaica	Tarapacá	20°26′22″S	69°32′15″W	Chile	
EP440	KF164516		*T. pallidior*	Quebrada Camarones	Arica-Parinacota	19°11′25.3″S	70°16′07″W	Chile	
EP434	KF164517	KF164582	*T. pallidior*	Quebrada Camarones	Arica-Parinacota	19°11′25.3″S	70°16′07″W	Chile	
RRG34	KF164518		*T. pallidior*	Aplao, Beringa, Castilla	Arequipa	16°20′44.09″S	72°28′37.64″W	Perú	680
HZP2691	KF164519		*T. pallidior*	Quebrada Canchimayo, Chiguata	Arequipa	16°24′ 11.8512″	71° 24′ 14.115″	Perú	
MN38	KF164520	KF164583	*T. pallidior*	Estancia San Lorenzo	Chubut	42°06′53″S	63°55′23″W	Argentina	
UP397 (CNP1921)	HM583413*		*T. pallidior*	Cerrito Piñón, Estancia Collón Curá	Neuquén	40°14′57″S	70°37′54″W	Argentina	608
LTU77 (CNP1919)	HM583411*		*T. pallidior*	Campamento Base, Sierra de la Ventana	Buenos Aires	38°4′ 8.4″S	62°1′ 22.8″W	Argentina	50
UP793 (CNP541)	KF164521		*T. pallidior*	Establecimiento San Nicolás, Meseta de Somuncurá	Río Negro	41°43′50″S	67°09′49″W	Argentina	884
DUS29 (CNP1409)	KF164522		*T. pallidior*	Bahía Cracker	Chubut	42°57′02″S	64°28′45″W	Argentina	
PNG1009 (CNP1678)	KF164523		*T. pallidior*	Piedra Grande	Chubut	42°57′02″S	64°28′45″W	Argentina	
AC47	HM583392*	KF164584	*T. pallidior*	La Tapera, Pampa de Achala	Córdoba	31°37′19.2″S	64°54′39.7″W	Argentina	1959
JPJ1292	KF164524		*T. pallidior*	Agua del Gauchi, Capayán	Catamarca	28°46′52′′S	66°18′43′′W	Argentina	2024
PNG1055 (CNP1693)	KF164525		*T. pallidior*	Las Plumas	Chubut	43°43′48″S	67°15′48″W	Argentina	
OCGR2153	HM583393*		*T. pallidior*	11 km E Humahuaca, 2 km E Pucará on road to Cianzo	Jujuy	23°11′59.99″S	65°14′34.799″W	Argentina	3505
OCGR 7390	HM583408*		*T. pallidior*	8.2 km S Sey	Jujuy	24°0′ 46.8″S	66°30′ 54″W	Argentina	4167
OCGR 7279	HM583406*		*T. pallidior*	16 km S and 1.8 km W Barrancas, along Río de las Burras	Salta	23°24′ 57.6″S	66°12′ 21.6″W	Argentina	3521

Table 1 Continued

Voucher	GenBank access cyt *b*	GenBank Access TTR	Species	Locality	Region/ Province	Latitude	Longitude	Country	Elevation
OCGR 3957	HM583403*		*T. pallidior*	17 km NW Cachi	Salta	25°1′19.1994″S	66°14′16.7994″W	Argentina	3155
OCGR 43	HM583397*		*T. pallidior*	3 km W Refugio Militar General Alvarado	Mendoza	34°16′ 12″S	69° 21′ 46.799″W	Argentina	
OCGR 230	HM583394*		*T. pallidior*	Salinas del Diamante RR Station	Mendoza	34°58′1.1994″S	68°49′58.7994″W	Argentina	
NK95436	KF164526	KF164585	*T. elegans*	Rinconada de Maipu	Santiago	33°29′40.92″S	70°53′34.5006″W	Chile	
NK95354	KF164527		*T. elegans*	Rinconada de Maipu	Santiago	33°29′40.92″S	70°53′34.5006″W	Chile	
NK95691	KF164528		*T. elegans*	San Carlos de Apoquindo	Santiago	33°28′8.2806″S	70°29′18.5388″W	Chile	
NK95677	KF164529		*T. elegans*	San Carlos de Apoquindo	Santiago	33°28′8.2806″S	70°29′18.5388″W	Chile	
NK95971	KF164530		*T. elegans*	Rinconada de Huechún, Colina	Santiago	33°1′3.8994″S	70°49′1.3116″W	Chile	
NK27583	HM583376*		*T. elegans*	Parque Nacional Fray Jorge	Coquimbo	30°40′1.2″S	71°40′1.2″W	Chile	
NK96879	KF164531		*T. elegans*	Parque Nacional Fray Jorge	Coquimbo	30°38′18.276″S	71°39′16.596″W	Chile	
SSUC519	KF164532		*T. elegans*	Desembocadura Rio Loa	Antofagasta	21°25′33.1449″S	70°03′22.32691″W	Chile	
SSUC520	KF164533	KF164586	*T. elegans*	Desembocadura Rio Loa	Antofagasta	21°25′33.1449″S	70°03′22.32691″W	Chile	
NK160526	KF164534		*T. elegans*	Vilches Alto	Maule	35°35′4.7004″S	71°5′27.999″W	Chile	
NK160518	KF164535		*T. elegans*	Vilches Alto	Maule	35°35′4.7004″S	71°5′27.999″W	Chile	
NK160466	KF164536	KF164588	*T. elegans*	Tregualemu	Maule	35°56′59.5998″S	72°44′38.3994″W	Chile	
NK106178	KF164537	KF164587	*T. elegans*	Duao, Licanten	Maule	34°52′55.56″S	72°9′15.0834″W	Chile	
NK160945	KF164538		*T. elegans*	Lipimávida, Vichunquén	Maule	34°52′12.2″S	72°08′50.6″W	Chile	
NK160972	KF164539		*T. elegans*	Lipimávida, Vichunquén	Maule	34°52′12.2″S	72°08′50.6″W	Chile	
NK105928	KF164540		*T. elegans*	Las Peñas, San Fernando	O′Higgins	34°45′59.2992″S	70°46′34.3992″W	Chile	
HZP3576	KF164541		*T.* sp.	Acho, Ayo, Valle de los Volcanes, Castilla	Arequipa	15°39′47.67″S	72°18′16.02″W	Perú	1900
MUSA1787	KF164542	KF164592	*T.* sp.	Andagua, Valle de los Volcanes, Castilla	Arequipa	15°29′04.01″S	72°20′47.75″W	Perú	3500
MUSA1789	KF164543		*T.* sp.	Tapay, Valle del Colca, Caylloma	Arequipa	15°35′34.33″S	71°57′01.07″W	Perú	2700
MUSA1791	KF164544		*T.* sp.	Tapay, Valle del Colca, Caylloma	Arequipa	15°35′34.33″S	71°57′01.07″W	Perú	2700
RRG68	KF164545		*T.* sp.	Chuquibamba, Castilla	Arequipa	15°50′04.71″S	72°38′21.69″W	Perú	2800
RRG69	KF164546		*T.* sp.	Chuquibamba, Castilla	Arequipa	15°50′04.71″S	72°38′21.69″W	Perú	2800
HZP3487	KF164547		*T.* sp.	Hacienda el Carrizal, Caravelí	Arequipa	15°29′04.01″S	72°20′47.75″	Perú	1750
HZP3481	KF164548		*T.* sp.	La Cueva, Altos de Caravelí	Arequipa	15°39′17.09″S	73°13′51.49″W	Perú	3500
HZP2582	KF164549		*T.* sp.	Lomas de Atiquipa	Arequipa	15°45′34.55″S	74°22′33.49W	Perú	900
HZP2595	KF164550		*T.* sp.	Quebrada de la Waca, Lomas de Taymara	Arequipa	15°43′ 26.043″	74°18′ 52.6638″	Perú	350
MVZ116614	HM583420*		*T.* sp.	3 mi W Atico	Arequipa	16°13′58.8″S	73°39′0″W	Perú	30
MVZ137896	HM583423*		*T.* sp.	15 mi WNW Puquio	Ayacucho	14°37′1.2″	74°20′20.3994″W	Perú	3657
HZP37	KF164551	KF164589	*T.* sp.		Lima	12°25′36″S	75°50′43″W	Perú	2680

Table 1 Continued

Voucher	GenBank access cyt *b*	GenBank Access TTR	Species	Locality	Region/ Province	Latitude	Longitude	Country	Elevation
				Tinco, Huantán, Provincia de Yauyos					
HZP51	KF164552	KF164590	*T.* sp.	Tinco, Huantán, Provincia de Yauyos	Lima	12°25′36″S	75°50′43″W	Perú	2680
HZP48	KF164553		*T.* sp.	Tinco, Huantán, Provincia de Yauyos	Lima	12°25′39″S	75°51′14″W	Perú	2628
HZP30	KF164554	KF164591	*T.* sp.	Tinco, Huantán, Provincia de Yauyos	Lima	12°25′39″S	75°51′14″W	Perú	2628
MUSM23121	KF164555		*T. tatei*	Pallasca, Pampas, 10 Km. to Pallasca	Ancash	8°13′45.984″S	77°54′18.684″W	Perú	2650
MUSM23253	KF164556	KF164593	*T. tatei*	Pallasca, Pampas, Río Conchucos	Ancash	8°12′50.364″S	77°55′29.6754″W	Perú	2650
MVZ135504	HM583449*		*T. tatei*	Ancash, 1 km N and 12 km E Pariacoto	Ancash	9°30′3.5994″S	77°46′26.4″W	Perú	2591
MUSM10738	KF164557		*T. tatei*	Chancay, Reserva Nacional Lachay	Lima	11°21′30″S	77°22′10″W	Perú	150
OCGR1525	HM583369*		*T. pulchellus*	Chumbicha, 0.5 km E Highway 38 on Highway 60	Catamarca	28°52′1.2″S	66°13′58.8″W	Argentina	457
JPJ1743	KF164558	KF164596	*T. pulchellus*	Trancas, intersection between H. 9 & India Muerta stream	Tucumán	26°33′16.5′′S	65°16′44.0′′W	Argentina	658
OCGR4240	HM583371*		*T. pulchellus*	Salinas de Ambargasta, ca. 8 km SE Cerro Rico	Santiago del Estero	29°4′1.2″S	64°37′58.7994″W	Argentina	141
OCGR1984	HM583370*		*T. pulchellus*	Virgen del Valle picnic area on Highway 64	Santiago del Estero	28°7′58.7994″S	64°49′58.7994″W	Argentina	701
OCGR3770	HM583372*		*T. pulchellus*	Bella Vista	Catamarca	28°37′37.1994″S	65°29′49.1994″W	Argentina	974
LIF091	KF164559		*T. pulchellus*	junction H. 95 & Riacho Pirane, 7 Km N junction H. 95 & 81	Formosa	25°13′14.8′′S	59°42′49.1″W	Argentina	
LTU539	HM583374*		*T. citellus*	Estancia Santa Ana de Carpinchorí	Entre Ríos	30°47′45.5994″S	58°38′38.3994″W	Argentina	134
BMH9881912	HM583373*		*T. citellus*	Goya	Corrientes	29°7′58.7994″S	59°16′1.2″W	Argentina	
NK25136	KF164560		*T. pusillus*	Estancia Bolivar	Tarija	21°38′S	62°37′W	Bolivia	400
NK23289	KF164561	KF164594	*T. pusillus*	53 km E of Boyuibe	Santa Cruz	20°27′S	62º50′W	Bolivia	
NK12574	HM583415*	KF164595	*T. pusillus*	3.8 km E (by road) Carandayti	Chuquisaca	20°46′S	63°03′W	Bolivia	480
NK25139	HM583416*		*T. pusillus*	Estancia Bolivar	Tarija	21°38′S	62°37′W	Bolivia	400
TK66476	HM583419*		*T. pusillus*	Parque Nacional Teniente Enciso	Boquerón	21°3′0″S	61°45′0″W	Paraguay	
TK65612	HM583418*		*T. pusillus*	Fortín Pikyrenda	Alto Paraguay	20°4′58.7994″S	61°46′58.8″W	Paraguay	
NK12539	HM583414*		*T. pusillus*	Tita	Santa Cruz	18°15′0″S	62°6′0″W	Bolivia	300
NK27536	HM583383*	KF164597	*T. macrurus*	7 km NE Escuela Agropecuaria	Concepción	23°21′0″S	57°22′58.8″W	Paraguay	
APC932	HM583382*		*T. macrurus*	Fazenda Califórnia	Mato Grosso do Sul	20°40′58.7994″S	56°52′ 1.1994″W	Brazil	650
PIDBA1227	KF164562		*T. sponsorius*	El Siambon on the edge of Rio Grande	Tucumán	26°46′3′′S	65°28′22′′W	Argentina	

Table 1 Continued

Voucher	GenBank access cyt *b*	GenBank Access TTR	Species	Locality	Region/ Province	Latitude	Longitude	Country	Elevation
PIDBA1228	KF164563	KF164602	*T. sponsorius*	El Siambon on the edge of Rio Grande	Tucumán	26°46′3″S	65°28′22″W	Argentina	
PIDBA1208	KF164564		*T. sponsorius*	El Siambon on the edge of Rio Grande	Tucumán	26°46′3″S	65°28′22″W	Argentina	
LIF168	KF164565		*T. sponsorius*	on the foothills of Chasquivil, 5 km upstreams La Hoyada on Rio Hoyada	Tucumán	26°39′28″S	65°33′32.7″	Argentina	
LIF134	KF164566		*T. sponsorius*	on the foothills of Chasquivil, 5 km upstreams La Hoyada on Rio Hoyada	Tucumán	26°39′28″S	65°33′32.7″	Argentina	
JPJ1010	KF164567		*T. sponsorius*	10 km S Hualinchay, on the road to Lara, Trancas	Tucumán	26°19′20.2″S	65°36′45.5″W	Argentina	2300
PIDBA1300	KF164568		*T. sponsorius*	21.7 km E Santa Clara, road to El Fuerte	Jujuy	24°17′42″S	64°27′58″W	Argentina	
PIDBA1166	KF164569	KF164603	*T. sponsorius*	road to Isla de Cañas, 43.7 km NW of the junction between 50 and 18 highways	Salta	23°00′S	64°33′W	Argentina	
NK23952	KF164570	KF164604	*T. sponsorius*	5 km N NW Entre Ríos	Tarija	21°39′36″S	64°12′0″W	Bolivia	
PIDBA1168	KF164571		*T. sponsorius*	road to Isla de Cañas, 43.7 km NW of the junction between 50 and 18 highways	Salta	23°00′S	64°33′W	Argentina	
JPJ1807	KF164572		*T. venustus*	Finca Las Moras, Santa Bárbara	Jujuy	23°49′38.8″S	64°31′33.5″W	Argentina	460
PIDBA1141	KF164573	KF164598	*T. venustus*	Piquirenda Viejo	Salta	22°20′S	63°47′W	Argentina	
JPJ1820	KF164574		*T. venustus*	Finca Las Moras, Santa Bárbara	Jujuy	23°49′38.8″S	64°31′33.5″W	Argentina	460
NK21815	HM583492*		*T. venustus*	Río Limón	Chuquisaca	19°33′S	64°08′W	Bolivia	1300
NK12671	KF164575		*T. venustus*	Porvenir	Chuquisaca	20°45′S	63°13′W	Bolivia	675
JPJ1022	KF164576	KF164599	*T. venustus*	El Piquete farm, on the edge of Arroyo Volcán, Santa Bárbara	Jujuy	24°18′S	64°56′W	Argentina	
FDA2291	KF164577	KF164600	*T. venustus*	Vila Vila – Parotani	Cochabamba	17°27′58″S	66°19′17″W	Bolivia	2480-2558
FDA1547	KF164578	KF164601	*T. venustus*	Chawarani – Tarata	Cochabamba	17°38′21″S	66°01′50″W	Bolivia	2790-2842
OMNH22284	HM583450*		*T. velutinus*	25 km S Brasilia	D Federal, Brasilia	16°0′32.4″S	47°55′1.2″W	Brazil	
OMNH37216	HM583451*		*T. velutinus*	Jardín Botánico	D Federal, Brasilia	15°46′58.7994″S	47°55′1.2″W	Brazil	1100
APC1561	HM583381*		*T. karimii*	Rio da Conceição	Tocantins	11°11′2.3994″S	46°50′38.4″W	Brazil	
MN36926	EF051700*		*T. karimii*	55 km N Niquelandia	Goias	14°28′S	48°27′W	Brazil	
PNG1399	KF164579	KF164605	*Lestodelphys halli*	Fofo Cahuel	Chubut	42°22′31.281″S	70°29′39.0012″W	Argentina	
MVZ171408	U34669*		*Marmosops impavidus*	72 km NE Paucartambo (by road), km 152	Cusco	13°10′42.16″S	71°35′05.48″W	Perú	1460
AMNH272760		AJ628398*	*Marmosops impavidus*		Loreto			Perú	

*Sequences previously available in GenBank.

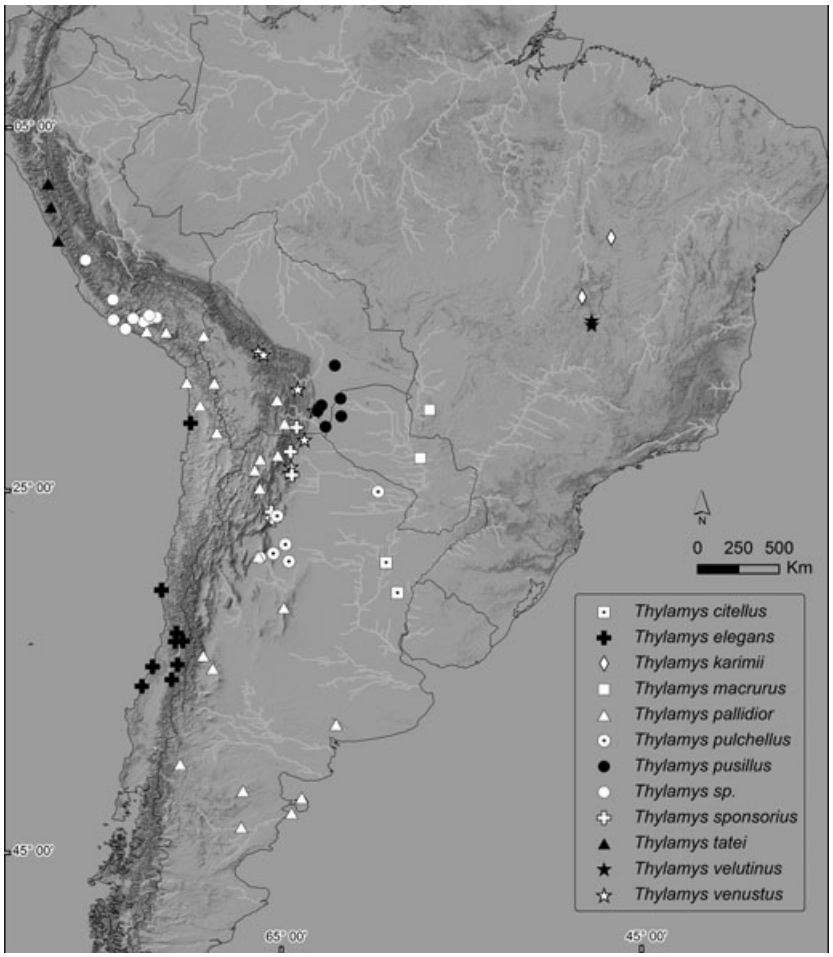

Fig. 1 Map of collecting localities for specimens of *Thylamys* sequenced for this study. Symbols on the map represent collecting localities for each of the species analysed in this study.

Table 2 Distance values among *Thylamys* spp. based on the Kimura-2-parameters model of sequence evolution

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*T. pallidior*_A													
2	*T. pallidior*_B	0.053												
3	*T. elegans*_north	0.106	0.104											
4	*T. elegans*_south	0.096	0.097	0.057										
5	*T.* sp.	0.108	0.110	0.110	0.106									
6	*T. tatei*	0.116	0.121	0.125	0.119	0.082								
7	*T. pulchellus*	0.136	0.140	0.157	0.149	0.143	0.147							
8	*T. citellus*	0.146	0.152	0.160	0.160	0.155	0.161	0.057						
9	*T. pusillus*	0.137	0.138	0.157	0.150	0.152	0.151	0.077	0.084					
10	*T. macrurus*	0.164	0.165	0.170	0.170	0.160	0.170	0.181	0.168	0.153				
11	*T. sponsorius*	0.196	0.199	0.184	0.200	0.188	0.201	0.202	0.194	0.188	0.168			
12	*T. venustus*	0.165	0.164	0.166	0.159	0.168	0.179	0.173	0.168	0.164	0.165	0.150		
13	*T. velutinus*	0.171	0.171	0.194	0.185	0.179	0.184	0.194	0.190	0.184	0.196	0.200	0.164	
14	*T. karimii*	0.172	0.177	0.190	0.177	0.157	0.166	0.173	0.172	0.177	0.165	0.206	0.194	0.164

with 100 random additions and branch swapping via tree-bisection–reconnection (TBR—Nei & Kumar 2000). A strict consensus tree was estimated when more than one equally parsimonious tree was obtained, and we obtained the consistency index (CI) and the retention index (RI) for the most parsimonious tree. The reliability of nodes was

estimated by nonparametric bootstrap (Felsenstein 1985) with 1000 pseudoreplications. Maximum likelihood (ML) searches were performed with the Treefinder version of October 2008 (Jobb 2008). We selected the best-fitting model of nucleotide substitution using the corrected Akaike Information Criterion (AICc—Akaike 1974) in Treefinder. We evaluated support for the nodes with 1000 bootstrap replicates (Felsenstein 1985). For cyt *b* sequences, the AICc identified the GTR + I + Γ model (Tavaré 1986) as the best model of base substitution. The proportion of invariable sites value was 0.5520, and the gamma shape parameter was =1.7661. The proportions of nucleotides were A = 0.2806, C = 0.2867, G = 0.1287 and T = 0.3038. For the concatenated sequences, the AICc identified the GTR + Γ (Tavaré 1986) as the substitution model. The gamma shape parameter was 0.1116, and the proportions of nucleotides were A = 0.3003, C = 0.2306, G = 0.1504 and T = 0.3185. Sequences also were analysed in a Bayesian framework to estimate the posterior probabilities of phylogenetic trees. Ten million phylogenetic trees were generated, sampling every 1000 trees to assure that successive samples were independent. The first 1000 trees of the sample were removed to avoid including trees before convergence of the Markov Chain. Given that we used two independent molecular markers, we applied a general likelihood-based mixture model (MM) as described by Pagel & Meade (2004, 2005), based on the general time-reversible (GTR) model (Tavaré 1986; Rodríguez *et al.* 1990) of sequence evolution. This model accommodates cases in which different sites in the alignment evolved in qualitatively distinct ways but does not require prior knowledge of these patterns or partitioning data. These analyses were conducted using the Bayes Phylogenies software (http://www.evolution.reading.ac.uk/BayesPhy.html). To find the best mixture model of evolution, we estimated the number of GTR matrices using a reversible-jump Markov Chain Monte Carlo method (RJMCMC—Pagel & Meade 2006). The RJMCMC visits the different mixtures of GTR matrices in proportion to their posterior probabilities, 'jumping' from simple to complex models or vice versa, making a direct estimate of the support of 1GTR, 2GTR, 3GTR, and so on. Only the combination of matrices with the fewest number of parameters that significantly increased the likelihood was used (1GTR + Γ for cytochrome *b* data; 2GTR + Γ for concatenated data) to compute a 50% majority rule consensus tree. The percentage of samples that recover any particular clade on this tree represents the posterior probability of that clade; these are the *P* values, and $P \geq 95\%$ was considered evidence of significant support for a clade (Huelsenbeck & Ronquist 2001). Finally, and to compare our results with previous studies of didelphid cyt *b* variation (*e.g.*, Patton *et al.* 1996; Giarla *et al.* 2010), we computed the Kimura-2-Parameter (K2P) among pairwise sequences of *Thylamys* spp.

The divergence times of *Thylamys* spp. were estimated using BEAST 1.6.2 software (Drummond & Rambaut 2007). As prior information, we used a GTR + Γ + I model of sequence evolution, the Yule process of speciation, and two points of fossil calibration: (i) *Thylamys pinei* from the Huayquerian (Cerro Azul Formation) of La Pampa, Argentina (6 My, upper Miocene; Goin *et al.* 2000), with affinities to *T. venustus;* and (ii) *Thylamys contrerasi* from the Montehermosan (Monte Hermoso Fm.) of Buenos Aires, Argentina (4–15 My, early Pliocene; Deschamps *et al.* 2012), with affinities to *T. pusillus*. These fossil points were treated as soft bonds and represented as a normal distributions. The 95% of the prior probabilities represent the whole SALMA age where the fossil was dated (*i.e. T. pinei* 7.5–2.3 My; *T. contrerasi* 5–4 My). Analyses were based on two models of mutation rate: an uncorrelated lognormal relaxed clock and an uncorrelated exponential relaxed clock. To find the best molecular clock model, we used Bayes factor to compare the two clock models. The MCMC chain was run for 20 000 000 generations (10 000 generations were discarded as burn in, before the posterior probability distribution of the model converged), with parameters sampled every 10000 steps. Examination of MCMC samples using TRACER v1.5 software (Rambaut & Drummond 2007) suggested that the independent chains were each adequately sampling the same probability distribution and that effective sample sizes for all parameters of interest were >500.

Ancestral distribution of Thylamys

We used the package BioGeoBEARS ('BioGeography with Bayesian Evolutionary Analysis in R Scripts'; Matzke 2013; http://cran.r-project.org/web/packages/BioGeoBEARS/index.html) to reconstruct the ancestral distribution of each node of our cyt b calibrated phylogenetic hypothesis of *Thylamys*, with the intraspecific diversity collapsed. Specifically, we used the DEC (dispersal–extintion–cladogenesis; Ree & Smith 2008) and DEC+j (Matzke 2013) model to obtain a probability distribution of the most probable ancestral areas. The DEC model has two free parameters, *d* (dispersal rate, *i.e.* the rate of range addition along a phylogenetic branch) and *e* (extinction rate, *i.e.* the rate of local range loss along a phylogenetic branch); and the DEC+j model has three free parameters, *d*, *e* and j (weighting factor for jump dispersal or founder-event speciaton). For this analysis, we recorded the exact geographical distribution of *Thylamys* species included in the phylogenetic analysis. For this, we made an exhaustive literature search, and then we coded species' distribution according to the ecoregions recognized by Morrone (2002, 2006). We used the Likelihood Ratio Test to select the best fit model to

generate the output of the biogeographical events and most probables areas of ancestral distribution.

Results

Phylogenetic relationships

All trees based on cyt *b* sequences recovered the same relationships among *Thylamys* taxa; thus, we show a single tree with bootstrap and posterior probability values for parsimony, likelihood and Bayesian phylogenies (Fig. 2). The maximum parsimony analysis recovered 384 trees, 1784 steps long, CI = 0.3700 (excluding uninformative characters), RI = 0.3223. Of a final cyt *b* matrix with 1149 characters, 430 were parsimony informative. The cyt *b* phylogeny shows a very well resolved and supported phylogenetic tree in which the species *T. karimii* occupies the most basal position in the phylogeny, followed by a clade that joined the two representatives of *T. velutinus* from Brazil (Fig. 2). Then, we obtained two major reciprocally monophyletic splits in the phylogeny: one representing *T. venustus* and *T. sponsorius* as sister species. The other major grouping of clades recovered a subdivided *T. pusillus* between Argentina and Bolivia–Paraguay, conforming a Chacoan clade, and a major clade including species from the Andes and the Coastal Desert of Peru and Chile that may well represent what Giarla *et al.* (2010) recognized as an Andean clade. However, the latter should be an Andean-coastal clade because this arrangement joined samples from the Andes and from the coast of the Pacific Ocean. *T. macrurus* joined this 'Chacoan/Andean-coastal clade' at a basal position. However, the phylogenetic position (cyt *b* and concatenated sequences; Figs. 2 and 3) of *T. macrurus* is uncertain, because this form was either recovered at the base of the *sponsorius-venustus* clade (parsimony analysis) or at the base of the Chacoan/Andean-coastal clade (with likelihood and Bayes; Figs. 2 and 3). In none of the analyses, the *macrurus* node recorded a significative support value. The Chacoan clade, on the other hand, recovered two subclades, one representing forms from the Bolivian Chaco that correspond to *T. pusillus*, *sensu stricto*, and a second subclade representing forms from the humid Argentinean Chaco that correspond to *T. pulchellus* (Teta *et al.* 2009). In the latter, two specimens from the localities of Entre Ríos and Corrientes in Argentina represent what Teta *et al.* (2009) recognized as *T. citellus*. Next in the tree the Coastal Andean forms: a sister relationship representing *T. tatei* from the Coastal Desert of Peru (Lima) and a new species represented by several specimens from Arequipa and Lima Peru. This new form of *Thylamys* was trapped in several localities of Arequipa and Lima between 250 and 3800 m. Finally, we recovered a clade representing the sister relationship of *T. pallidior* and *T. elegans*. As hypothesized by Braun *et al.* (2005), the *T. pallidior* clade recovered two subgroups: one constituted by specimens from the Andean Altiplano and transversal valleys in the Atacama Desert representing *T. pallidior* 'A' and the other group that included forms from the Patagonia of Argentina, *T. pallidior* 'B'. A similar topology was obtained for *T. elegans* of the Mediterranean ecoregion of Chile because we obtained a north and a south clade in the topology. In Fig. 3, we show the phylogeny between the concatenated cyt *b* and TTR sequences recovering a similar topology to that obtained with the cyt *b* data, with good support both in the bootstrap and posterior probability values.

Molecular clock

We performed the molecular clock calibration using the cytochrome *b* gene sequences for which we have sequences of all species considered in this study. The divergence time for *Thylamys* and the rest of mouse opossums (represented here for outgroups *Lestodelphys halli* and *Marmosops impavidus*) is hypothesized to have occurred about 24.19 My, late Oligocene, with *Thylamys* differentiation about 18.58 My (Fig. 4). The same topology shows the mean values for divergence times of other taxa: *T. sponsorius* and *T. venutus* would have diverged about 6.5 My, whereas *T. macrurus* from the rest of *Thylamys* from the Andean-coastal clade is hypothesized to have speciated about 12 My. Among the 'most recent' speciation events in the radiation of *Thylamys*, it is the 4.7 My for the new species of *Thylamys* and *T. tatei*, the 4.6 mya split between *T. pusillus* and *T. pulchellus*, and the 6.11 My for the differentiation between sister taxa *T. elegans* and *T. pallidior*, late Miocene times.

Ancestral distribution of Thylamys

The model that best fits the *Thylamys* phylogeny and distribution is the DEC model (lnL = −24.7), with $d = 0.01$ and $e = 0$ (Fig. 5). The most recent common ancestor's (MRCA) area of distribution of the genus *Thylamys* would be the Chaqueña ecoregion that according to Morrone (2002, 2006) includes the Caatinga, Cerrado, Chaco, Pampa and Monte ecoregions with species *T. velutinus* and *T. karimii* (Fig. 5). From these areas, four major routes may have followed (see below): (i) dispersal (range expansion) of the MRCA of *T. sponsorius-T. venustus* to what today constitute the Chaqueña-Amazonica ecoregion that comprise among others the Yungas and Chaco biomes (sensu Morrone 2002, 2006); (ii) dispersal of *T. macrurus* to the Chaqueña-Paranaenense ecoregions that includes the Brazilian Atlantic Forest; (iii) a vicariant and posterior dispersal event of the MRCA to the boreal, dry and wet Chaco ecoregion where *T. pusillus*, *T. pulchellus* and *T. citellus* diversified by sympatric processes, respectively; (iv) to the Paramo-Puneña (western slope af the Andes) ecoregion that comprises the Puna, Prepuna and the Coastal Desert of Peru and Chile with the MRCA of *T. pallidior-T. elegans*

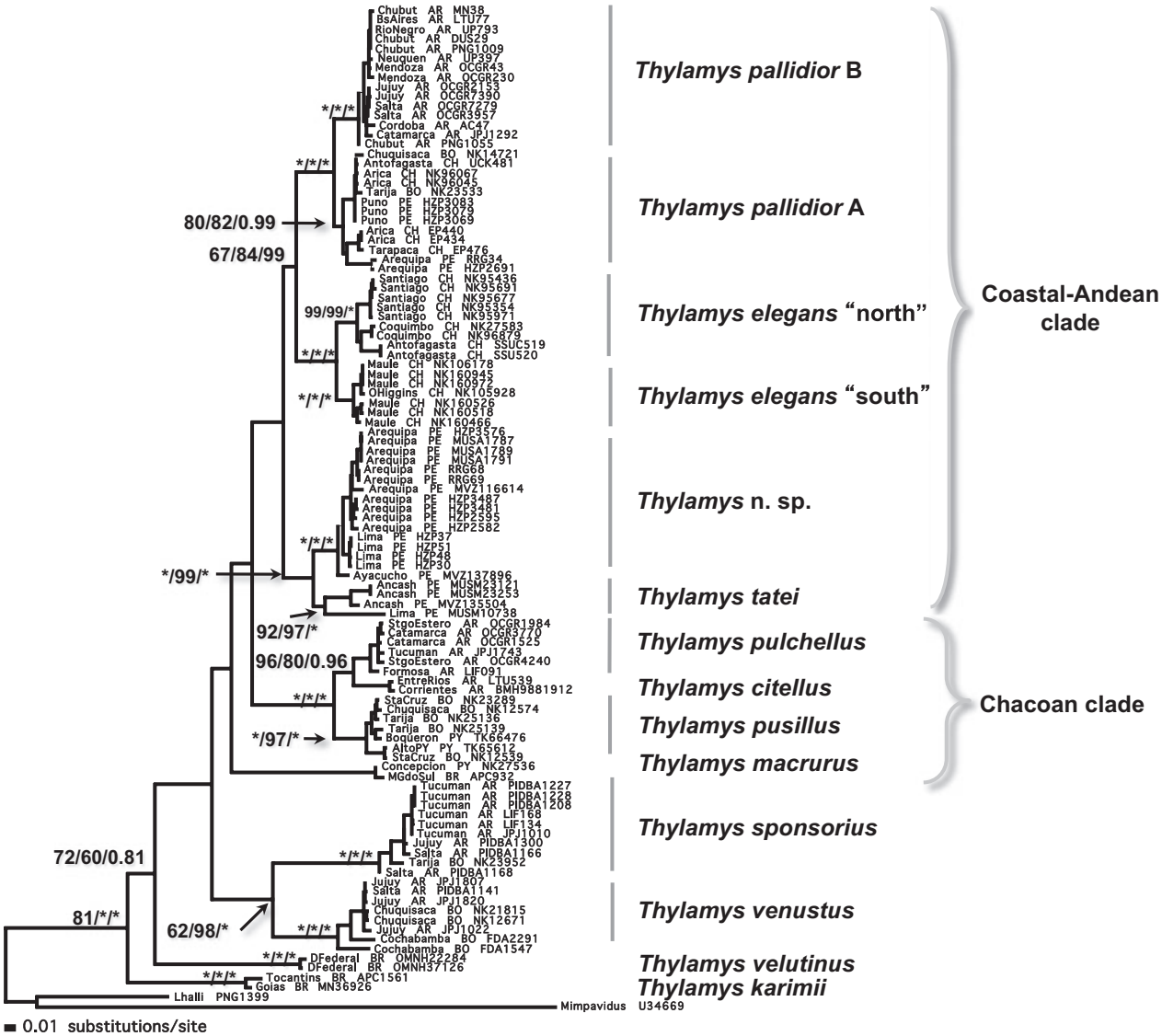

Fig. 2 Phylogenetic relationships of *Thylamys* based on the complete mitochondrial cytochrome *b* gene. Numbers on the nodes represent bootstrap support values for maximum parsimony and maximum likelihood analyses, as well as posterior probability support scores for Bayesian analysis (asterisks represent 100 support value either for parsimony and/or likelihood).

and *T. tatei*-*T.* sp. A final range expansion ocurred during the evolution of *T. pallidior* to the Patagonia (Fig. 5). Thus, the biogeographical history of the genus *Thylamys* was mostly dominated by range expansion to nearest areas of the diverse MRCAs and by a single vicariant event. No founder events and range contractions were detected.

Discussion

Phylogenetic relationships

The cytochrome *b*-based tree recovered the Cerrado form *Thylamys karimii* as the most basal taxon followed by the other Brazilian species *T. velutinus*. These results slightly contrast with Giarla *et al.* (2010) study that recovered the latter species as most basal based on cyt *b* sequences, although when they concatenated the latter sequence data with two other mitochondrial markers, *T. velutinus* and *T. karimii*, appeared at the base of the phylogenetic tree, as sister taxa, as showing our results. Then, two major clades are recovered with good support: one that includes the *T. venustus*-*T. sponsorius* relationship and the other major clade that includes *T. macrurus* as basal to a relationship consisting of *T. pusillus*, *T. citellus*, *T. pulchellus*, *T. tatei*, *T.* sp., *T. elegans* and.*T. pallidior*. The uncertain phylogenetic position of *macrurus* as either at the base of the *venustus-sponsorius* clade or at the base of the Andean clade was also recovered by Giarla *et al.* (2010) study. Needless to

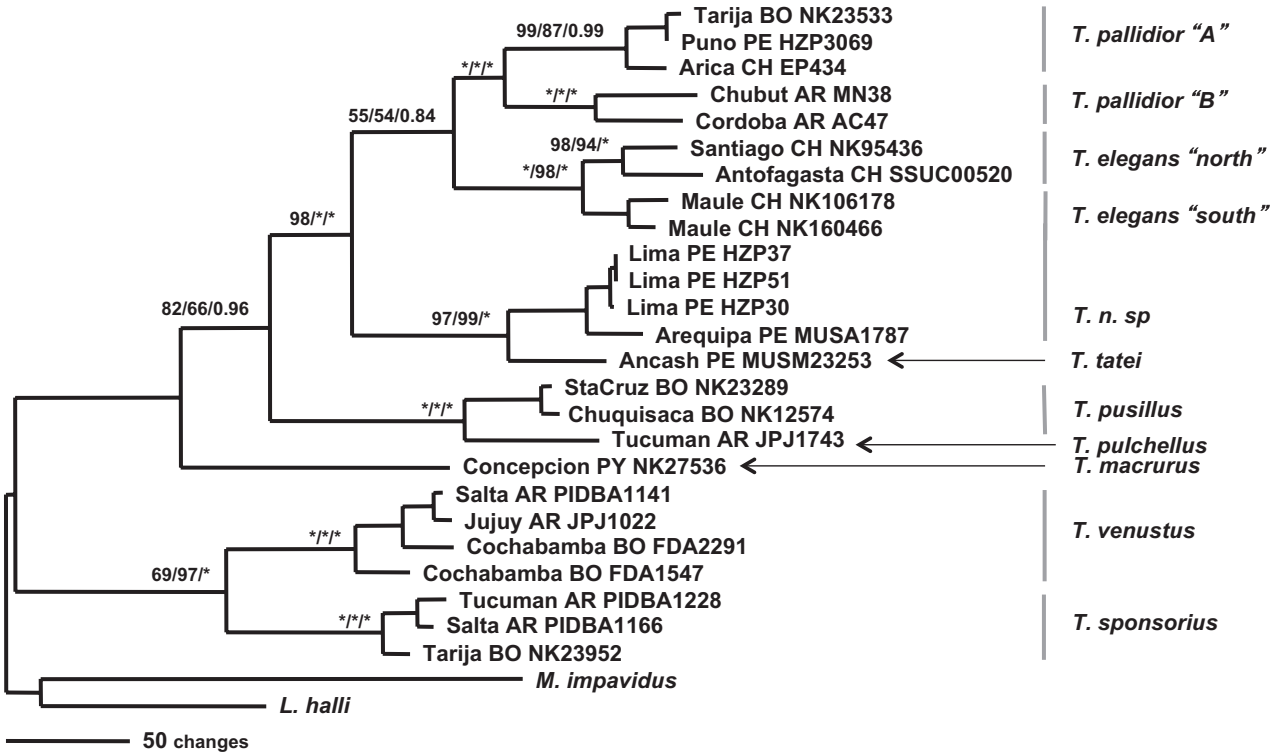

Fig. 3 Phylogenetic relationships of *Thylamys* based on the concatenated cytochrome *b* and TTR intron genes. Numbers on the nodes represent bootstrap support values for maximum parsimony and maximum likelihood analyses, as well as posterior probability support scores for Bayesian analysis (asterisks represent 100% support value either for parsimony and/or likelihood). *Marmosops impavidus* sequence is chimerical between two different individuals obtained from GenBank (see Table 2 for accession numbers).

say, additional specimens of *T. macrurus* would be useful to correctly allocate its phylogenetic position in the *Thylamys* reconstruction.

The association *venustus-sponsorius* is well supported, and our results confirm *sponsorius* as a valid species, different from *venustus*. Tate (1933) recognized *cinderella* and *sponsorius* as subspecies of *T. venustus*, but Flores *et al.* (2000) raised the taxonomic status of *cinderella* to species, proposition later supported by Braun *et al.* (2005). In fact, Flores *et al.* (2000) based on morphological features, recognized *T. venustus*, *T. cinderella* and *T. sponsorius* as valid species, but Braun *et al.* (2005) based on partial cyt *b* sequences synonymized *cinderella* and *sponsorius*, recognizing *T. venustus* and *T. cinderella*. Later, Gardner (2005) and Creighton & Gardner (2008) recognized *T. cinderella*, *T. sponsorius* and *T. venustus* as valid species following Flores *et al.* (2000); however, Voss & Jansa (2009) recognized *T. cinderella* (including *sponsorius*) as earlier proposed by Braun *et al.* (2005). Our cyt *b* phylogeny did not recover the two subclades or haplogroups obtained by Giarla *et al.* (2010, 2014) for *T. sponsorius*. We believe that this is probably due to a more wide sampling that finally recovered both haplogroups and that might represent two different subspecies (*sponsorius* and *janetta*) as Giarla *et al.* (2014) proposed. Giarla *et al.* (2010) recovered three cyt *b* haplogroups ('A', 'B' and 'C') within the *T. venustus* clade that seems to represent a single species. These authors proposed that haplogroup 'C' that includes representatives from the type locality of *cinderella* (Tucumán, Argentina) could be *cinderella*; haplogroup A could be *venustus* as that grouping included specimens from Cochabamba, the type locality, whereas, for the haplogroup B, there would be no available name. Furthermore, Giarla *et al.* (2010) proposed that *T. venustus* and *T. sponsorius* constitute sister and valid species based on molecular (cyt *b*) and morphometric data. The latter authors stated that *cinderella* and *venustus* would be unambiguously assignable to a morphometric small-sized clade that reunited *venustus*, in contrast to the large-sized morphometric clade that grouped *sponsorius*. Taxonomically, the older available name between *cinderella* and *venustus* is *venustus*; thus, this would be the species recognized in the clade. Based on morphological and geographical data, our study included several specimens recognized as either *sponsorius* or *venustus*, and the phylogeny consistently recovered both taxa as well-differentiated sister species. However, we still believe that a careful revision of

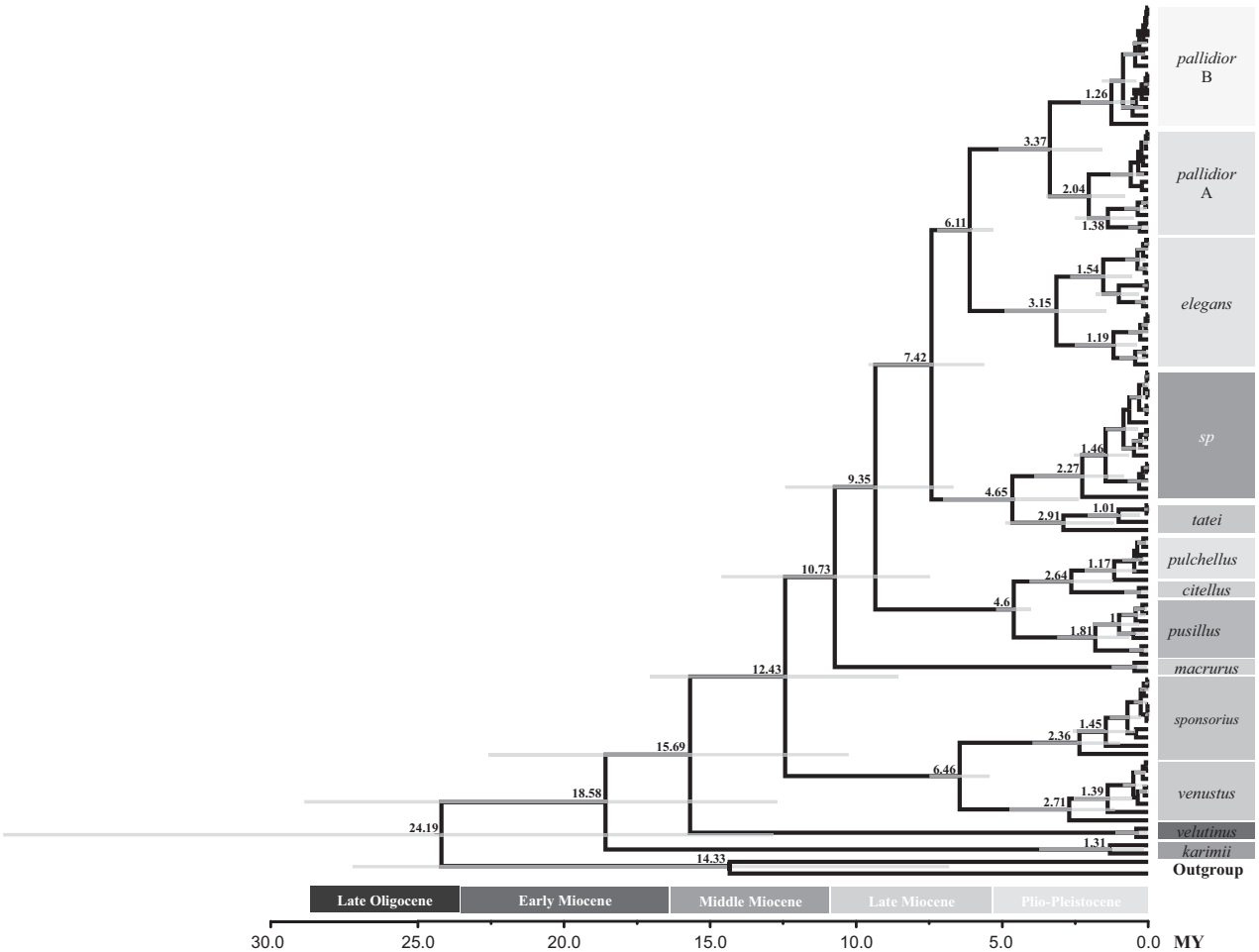

Fig. 4 Molecular clock calibration for the different nodes on the cyt *b* sequences of *Thylamys*. Numbers on the nodes reflect the mean value for the time of differentiation, and the grey bars reflect the 95% of the highest posterior density.

voucher specimens, holotypes and localities would be still necessary to clarify the taxonomy of *Thylamys* from northwestern Argentina and southern Bolivia.

The *T. pusillus* clade exhibited two major subclades, one representing forms from the boreal Chaco (Santa Cruz, Chuquisaca, Tarija, Alto Paraguay and Boquerón) that would correspond to *T. pusillus sensu stricto*. The latter subclade is in sister relationship to another subclade that recovered specimens from the dry Chaco of Argentina that would represent *T. pulchellus* (Santiago del Estero, Catamarca, Tucumán and Formosa; Teta *et al.* 2009). Even the *pulchellus* subclade recovered two other specimens from Entre Ríos and Corrientes provinces in Argentina, part of the humid Chaco, that must correspond to *T. citellus*. In a recent paper, Teta *et al.* (2009) proposed that *T. pusillus* is a complex of three species: *T. pusillus*, *T. pulchellus* and *T. citellus* all of them distinguished morphologic and biogeographically. Although in our phylogeny, we did not have a good sampling of what Teta *et al.* (2009) recognized as *T. citellus* (from the humid subtropical areas of Entre Ríos and Corrientes, coast of Paraná river and neighbour areas, Argentina), the latter form exhibited a sequence divergence of K2P 5.7% with respect to *T. pulchellus* from the dry Chaco (west of Paraná river). Distance values between well-recognized species within *Thylamys* fluctuate between K2P 7–20% (this work, Table 2; Patton *et al.* 1996; Giarla *et al.* 2010). Besides the low nucleotide divergence between *pulchellus* and *pusillus*, there are marked differences between the morphology of both taxa, particularly cranial, dental and body morphology of *citellus* with respect to *T. pulchellus* and even with *T. pusillus*, the other related taxon (Teta *et al.* 2009). The still low nucleotide divergence between *citellus-pulchellus*, coupled to the morphological divergence between both sister taxa, make *citellus* to be recognized at the specific level as Teta proposed earlier (Teta *et al.* 2009). Giarla *et al.* (2010) also recovered three well-

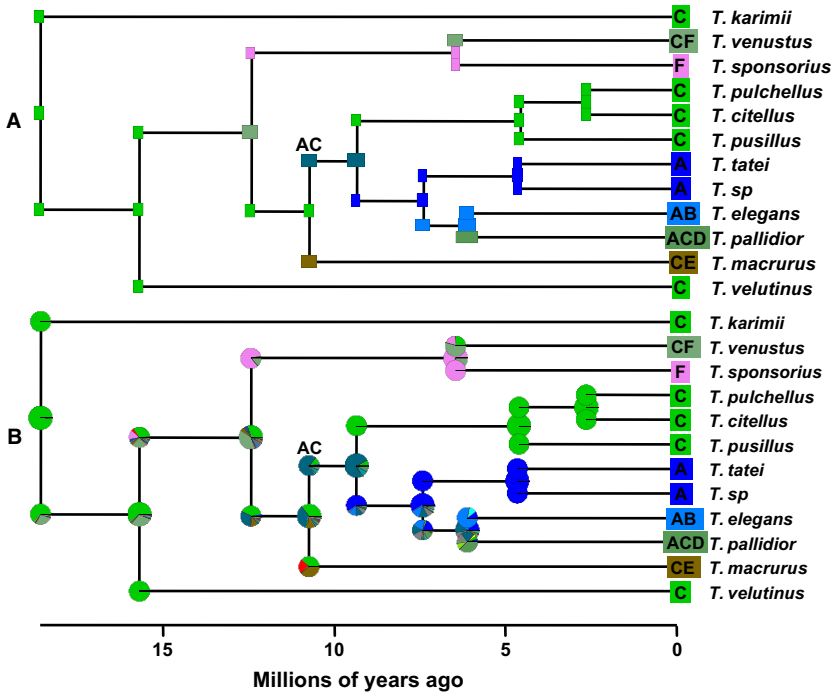

Fig. 5 Ancestral Distribution of *Thylamys*. Node's ancestral distribution has been estimated under the DEC model (Ree & Smith 2008) available at the BioGeoBEARS package for the software R. —A: the most probable event-based scenario of historical biogeography of *Thylamys*. —B: the pie charts represent the probability of the ancestral areas. The colour code is consistent with the tips' current distribution data (except for AC code). Ecogeographic zones according to Morrone (2002, 2006): A: Paramo Punan; B: Central Chilean; C: Chacoan; D: Patagonian; E: Parana; F: Amazonian.

supported clades within *pusillus* that they called Haplogroups A, B and C. They, however, did not consider these haplogroups as different species due to no sequence data are available for large intervening areas in the range of *pusillus*. Giarla *et al.* (2010), instead, consider that a biogeographical sampling bias could explain the difference among the three haplogroups. In any case, Giarla *et al.* (2010) proposed the application of available names for the three haplogroups as follows: Haplogroup A would correspond to *T. pusillus sensu stricto*, Haplogroup B to *T. pulchellus* and Haplogroup C to *T. citellus*.

The phylogeny of *Thylamys* recovered a new taxon that deserves specific status represented by specimens from Arequipa and Lima, Peru. This new species was recovered in sister relationship with specimens of *T. tatei* from the departments of Ancash and Lima, Perú. The new *Thylamys* species occurs in an altitudinal gradient between 250 up to 4800 m, being an endemic form from the Puna and the western slopes of the Peruvian Andes. Although we do not have a proper sample size representing *T. tatei*, as earlier proposed by Solari (2002, 2003) and more recently by Braun *et al.* (2005), we here recognize *T. tatei* as a valid species, phylogenetically the sister taxon to the new species of *Thylamys* from Arequipa and Lima, Perú. Thus, our results clarify the taxonomic problem mentioned by Giarla *et al.* (2010), because they could not properly resolve the phylogenetic relationships between *tatei* and the new taxon.

The last part of the tree depicted the sister relationship between *T. pallidior* and *T. elegans*. The clade representing the latter form is mostly restricted to Mediterranean Chile, showing two well-supported subclades that may well represent two subspecies. One of these subclades is represented by samples from O'Higgins and Maule regions, south of the Santiago Metropolitan region in Chile. The other subspecies of *T. elegans* grouped samples north of Santiago up to the Rio Loa mouth (about 22°S). This northern subspecies of *T. elegans* would correspond to *T. e. elegans* whose type locality is Valparaíso, Chile (Tate 1933). Although we do not have samples from the type locality, biogeographically the clade representing this form includes the Valparaíso region. We do not believe that this northern subspecies of *T. elegans* be *T. e. coquimbensis* because the type locality of the latter is Paiguano (30°S) an area located 120 kms E of Coquimbo at about 1600 m. The southern subspecies of *T. elegans* it might correspond to *T. e. soricina*, although the type locality for this taxon is located in Valdivia province, Los Rios region (40°S). The holotype of *soricina* was recorded by R. A. Philippi (1894), and no further

records of *Thylamys* exist in the Valdivia area, even no records of *Thylamys* have been reported south of the Biobio river (38° S) except for those of Tate (1933) and Greer (1966) in the Araucanía region (Angol, Malleco province 37°S). More recent records of *T. elegans* in southcentral Chile have been reported in the Maule region (e.g. Rio Maule and Siete Tazas) being those phenetically ascribed to *T. e. soricina* (Pine *et al.* 1979).

For *T. pallidior*, we also recognize two subclades representing two subspecies as proposed by Braun *et al.* (2005). The upper subclade 'A' recovered samples from the Puna of Peru and Chile, and the transversal valleys that cross the Atacama Desert in northern Chile, whereas subclade 'B' within *T. pallidior* corresponds to specimens from the southern portion of northwestern Argentina, the Monte, the Pampas and the Patagonia of that country. *T. pallidior* may be found in the lowlands of northern Chile such as Quebrada de Camarones (Arica-Parinacota region, 19°S; this paper) or La Huayca, Pampa del Tamarugal (20°S; Tarapacá region; this paper). This northernmost taxon of *pallidior* (the 'A' form) would clearly correspond to the subspecies *T. p. pallidior* because the type locality of this is Lago Poopo, Oruro, Bolivia (Tate 1933). On the other hand, the *pallidior* form that ranges east of the Andes, from NW Argentina, as far south as the Monte Desert, the Pampas and the Patagonia far south to southern Chubut (Formoso *et al.* 2011) would also constitute a subspecific taxon of *T. pallidior*. A recent work by Martin (2009) distinguished *T. fenestrae* from *T. pallidior* as a valid species based on morphological characters. However, some of the geographical localities of the specimens recognized as *fenestrae* by Martin (2009) correspond to the localities analysed by us (Abra de la Ventana, Buenos Aires and La Tapera, Córdoba, Argentina). We thus believe that the morphological differences between both nominal taxa are not strong enough and we believe that what Martin (2009) recognized as a different species would be the southern subspecies of *T. pallidior*. In this context, the name *fenestrae*, with type locality in Abra de la Ventana (Buenos Aires province, Argentina; Marelli 1931), remains available for subspecific treatments. At the time of finishing this work, a new paper by Giarla *et al.* (2014) that used different nuclear loci tested the 'species limits' (genetic isolation) using coalescent-based approaches to validate or refute morphologically cryptic lineage diversity within *T. pallidior*, *T. sponsorius* and *T. venustus*, previously detected with mtDNA (Giarla *et al.* 2010). They concluded that all mtDNA haplogroups should be recognized as genetically isolated lineages (Giarla *et al.* 2014).

Biogeographical scenario

Our results hypothesized that the majority of species of *Thylamys* differentiated during Miocene times and some others speciating during Plio-Pleistocene times. These epochs of time in South America (middle-late Cenozoic) characterized by dramatic changes in the landscape and temperature shifts (Potts & Behrensmeyer 1992). Indeed, by the end of Oligocene, humid forests were common in the southern part of the continent, whereas by middle Miocene, gradual cooling and drying started changing the landscape to a mixed forest and savanna habitats. In addition, it is a time for the rise of the Andes mountains with the consequent formation of a rain shadow in south-east South America (Potts & Behrensmeyer 1992). The biogeographical changes continued through the Plio-Pleistocene with an increased uplift and vulcanism in the Andes mountains, with the formation of the Panamanian Land Bridge, the arid-wet glacial cycles and the expansion and contraction of forests and savannas due to climate changes (Potts & Behrensmeyer 1992; Brown & Lomolino 1998; Scotese 2004). Is in the former biogeographical scenario that we have to understand the radiation of mouse opossums, particularly for the species of the genus *Thylamys*

The phylogenetic and the ancestral distribution analyses are consistent in showing *T. karimii* and *T. velutinus* as the most basal species in the *Thylamys* radiation, hypothesizing the Chaqueña ecoregion that comprises the Cerrado and the Caatinga biomes as the most ancestral areas for the origin of the genus, leaving *T. karimii* in both ecoregions and restricting *T. velutinus* to the Cerrado (Carmignotto & Monfort 2006). From these areas, four major radiations may have occurred within *Thylamys* that may have caused the divergence of different lineages in different biomes: (i) the MRCA of *T. sponsorius*-*T. venustus* clade that gradually invaded from the Cerrado and Caatinga to southern Bolivia and the Yungas triggering the speciation of sister taxa *T. venustus* in the eastern slope of the Andes at intermediate elevations (from Cochabamba, Bolivia to Salta Argentina) and *T. sponsorius* in the Argentinean portion of the Yungas; (ii) to the most humid and open areas of southern Amazonia: the Atlantic Forests of Mato Grosso do Sul, Brazil and eastern Paraguay with the species *T. macrurus*; (iii) a more complex scenario of dispersal and posterior vicariance of the MRCA triggering the diversification of *T. pusillus* in the boreal Chaco ecoregion of Bolivia and Paraguay), *T. pulchellus* in the dry Chaco of Argentina (e.g. Catamarca, Formosa provinces) and *T. citellus* in the most humid portion of the Argentinean Chaco (Entre Ríos, Corrientes provinces); and (iv) across the Andes where *T. pallidior*, *T. elegans*. *T. tatei* and *T.* sp. diversified in the Altiplano and Coastal Desert of Chile and Peru. Our results do not agree with Solari (2003) and Carmignotto & Monfort (2006) in the sense that the Brazilian forms of *Thylamys* (*karimii* and *velutinus*) derived from the Paraguayan forms *T. macrurus* and *T. pusillus*, from where they might have

dispersed into the open formations of Brazil. Instead, our data consistently showed that the Brazilian taxa are the most basal in the radiation of the genus, whereas the origin of Paraguayan forms would be much more recent.

With respect to the speciation of sister taxa *T. elegans* and *T. pallidior*, our results suggest a time of differentiation of about 6 mya which is late Miocene, a period marked by the rise of the Andes and marine transgressions (Ortiz-Jaureguizar & Cladera 2006). It is important to note that several fossil species of *Thylamys*, such as *T. pinei*, *T. contrerasi* and *T. zettii*, have been recorded from late Miocene-early Pliocene deposits in Central Argentina. According to Goin *et al.* (2000); see also Goin (1997), these species are morphologically close to the '*elegans* group' sensu Tate (1933). Our results propose a scenario of dispersal for the speciation of *elegans* and *pallidior* lineages. In fact, *T. pallidior* occurs at high altitudes in the Altiplano which might have occurred when the ancestral form occupied a wide distribution in what is now southern/northern Peru and Chile. That split might have occurred by vicariance leaving *T. pallidior* in the Andes and *T. elegans* in the lowlands. The occurrence of *T. pallidior* in the canyons of the lowlands of southern Peru and northern Chile could be a posterior dispersal from the Puna Andes, from where they might also have colonized eastward and southward through the Monte Desert, Pampas and Patagonia of Argentina. On the other hand, *Thylamys elegans* may have characterized by a wider distribution in the past (today restricted to Mediterranean Chile in the central portion of the country), including an important area of northern Chile, because we have found relictual populations at the mouth of Río Loa (21°S, Antofagasta region, this study), which is 1000 km north of the northernmost distribution of this species in the Coquimbo region of Chile (33°S).

The rivers seem to be an important barrier for the differentiation of *Thylamys* spp. Teta *et al.* (2009) stated that major rivers might have played an important role delimiting local species of *Thylamys*. Teta's *et al.* (2009) work proposed that what they recognized as *T. citellus* (the subclade within *T. pulchellus* in our study) is geographically separated from *T. pulchellus* by the Paraná river in central South America, and the latter would be separated from *T. pusillus* by the Bermejo river. On the other hand, *T. pusillus* seems not to cross the Paraguay river to the east where *T. macrurus* occurs (Voss *et al.* 2009), although *pusillus* and *macrurus* are not sister taxa. Finally, a similar scenario of rivers as barriers could explain the differentiation between *T. tatei* and the new species of *Thylamys* from Arequipa, Peru. Indeed, both taxa have an important altitudinal distribution, between 300 up to 3000 m for *T. tatei* (Solari 2002, 2003), and between 250 and 4800 m for *T.* sp. (H. Zeballos, personal observation). Indeed, between both major ranges, there are a series of major and narrow rivers that run across the Andes (e.g. the Cañete river) that separates the distribution of both taxa, with no data of sympatric populations, thus being the canyons and rivers an effective barriers for dispersal. The river differentiation could also apply for some of the subspecies that we are recognizing in this study, for example the split within *T. elegans*. The 'north' subclade in this species is geographically separated from the 'south' clade by the Maipo River in Chile, in the Metropolitan region of Santiago. The 'north' form ranges north to the river that may correspond to *T. elegans elegans*, whereas the south form would be an unnamed taxon of subspecific rank.

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