



# Phylogeny of *Marmosops* and the occurrence of *Marmosops pinheiroi* (Pine, 1981) (Didelphimorphia, Didelphidae) in the Cerrado savanna of Maranhão, Brazil

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**ABSTRACT.** The present study reviews the phylogeny of the genus *Marmosops* and expands the geographic range of *Marmosops pinheiroi* to the Brazilian State of Maranhão. Five specimens of *M. pinheiroi* were collected from the Inhamum Municipal Environmental Protection Area in Caxias, Maranhão. Total DNA was extracted and the mitochondrial cytochrome *b* gene was sequenced in an ABI PRISM 3500. Additional sequences of

*Marmosops* were obtained from GenBank for specimens from southeastern and northeast Brazil and the northern Amazon Forest. The results of the analysis indicated that *M. incanus* and *M. paulensis* form a well-supported clade, and that *M. pinheiroi*, *M. pakaraimae*, and *M. parvidens* are sister groups, with 71% support. The *M. pinheiroi* specimens from different regions grouped with high bootstrap values (99 and 100%). Interspecific genetic divergence varied from 8.3 to 21.5%, while intraspecific divergence ranged from 0.2 to 6.3% in *M. pinheiroi*, 0.3 to 2.9% in *M. parvidens*, and 0.4 to 6.9% in *M. incanus*. Given the taxonomic complexity of the genus, the results of the present study offer important insights into the taxonomic status of *M. pinheiroi*, as well as extend its known distribution 670 km south and east to the Brazilian State of Maranhão.

**Key words:** Genetic diversity; Genetic variability; Maranhão; Marsupial; *Marmosops pinheiroi*; Phylogeny

## INTRODUCTION

A total of 16 *Marmosops* species are currently recognized, and with the exception of *Marmosops invictus*, which is endemic to Panama, all known species are found in South America (Gardner, 2008; Voss et al., 2013). In Brazil, nine species are known to be present in the country's different terrestrial biomes (Paglia et al., 2012) - Atlantic Forest (*Marmosops paulensis* and *Marmosops incanus*), Cerrado (*M. incanus* and *Marmosops ocellatus*), Caatinga (*M. incanus*), Pantanal (*M. ocellatus*), and the Amazon Forest (*Marmosops bishopi*, *Marmosops impavidus*, *Marmosops neblina*, *Marmosops noctivagus*, *Marmosops parvidens*, and *Marmosops pinheiroi*).

The nomenclature of the taxon *M. pinheiroi* has undergone a series of modifications, being described initially as a subspecies (*Marmosops parvidens pinheiroi*) of the *M. parvidens* complex by Pine (1981). More recently, Voss et al. (2001) elevated this taxon to a full species. *M. pinheiroi* is found in the northern Amazon basin, ranging from southeastern Venezuela to the north of the Brazilian state of Pará (Gardner, 2008), in areas where two other species - *M. parvidens* and *Marmosops pakaraimae* - may also occur (Voss et al., 2013).

The species *M. pinheiroi*, *M. parvidens*, and *M. pakaraimae* share a lateral tubercle on the carpus, which differentiates them from other members of the genus. However, they are also differentiated from one another by other morphological characteristics. In particular, *M. pinheiroi* is characterized by a grayish-brown pelage, taller canines, a wider cranium and more accentuated lachrymals, whereas *M. parvidens* has reddish pelage, shorter canines, a narrower cranium, and reduced lachrymals; *M. pakaraimae* has dark brown pelage and mean craniodental measurements higher than those of other species (Voss et al., 2001, 2013).

However, while *M. pinheiroi* is well differentiated morphologically from the other species of the genus, traditional taxonomic approaches have not been conclusive in determining its species status (Dayrat, 2005). The small neotropical marsupials are an especially poorly-known group, specifically in regard to the delimitations of species ranges (Miranda et al., 2005). The use of molecular data, in particular DNA barcodes, has become increasingly important for the identification of species in taxonomic studies (Hebert et al., 2004). In the specific case of the didelphids, the mitochondrial cytochrome oxidase I (COI) and cytochrome

*b* genes have been extremely effective for species determination (Agrizzi et al., 2012).

Cytochrome *b* is widely used in phylogenetic studies of vertebrates, and the evolutionary and biochemical dynamics of the protein encoded by this gene are well understood. Divergence in this gene, associated with sister species, congeners, and confamilial genera, is phylogenetically informative, with a reduced likelihood of saturation in the substitution of nucleotides (Meyer, 1994; Russo et al., 1996; Johns and Avise, 1998). Gutiérrez et al. (2010) considered molecular data to be especially useful for the evaluation of the monophyly of species that are well-defined morphologically, in order to evaluate intraspecific genetic divergence; this has provided reliable support for the majority of the clades of sister species in the genera *Micoureus* and *Marmosa*.

Molecular analyses of the phylogenetic relationships among *Marmosops* species have focused on populations from southeastern Brazil, the western Amazon basin, the Guyanas, and Bolivia (Mustrangi and Patton, 1997; Patton et al., 2000; Voss et al., 2001, 2004b), but none of these studies have included specimens from the Brazilian State of Maranhão. In the present study, based on the analysis of the cytochrome *b* gene, we expanded the geographic distribution of *M. pinheiroi* to include Maranhão, and the phylogenetic relationships between this species and its congeners that occur within the same geographic region, with the primary aim of ensuring the correct identification of the different species.

## MATERIAL AND METHODS

Specimens were collected in the Inhamum Municipal Environmental Protection Area (Inhamum MEPA) using pitfall traps between July, 2010 and January, 2012. This conservation unit has a total area of 4500 hectares (Barros, 2012), and is located in the central-eastern portion of the Brazilian State of Maranhão, adjacent to the BR-316 federal highway, approximately 2 km from the urban perimeter of the municipality of Caxias, Maranhão (4°53'30"S, 43°24'53"W). The vegetation is typical of the Cerrado savanna, with two main strata, one arboreal/shrubby and the other grassy on the flatter terrain, and dense stands of buriti palm in the depressions, accompanying the local water courses (Neres and Conceição, 2010).

The DNA was extracted from muscle tissue using the standard phenol-chloroform protocol described by Sambrook and Russel (2001). The cytochrome *b* gene was amplified by polymerase chain reaction (PCR) using the forward primer MVZ05 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and the reverse primer MVZ16 (5'-AAATAGGAARTATCAYTCTGGTTTRAT-3') (Smith and Patton, 1993) under the following PCR regime: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 45°C for 1 min, 72°C for 2 min, and a final cycle of 72°C for 10 min. PCR products were purified using enzymes ExoProStar 1-Step (GE Healthcare) according to the manufacturer protocol, and cycle sequenced using BigDye Terminator 3.1 (Applied Biosystems) during 30 cycles of 96°C for 15 s, 50°C for 15 s, 60°C for 4 min. After precipitation in EDTA/sodium acetate/ethanol, the product was sequenced using an automated capillary sequencer ABI 3500/Life Technologies (Applied Biosystems).

The sequences were aligned using ClustalW (Thompson et al., 1994) using standard parameters, and then converted into the Fasta format. Where necessary, the sequence was adjusted using the BioEdit sequence editor, version 7.0.5.2 (Hall, 1999). Nucleotide saturation was evaluated through plots of transitions and transversions with the Tamura-Nei distance parameter in DAMBE, version 5 (Xia, 2013). The evolutionary model that was best adjusted

to the data, phylogenetic reconstructions based on the maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) approaches, and the matrix of genetic divergence were obtained in MEGA 6.0 (Tamura et al., 2013). Bootstrap support (Felsenstein, 1985) was obtained through an analysis with 1000 replications.

## RESULTS

A total of five specimens of *M. pinheiroi* were collected in the Inhamum MEPA (Table 1). For molecular analyses, a 737 base pair (bp) fragment was obtained from each of these five specimens and a further 34 sequences were obtained from GenBank, including eight from *M. pinheiroi* and 26 from *M. pakaraimae*, *M. incanus*, *M. paulensis*, and *M. parvidens*. The phylogenetic analyses were based on the haplotypes, and the matrix of genetic divergence was organized in groups, with means being calculated for the divergence within and between groups (Table 2).

**Table 1.** Details of the *Marmosops pinheiroi* specimens collected in the Inhamum Municipal Environmental Protection Area, Caxias, MA, Brazil.

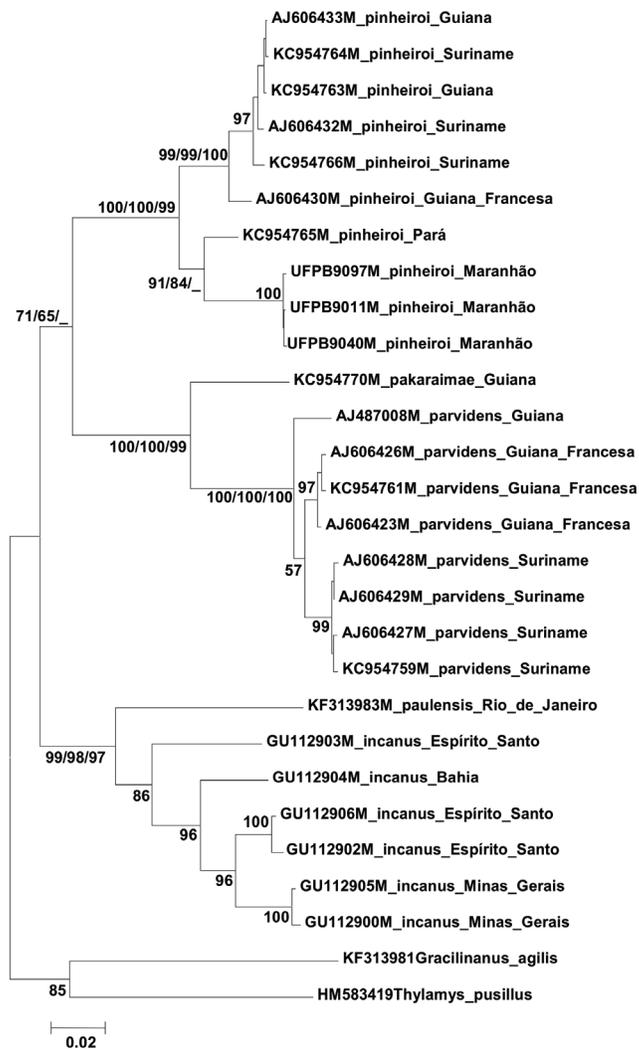
Voucher	Date of collection	Age/Sex	TL	TA	HL	HBL
UFPB 9037	08/03/11	Adulto ♂	235	135	32	100
UFPB 9011	18/07/11	Jovem ♂	182	105	30	77
UFPB 9012	16/07/11	Jovem ♂	190	100	28	90
UFPB 9040	08/09/11	Jovem ♂	195	110	28	85
UFPB 9097	19/10/11	Adulto ♂	220	135	25	85

TL = total length (mm); TA = tail length (mm); HL = head length (mm); HBL = length of head-and-body (mm).

**Table 2.** Localities and access numbers of the *Marmosops* specimens analyzed in the present study.

Species (haplotypes)	Locality	Coordinates	GenBank accession Nos.
<i>M. pinheiroi</i>	Maranhão, BR	4°53'S, 43°24'W	-
<i>M. pinheiroi</i>	Maranhão, BR	4°53'S, 43°24'W	-
<i>M. pinheiroi</i>	Maranhão, BR	4°53'S, 43°24'W	-
<i>M. pinheiroi</i>	Pará, BR	3°39'S, 52°22'W	KC954765
<i>M. pinheiroi</i>	Guiana	4°44'N, 58°43'W	KC954763
<i>M. pinheiroi</i>	Guiana	4°17'N, 58°31'W	AJ606433
<i>M. pinheiroi</i>	Suriname	2°02'N, 56°08'W	KC954766
<i>M. pinheiroi</i>	Suriname	4°29'N, 57°02'W	KC954764
<i>M. pinheiroi</i>	Suriname	5°08'N, 55°29'W	AJ606432
<i>M. pinheiroi</i>	French Guiana	4°05'N, 52°40'W	AJ606430
<i>M. parvidens</i>	Suriname	4°56'N, 55°12'W	AJ606427
<i>M. parvidens</i>	Suriname	4°56'N, 55°12'W	AJ606429
<i>M. parvidens</i>	Suriname	4°33'N, 57°04'W	KC954759
<i>M. parvidens</i>	Suriname	4°56'N, 55°12'W	AJ606428
<i>M. parvidens</i>	French Guiana	5°17'N, 52°55'W	AJ606426
<i>M. parvidens</i>	French Guiana	4°51'N, 53°04'W	AJ606423
<i>M. parvidens</i>	French Guiana	5°17'N, 52°55'W	KC954761
<i>M. parvidens</i>	Guiana	3°45'N, 59°18'W	AJ606424
<i>M. incanus</i>	Rio de Janeiro, BR	22°57'S, 44°02'W	KC954771
<i>M. incanus</i>	Espírito Santo, BR	20°28'S, 40°52'W	GU112906
<i>M. incanus</i>	Espírito Santo, BR	19°23'S, 40°79'W	GU112903
<i>M. incanus</i>	Espírito Santo, BR	19°96'S, 40°54'W	GU112902
<i>M. incanus</i>	Minas Gerais, BR	17°13'S, 42°76'W	GU112905
<i>M. incanus</i>	Minas Gerais, BR	20°08'S, 43°50'W	GU112900
<i>M. incanus</i>	Bahia, BR	12°80'S, 41°26'W	GU112904
<i>M. pakaraimae</i>	Guiana	5°07'N, 59°49'W	KC954770
<i>M. paulensis</i>	Rio de Janeiro, BR	22°24'S, 57°59'W	KF313983

Overall, 278 sites were variable, of which 247 were information for parsimony analysis. Haplotype diversity was 0.98, and nucleotide diversity, 0.12, and there was no evidence of saturation in the transition/transversion plot. The Tamura-Nei model was the most relevant here, with a discrete gamma distribution, considering the five taxa as categories and assuming that a certain proportion of the sites are evolutionarily invariable (TN93+G+I). The same topology was obtained for all the different approaches (Figure 1), with bootstrap values of over 50% at most nodes. The species *Gracilinanus agilis* (KF313981) and *Thylamys pusillus* (HM583419) were used as the outgroup owing to the fact that their relationships had been reconstructed in previous studies of didelphimorph phylogeny (Voss and Jansa, 2009).



**Figure 1.** Phylogenetic tree obtained by neighbor-joining analysis using the Tamura-Nei parameter for *Marmosops* specimen cytochrome b sequences, with bootstrap values for the ML/NJ/MP methods (left to right).

The phylogenetic trees reveal a well-supported (99/98/97%) clade between *M. incanus* and *M. paulensis*. The species *M. pinheiroi*, *M. pakaraimae*, and *M. parvidens* appear as sister groups, with 71% bootstrap support. However, whatever the locality or the method used, the *M. pinheiroi* specimens form a strongly supported (bootstrap scores of 99 and 100%) monophyletic group, as observed in the sister group formed by *M. pakaraimae* and *M. parvidens* (Figure 1).

Interspecific genetic divergence varied between 8.3 and 21.5% (Table 3), with the lowest value being recorded between *M. pakaraimae* and *M. parvidens*, both from the Guyanas, and the maximum values being found between *M. pinheiroi* from Maranhão and *M. incanus* from Minas Gerais, with a mean value of 14.6%. Intraspecific divergence in *M. pinheiroi* ranged from 0.2 to 6.2%, with the lowest values being recorded among the specimens from Maranhão, and the highest between those from Maranhão and French Guiana. Intraspecific divergence varied from 0.3 to 2.9% in *M. parvidens* and from 0.4 to 6.9% in *M. incanus*. While the intraspecific divergence recorded in *M. pinheiroi* was as high as 6.3%, it was consistent with that found in *M. incanus*, and lower than the values for interspecific divergence involving this species.

**Table 3.** Matrix of genetic divergence (group means) for the *Marmosops* species analyzed in the present study, based on sequences of the mitochondrial cytochrome *b* gene, using the Tamura-Nei parameter.

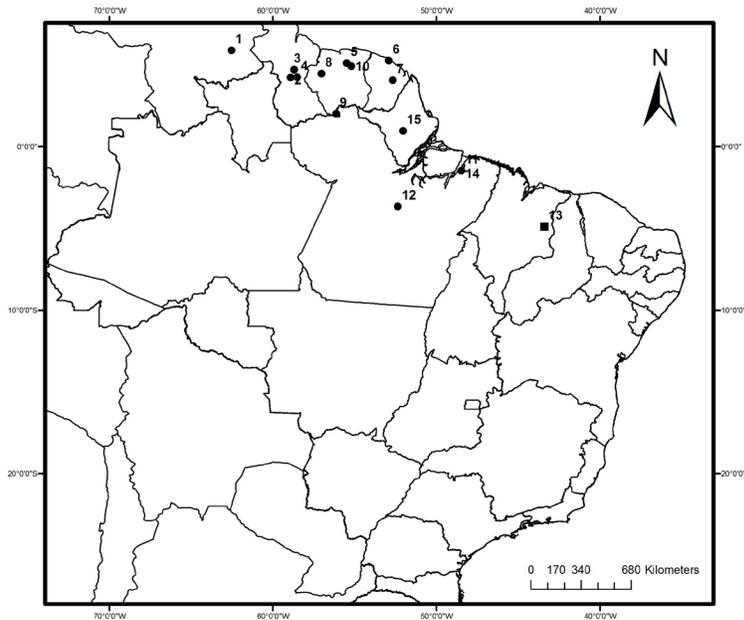
	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]
[1]	<b>0.2</b>														
[2]	4.2	-													
[3]	6.0	5.6	<b>0.3</b>												
[4]	6.0	5.5	0.5	<b>0.7</b>											
[5]	6.3	6.0	2.2	2.2	-										
[6]	18.0	16.0	17.0	17.0	15.9	<b>0.3</b>									
[7]	18.0	15.7	18.0	18.1	17.4	2.9	-								
[8]	16.9	15.0	16.5	16.5	15.4	1.9	2.8	<b>0.4</b>							
[9]	20.3	17.5	17.7	17.7	16.4	19.1	18.8	18.2	<b>5.5</b>						
[10]	21.5	18.4	18.9	19.2	19.0	19.8	19.1	19.0	5.4	<b>0.4</b>					
[11]	19.1	16.5	18.3	18.4	17.0	19.3	18.1	18.0	6.9	5.8	-				
[12]	15.7	13.6	14.9	14.8	14.6	9.2	8.6	8.3	18.6	19.6	18.5	-			
[13]	19.0	18.9	19.3	19.4	19.4	20.8	20.7	20.3	13.6	12.4	11.7	20.7	-		
[14]	21.3	18.3	21.4	20.9	21.5	23.7	23.4	23.1	19.8	20.6	20.2	20.5	21.7	-	
[15]	19.3	19.2	17.1	17.1	18.7	24.3	23.4	24.0	25.0	25.9	22.6	21.9	21.4	18.9	-

Number of haplotypes recorded for each group (locality) is shown within parentheses. [1] *M. pinheiroi*\_Maranhão, Brazil (3); [2] *M. pinheiroi*\_Pará, Brazil (1); [3] *M. pinheiroi*\_Guyana (2); [4] *M. pinheiroi*\_Suriname (3); [5] *M. pinheiroi*\_French Guiana (1); [6] *M. parvidens*\_Suriname (4); [7] *M. parvidens*\_Guyana (1); [8] *M. parvidens*\_French Guiana (3); [9] *M. incanus*\_Espírito Santo, Brazil (3); [10] *M. incanus*\_Minas Gerais, Brazil (2); [11] *M. incanus*\_Bahia, Brazil (1); [12] *M. pakaraimae*\_Guyana (1); [13] *M. paulensis*\_Rio de Janeiro, Brazil (1); outgroup: [14] *G. agilis* [15] *T. pusillus*. Values in the diagonal represent the intraspecific divergence for each group represented by more than one specimen.

The lowest interspecific divergence recorded in this study (8.3%) is only two percentage points higher than the greatest intraspecific variation (6.3%) for the *M. pinheiroi*, and even lower in the case of *M. incanus* (1.4%), emphasizing the taxonomic complexity of the group. Despite the relatively high intraspecific divergence recorded for *M. pinheiroi*, interspecific values were much higher, ranging from 15.7% for the comparison with *M. pakaraimae* to 19.1% in the case of *M. incanus*.

These values, together with the results of the phylogenetic analyses, reinforce the species status of *M. pinheiroi* as indicated by the morphological data. The confirmation of the

occurrence of the species in the Brazilian State of Maranhão represents a range extension of the order of 670 km (Figure 2) to the south and east of its previously known distribution.



**Figure 2.** Map of the geographic distribution of *Marmosops pinheiroi* with the new record from Maranhão (Brazil) shown as a black square: 1: Bolívar, Auyán-tepui (Venezuela); 2: Potaro-Siparuni, Iwokrama Forest, Canopy Walkway (Guyana); 3: Potaro-Siparuni, Iwokrama Forest, Turtle Mountain, 10 km NW Kurupukari (Guyana); 4: Potaro-Siparuni, Iwokrama Forest, Kabukalli Landing (Guyana); 5: Brokopondo, Finisanti (Suriname); 6: Paracou (French Guiana); 7: Les Nouragues (French Guiana); 8: Sipaliwini, Bakhuis Transect 9 (Suriname); 9: Nickerie, Sipaliwini Airstrip (Suriname); 10: Brokopondo, Brownsberg Nature Park, Jeep Trail (Suriname); 11: Utinga, near Belém, Pará (Brazil); 12: 52 km SSW Altamira, Pará (Brazil); 13: Inhamum, Maranhão (Brazil); 14: Belém, Pará (Brazil); 15: Serra do Navio, Amapá (Brazil).

## DISCUSSION

The current classification of the small-bodied didelphids (mouse opossum) should be considered with caution, given that the ample geographic ranges of many forms, together with the variation in their morphological characteristics, has led to the description of a number of new species (*Monodelphis handleyi*, *Monodelphis ronaldi*, *M. pakaraimae*, and *Marmosops creightoni*) in recent years, and even three new genera - *Tlacuatzin*, *Chacodelphis*, and *Criptonanus* (Voss and Jansa, 2003; Solari, 2003, 2007; Voss et al., 2004a,b, 2005, 2013). The phylogenetic analyses presented in this study provide a new perspective on this diversity, in particular the relationship between *M. pinheiroi* and other *Marmosops* populations, given that Inhamum is 670 km south and east of the nearest recorded locality for this species.

The mean total length of the adult specimens collected in the present study (227.5 mm) was lower than that of the *M. pinheiroi* specimens from French Guiana, which had mean lengths of 255 mm in the males and 244 mm in the females. With lower sized about 37 mm length of type specimen, a male of 265 mm from the Serra do Navio in the Brazilian State of

Amapá (Voss et al., 2001). Other species, such as *M. pakaraimae* (270 mm) and *M. parvidens* (250 mm), are also larger in size (Voss et al., 2013). A larger sample, together with quantitative craniodental analyses, would be necessary to define any pattern reliably.

The analysis presented here shows a strong relationship between the specimens from Maranhão and Pará, with the three northern Amazonian species forming a moderately well supported group, and *M. parvidens* and *M. pakaraimae* in a clear sister grouping, reinforcing the conclusions of Voss et al., (2013). High levels of intraspecific genetic diversity, like those found here in *M. pinheiroi* (6.3%) and *M. incanus* (6.9%), have been observed in other didelphids, such as *Gracilinanus microtarsus* (8.7%), *M. incanus* (9.1%), *Caluromys philander* (6.2%), and *Metachirus nudicaudatus* (6.1%) (Agrizzi et al., 2012). Patton and Costa (2003), analyzing cytochrome *b*, also found high levels of genetic divergence in the didelphids, in particular the smallest-bodied genera (*Monodelphis* and *Marmosops*), and concluded that they may represent an ancient radiation. Steiner and Catzefflis (2004) also recorded a high level of intraspecific divergence (7.2%) in *M. pinheiroi* from Pará and French Guiana, analyzing a fragment of the cytochrome *b* gene.

Like a number of other didelphids, the taxonomy of *Marmosops* has been the subject of a number of reviews since the classic revision of Tate (1933), and is now the second most diverse didelphid genus (Gardner, 2008). In the specific case of the *M. parvidens* complex (*sensu* Pine, 1981), Mustrangi and Patton (1997) found high levels of intraspecific divergence with clear differences in relation to other congener species. The molecular data presented here are important not only for the understanding of the taxonomy of *M. pinheiroi*, but also in understanding its genetic diversity over a wider area of distribution.

Prior to the present study, *M. pinheiroi* was known only from the Amazon forest, and there were no records from more open forest habitats (Gardner, 2008), although other animal species typically found in the rainforest have been recorded in the Brazilian Cerrado savanna (Lopes et al., 2007; Miranda et al., 2009). This appears to be related to the presence of gallery forests in these systems, which function as connecting corridors (Costa, 2003). In addition to these features, Inhamum is located within a transition zone, and is characterized by a mosaic of habitat types, which is reflected in a considerable heterogeneity of fauna, including amphibians and other mammals typical of the Amazon and Atlantic forests, and the Caatinga scrublands (Barros, 2012), reinforcing the value of this tract of Cerrado as a conservation area.

The geographic range of *Marmosops* encompasses an area that has undergone a number of important geological processes related to the uplifting of the Andes, marine transgressions, and climatic shifts over different geological epochs (Hoorn et al., 2010; Patterson et al., 2012). A more complete phylogeny based on molecular dating would help to clarify the evolutionary history of the genus, and the factors that determined the present-day distribution of the different species of the genus.

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