Conserv Genet (2010) 11:1579–1585
DOI 10.1007/s10592-009-9962-5

SHORT COMMUNICATION

Delineating geographic boundaries of the woolly mouse opossums, *Micoureus demerarae* and *Micoureus paraguayanus* (Didelphimorphia: Didelphidae)

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Received: 3 April 2009 / Accepted: 10 July 2009 / Published online: 29 June 2009

Abstract This paper reports on molecular classification of the woolly mouse opossum, *Micoureus* spp., in the southeastern Atlantic Forest in Brazil, a hotspot of critically threatened biodiversity. Phylogenetic analysis and character-based diagnosis were done using DNA sequences from the mitochondrial cytochrome *b* gene and cytochrome *c* oxidase subunit I genes, and exon 6 of the nuclear dentine matrix protein 1 gene (*DMP1*). Although the nuclear *DMP1* gene showed insufficient genetic variation for species diagnosis, the mtDNA analyses resulted in the robust grouping of samples of the *M. paraguayanus*/*M. demerarae* complex into three clades with distinct DNA sequence diagnostics for the species units in this study. The results support the species status of *M. paraguayanus* (Tate in Am Mus Novit 493: 1–13, 1931), which has a geographic distribution in the Atlantic Forest from the North and Northeast of Minas Gerais state in Brazil, going south along the coastal region of Brazil, to Paraguay and Argentina. Evidence of the boundary for this species and the provided diagnostics should facilitate and improve accuracy of studies that have been done in critical threatened fragments of the Atlantic Forest, especially in Minas Gerais and Bahia states, Brazil.

Electronic supplementary material The online version of this article (doi:10.1007/s10592-009-9962-5) contains supplementary material, which is available to authorized users.

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Keywords Atlantic Forest · Cytochrome *b* · Cytochrome *c* oxidase 1 · Didelphidae · *Micoureus* · Phylogeny · Taxonomy

Introduction

Because molecular classification as well as geographic ranges of *Micoureus* are not well known, it is difficult to address conservation strategies for species of this genus, especially considering the small amount of the Atlantic Forest, a biodiversity hotspot and critically endangered, left in Minas Gerais state, Brazil (Brito and Fonseca 2006). The genus *Micoureus* has never had a formal taxonomic review. Without a precise and proper taxonomy, species that are at risk of local extinction in certain areas (Brito and Grelle 2004; Viveiros de Castro and Fernandez 2004) do not have a defined conservation management program, nor can they be correctly listed on the Endangered Fauna Red List of Brazil (Biodiversitas Fundação 2005) or on the IUCN Red List—The World Conservation Union (IUCN 2008). This study attempts to address this important conservation problem.

The genus *Micoureus* is composed of small-sized species, which are arboreal and nocturnal (Charles-Dominique et al. 1981). The main taxonomic resources for mammals, Wilson and Reeder (2005) and Gardner (2007) suggest that the genus *Micoureus* has six species (see online supplementary Table S1). According to these lists, four species of *Micoureus* are found in Brazil. *Micoureus regina* and *M. constantiae* are restricted to western South America, the former to the Amazon and the latter to northwestern Argentina, Bolivia, and the west of the Brazilian State of Mato Grosso. Two other species, *M. paraguayanus* and *M. demerarae*, have less clear geographic range

descriptions, especially in the Atlantic Forest, since the first is described as inhabiting the Atlantic Forest from the South of Bahia State (Brazil) to Paraguay and the second is just referred to as "North Brazil".

Using DNA sequences of three genes, our objective was to clarify the molecular systematics of this group and to consider these results in the context of morphology and biogeography. For this reason, we were mostly concerned with the taxonomy of specimens collected in the Brazilian states of Minas Gerais and Bahia. In accordance with the geographic distribution previously proposed for species occurring in Brazil (Costa 2003; Wilson and Reeder 2005; Gardner 2007), we assumed that specimens collected in the Atlantic Forest south of Minas Gerais state are most likely *M. paraguayanus*, while samples from habitats other than the Atlantic Forest represent *M. demerarae*.

Materials and methods

Table 1 lists samples and mitochondrial DNA sequences used in this study with their respective GenBank accession numbers and localities. Genomic DNA extraction from samples and all PCR were performed according to protocols in Dias et al. (2008). Fragments containing part of the mitochondrial gene cytochrome *b* (*CytB*, 803 bp), part of the mitochondrial gene cytochrome *c* oxidase 1 (*COI*, 643 bp) and the exon 6 of the dentin matrix protein 1 gene (*DMP1*, 1,203 bp. See online supplementary Table S2 for GenBank accession numbers and localities) from the nuclear genome, were amplified using a combination of primers and PCR conditions as described in previous publications (Smith and Patton 1993; Borisenko et al. 2008; Ivanova et al. 2007; Jansa et al. 2006; Toyosawa et al. 1999). A longer fragment of the *CytB* gene (1,147 bp) was obtained for a subsample of 13 specimens to clarify the taxonomic position of a sample from Urucu, using primers (MVZ05, MVZ14, MVZ16 and MVZ39) and PCR conditions according to Smith and Patton (1993).

After sequencing in both directions, sequences were edited and aligned manually using Sequencher 4.2 (Gene Codes Corporation). They were characterized for a number of variable and informative bases using the program MEGA version 3.1 (Kumar et al. 2004) and DNASP version 4.20 (Rozas et al. 2003). The sequences of mitochondrial DNA and nuclear DNA were examined using Population Aggregation Analysis (PAA). This method identifies diagnostic sites on a species or population level (Davis and Nixon 1992). Attribute scores (presence/absence) for population samples of individuals were determined visually using MacClade version 4.08 (Maddison and Maddison © 2005). Trees were obtained using both maximum parsimony (MP) and maximum likelihood (ML) algorithms. The MP analyses were carried out using PAUP* version 4.10b (Swofford 2002). ML trees were obtained using the online version of PHYML (Guindon and Gascuel 2003).

Results

Partial *CytB* sequences from the 37 individuals in our study comprised 28 different haplotypes, or nearly one unique haplotype per individual. The partial *COI* sequences from the 19 individuals sequenced constituted 10 different haplotypes. The sequenced region of *DMP1* gene from 28 individuals comprised 17 different haplotypes defined by 25 variable sites of which only 8 were parsimony informative.

The PAA analysis comparing *M. demerarae* and *M. paraguayanus* revealed no diagnostic sites that were reliable for distinguishing these two species in *DMP1*. This result can be attributed to the very low number of variable sites found in this species pair (see online supplementary Table S2).

The PAA done using 803 bp of *CytB* gene sequences from total of 37 individuals showed 26 fixed and unique differences between 23 samples assigned to *M. demerarae* (including samples from localities 16, 17 18, and 21, Table 1; Fig. 2a) and 10 samples assigned to *M. paraguayanus* (from localities 22, 23, 24, 25, and 26, Table 1; Fig. 2a), including all nine samples from Minas Gerais in the latter species. These results substantiate species differentiation and were in agreement with the phylogenetic trees (Fig. 1a). The sequence of the sample from the Brazilian locality Urucu, in the Amazon (locality 13, Table 1), obtained from GenBank (Patton et al. 1996), was highly divergent from the samples remaining in the *M. paraguayanus*/*M. demerarae* clade (29 unique diagnostic sites when 1,147 bp were analyzed). This sequence was originally published as *M. demerarae*, but using the diagnostics we established with *CytB* we are unable to identify this specimen to either pre-established taxon.

The PAA analysis of 643 bp *COI* gene sequences from total of 16 individuals corroborated results obtained with *CytB* revealing 25 exclusive characters for *M. paraguayanus* (three samples) and 24 exclusive characters for *M. demerarae* (10 samples). We examined information from the Barcode of Life Data System (BOLD) website, where three specimens identified as *M. demerarae* have sequences available (ABSMS555-ROM104494, ABSMS565-ROM 105521, and ABSMS587-ROM113431). When compared to our data, these sequences had 30 exclusive characters that differentiated them from all the other *Micoureus* species.

MP and ML analyses of all 37 *CytB* sequences yield the trees shown in Fig. 1. In both trees the genus *Micoureus*

Table 1 Samples and localities of didelphid marsupials which mtDNA (*CytB* and *COI*) sequences were analyzed in the present study and GenBank sequences numbers

Locality[a]	Label	Sample code	GeneBank (cytb)	GenBank (*COI*)	Species	Locality	Source
1	Mreg1	DWF659 (AMNH273164)[c]	EF587310[c]	EU597272[c]	*M. regina*	Peru, Loreto: Rio Galvez	AMCC, AMNH
2	Mreg2	MVZ154766	1041859	–	*M. regina*	Peru, Huampami	Patton et al. (1996)
3	Mdem1	ISEM V-1570	47457397	–	*M. demerarae*	French Guiana: Paracou	Steiner and Catzeflis (2004)
4	Mdem2	ISEM V-1308	47457399	–	*M. demerarae*	French Guiana: Cayenne	Steiner and Catzeflis (2004)
4	Mdem3	ISEM V-1320	47457419	–	*M. demerarae*	French Guiana: Cayenne	Steiner and Catzeflis (2004)
5	Mdem4	ISEM V-1040	47457405	–	*M. demerarae*	French Guiana: Nouragues	Steiner and Catzeflis (2004)
6	Mdem5	ROM 97932	47457395	–	*M. demerarae*	Guyana: Potaro-Siparuni	Steiner and Catzeflis (2004)
7	Mdem6	ISEM N-644	49168684	–	*M. demerarae*	French Guiana: Pic Matecho	Steiner and Catzeflis (2004)
8	Mdem7	ISEM N-568	49168683	–	*M. demerarae*	French Guiana: Saul	Steiner and Catzeflis (2004)
9	Mdem8	ROM 98124	47457407	–	*M. demerarae*	Guyana: Takutu-Essequibo	Steiner and Catzeflis (2004)
10	Mdem9	USNM560731	1041847	–	*M. demerarae*	Venezuela: Cerro Neblina, Camp VII	Patton et al. (1996)
11	Mdem10	CRB 2101[c]	EF587290[c]	EU597275[c]	*M. demerarae*	Brazil, AM: Barcelos	CRB-RJ
12	Mdem11	CRB 2178[c]	EF587291[c]	EU597274[c]	*M. demerarae*	Brazil, AM: Santa Isabel	CRB-RJ
13	Mdem12	MNFS187	1041845	–	*M. demerarae*	Brazil, AM: Alto Rio Urucu	Patton et al. (1996)
14	Mdem13	MUSM 13294	47457409	–	*M. demerarae*	Peru: Loreto	Steiner and Catzeflis (2004)
14	Mdem14	DWF568 (MUSM15312)[c]	EF587309[c]	EU597273[c]	*M. demerarae*	Peru, Amazonas: Rio Galvez	AMCC, AMNH
15	Mdem15	CRB 2791[c]	EF587295[c]	EU597282[c]	*M. demerarae*	Brazil, MT: São José do Xingu	CRB-RJ
19	Unknown1	RM198[c]	EF587300[c]	–	?	Brazil, BA: Itamari, Fazenda Alto São Roque	Zoology-UFMG
17	Unknown2	RM109[c]	EF587297[c]	EU597277[c]	?	Brazil, BA: Itacaré, Fazenda Rio Capitão	Zoology-UFMG
17	Unknown3	RM108[c]	EF587299[c]	–	?	Brazil, BA: Itacaré, Fazenda Rio Capitão	Zoology-UFMG
18	Unknown4	RM116[c]	EF587298[c]	EU597276[c]	?	Brazil, BA: Uruçuca, Fazenda Caititu	Zoology-UFMG
16	Mdem16	CRB 2304[c]	EF587292[c]	EU597279[c]	*M. demerarae*	Brazil, GO: Mimoso	CRB-RJ
16	Mdem17	CRB 2320[c]	EF587293[c]	EU597280[c]	*M. demerarae*	Brazil, GO: Mimoso	CRB-RJ
16	Mdem18	CRB 2324[c]	EF587294[c]	EU597281[c]	*M. demerarae*	Brazil, GO: Mimoso	CRB-RJ
20	Unknown5	RM219[c]	EF587296[c]	EU597278[c]	?	Brazil, BA: Porto Seguro, Pau Brazil Nac. Park	Zoology-UFMG
21	Unknown6	JEQ01[c]	EF587303[c]	EU597284[c]	?	Brazil, MG: Berilo, Usina Hidrelétrica de Irapé	PUC-MG Museum
22	Unknown7	A1091[c]	EF587301[c]	–	?	Brazil, MG: Marliéria, PERD[b], Campolina	UFMG-PELD/PERD
22	Unknown8	A1427[c]	EF587302[c]	–	?	Brazil, MG: Marliéria, PERD[b], Campolina	UFMG-PELD/PERD
22	Unknown9	A890[c]	EF587308[c]	–	?	Brazil, MG: Marliéria, PERD[b], Campolina	UFMG-PELD/PERD
22	Unknown10	266[c]	EF587288[c]	EU597283[c]	?	Brazil, MG: Marliéria, PERD[b], Campolina	UFMG-PELD/PERD
23	Unknown11	A1317[c]	EF587304[c]	–	?	Brazil, MG: Marliéria, PERD[b], Vinhático	UFMG-PELD/PERD
23	Unknown12	A315[c]	EF587305[c]	–	?	Brazil, MG: Marliéria, PERD[b], Vinhático	UFMG-PELD/PERD
23	Unknown13	A1421[c]	EF587306[c]	–	?	Brazil, MG: Marliéria, PERD[b], Vinhático	UFMG-PELD/PERD
24	Unknown14	CB01[c]	EF587307[c]	–	?	Brazil, MG: Casa Branca, Rola Moça	PUC-MG Museum

Table 1 continued

Locality[a]	Label	Sample code	GeneBank (cytb)	GenBank (*COI*)	Species	Locality	Source
25	Mpar1	CRB 1287[c]	EF587289[c]	EU597285[c]	*M. paraguayanus*	Brazil, RJ: Guapimirim	CRB-RJ
26	Mmur	ROM F-41351	49168685	–	*Marmosa murina*	Suriname: Brownsberg	Steiner and Catzeflis (2004)
27	Mlep	ROM 107034	47457431	–	*Marmosa lepida*	Guyana: Potaro-Siparuni	Steiner and Catzeflis (2004)

AMCC, AMNH (The Ambrose Monell Cryo Collection at American Museum of Natural History-NY), CRB-RJ biology collection (Cibele Rodrigues Bonvincio-RJ), PUC-MG Museum biology collection (Museu de Ciências Naturais-Pontifícia Universidade Católica de Minas Gerais), UFMG-PELD/PERD field samples (Universidade Federal de Minas Gerais-Programa Ecológico de Longa Duração/Site: Parque Estadual do Rio Doce)

[a] Number of localities in the map of the Fig. 2a

[b] PERD: Rio Doce State Park, MG, Brazil

[c] New samples and sequences obtained uniquely for this study

appears as monophyletic. *Micoureus regina* is the first species to branch off in both analyses. The remaining samples, belonging to either *M. paraguayanus* or *M. demerarae*, clustered together.

One main difference between the two trees was the position of the sample from Urucu (Amazonas, Brazil). In the ML analysis this sample clustered with *M. demerarae* samples while in the MP analysis it clustered with *M. paraguayanus*, but neither result was supported by bootstrap analysis. Analyzing the complete *CytB* gene (1,147 bp) for a subset of 13 samples did not give extra support to either alternative arrangement (Fig. 1a).

Phylogenetic analyses of the *COI* sequences also support differentiation between *M. paraguayanus* and *M. demerarae* (Fig. 1b). In both the MP and the ML analyses of *COI* sequences *M. paraguayanus*, including specimens from Minas Gerais, and *M. demerarae*, including specimens from southern Bahia, clustered in two distinct monophyletic groups. The main difference between the phylogenetic results obtained with *COI* as compared to *CytB* is that with the former gene *M. regina* clusters with *M. paraguayanus* while with the latter gene this species clusters with *M. demerarae*. It is interesting to note that while *COI* shows higher support for species clades, it reveals less interspecific structure than *CytB*. Neither PAA nor phylogenetic analysis supports the identification of the three BOLD sequences as *M. demerarae*. In fact, given the high degree of divergence of these specimens from the ones examined in the present study, they may not even represent specimens in the genus *Micoureus*.

Discussion

Our results indicate a clear genetic distinction between *M. paraguayanus* and *M. demerarae*. Although the gene *DMP1* did not show enough variation to resolve the issue, both *CytB* and *COI* showed high levels of interspecific variation with a number of diagnostic characters for these two species. The PAA and phylogenetic analyses done on *CytB* sequences allowed us to group samples of the *M. paraguayanus*/*M. demerarae* complex into three clades. The three clades were highly supported in phylogenetic analyses using both MP and ML approaches. The discovery of multiple diagnostic differences between the two species found in the Atlantic Forest mirrors the strongly supported divergence of the clades in the phylogenetic analyses.

The first of the three clades consisted of one sample from Rio de Janeiro State and five samples from Minas Gerais State. This result confirms the phylogeny and phylogeographic structure proposed by Costa (2003), Patton and Costa (2003) and Steiner and Catzeflis (2004). The Southeastern Atlantic Forest clade has previously been

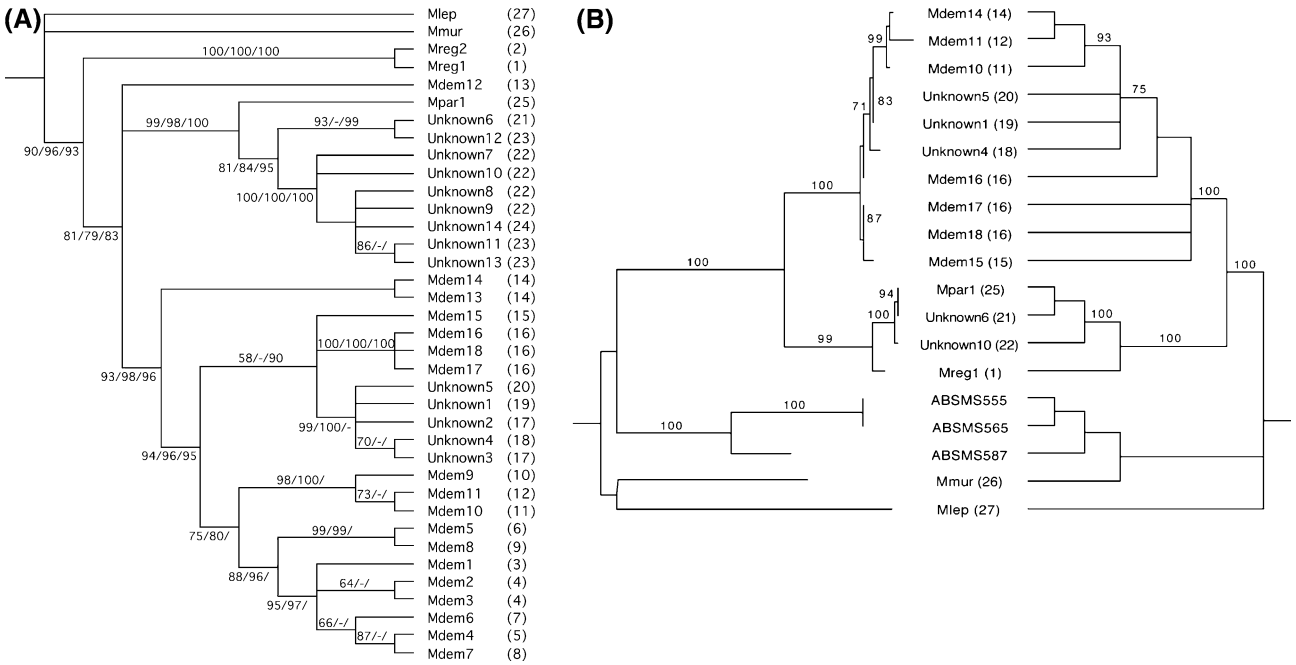

Fig. 1 **a** Parsimony bootstrap tree obtained with the *CytB* 803 bp data set using heuristic searches with 10 random stepwise additions and tree-bisection-reconnection (*TBR*) branch swapping. Numbers on the tree represent bootstrap support values for MP *CytB* 803 bp/ML *CytB* 803 bp/MP *CytB* 1,147 bp. A *dash* (-) represents values below 50 and *blank* represents absence of the node in the correspondent analysis. The number of informative bases for parsimony analysis was 194 bp from 803 bp, 174 bp of which were within *Micoureus* and 135 bp within the *M. paraguayanus*/*M. demerarae* complex. Thirty most parsimonious trees were recovered with length = 545 steps, CI = 0.624, RI = 0.828, and RC = 0.516. Bootstrap support values were obtained with 1,000 pseudo-replicates. The ML tree (log likelihood of 3,630.21) had very similar topology (not shown) and was obtained using the General Time Reversible model of site substitution with estimated proportion of invariable site and gamma distribution parameter (GTR+I+R). Parameters were estimated from the data: A = 0.306, C = 0.245, G = 0.130, T = 0.319; substitution rate matrix: [A-C] = 3.652, [A-G] = 18.392, [A-T] = 3.603, [C-G] = 2.492, [C-T] = 33.472, [G-T] = 1; proportion of invariable sites = 0.478; gamma distribution parameter = 1.002. Topology robustness of ML trees was assessed through nonparametric bootstrap over 500 replicates. Samples labels and localities numbers (in parenthesis) are in accordance with Table 1 and Fig. 2. **b** MP and ML trees obtained in the analyses of 643 bp of the *COI* gene. Two-hundred-five positions were variable, and 131 of which were transitions. MP analyses were based on 143 parsimony informative sites and retrieved 5 equally parsimonious trees. ML analyses was based on the GTR+I+R and used the following parameters as estimated from the data: base frequencies: A = 0.268, C = 0.222, G = 0.168, T = 0.341; substitution rate matrix: [A-C] = 6.384, [A-G] = 25.390, [A-T] = 11.701, [C-G] = 4.812, [C-T] = 48.103, [G-T] = 1; proportion of invariable sites = 0.133; gamma distribution parameter = 0.590. *Numbers* represent bootstrap values and were obtained as described above for *CytB*

called *Micoureus paraguayanus* Tate 1931 by Voss and Jansa (2003) and Jansa et al. (2006) and *Micoureus travassosi* Miranda-Ribeiro (1936) for the Middle-South Atlantic Forest by Patton and Costa (2003) and Costa (2003). Voss and Jansa (2003) had two arguments favoring the taxonomic identity for the southeastern clade as *M. paraguayanus*, which we agree with. The first was one of primacy; *M. paraguayanus* is the oldest name (Tate 1931). The second was that morphologically, the specimens from São Paulo (grayish with a conspicuously white-tipped tail) studied by them matched almost perfectly with Tate's (1931) original diagnostic description (very clear mouse gray; tail longer than head and body together). The same description also matched with our samples from Minas Gerais State. Our samples do not match the description of Miranda-Ribeiro (1936) for *M. travassosi*, in which the key specimens from Angra dos Reis (Rio de Janeiro State) had a brief description note: "Smaller tail, intense abdominal color, longer hairs".

The second clade consisted of samples from the Amazon (North of Brazil, Venezuela, and Peru), the Guyanas (French Guyana and Guyana), Central Brazil (Goiás and Mato Grosso states), and the northeastern Atlantic Forest (Bahia State). We suggest that this clade should be assigned to *M. demerarae* since it includes specimens from the type locality of this species (Guyana). The third "clade" included one sample from the Amazonian locality of Urucu. Our analyses suggest that this sample most likely represents a third taxon. More studies including a large number of samples from this locality will be necessary to determine the specific status of this clade.

The southern Bahia and northern Minas Gerais samples, despite their geographical proximity, were grouped into different clades. This observation contrasts with the results

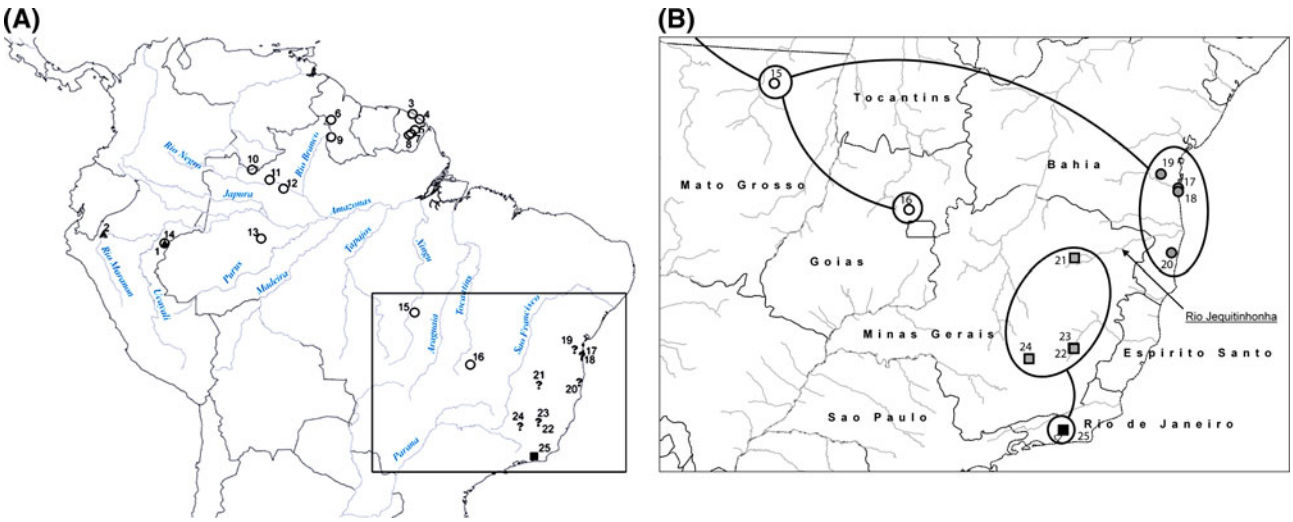

Fig. 2 Maps of sample localities. **a** Sample localities as listed in Table 1. Circles represent *M. demerarae*, square represents *M. paraguayanus*, triangles represent *M. regina*, and interrogation marks (?) represent our sample targets labeled as Unknown in Table 1. **b** This map shows in detail the Brazilian Central and Southeastern regions (square area delimited in map of Fig. 2a) and how samples of these regions were grouped in our phylogenetic analyses

found by Costa (2003) and the boundaries described by Wilson and Reeder (2005) and Gardner (2007), who include northern Minas Gerais samples in *M. demerarae* and southern Bahia samples *M. paraguayanus*.

While our sample sizes are relatively small, the results of character diagnosis discovery and phylogenetic analysis are clear. These results will provide a starting point for future studies with more specimens, considering that the morphological basis for the distinction between *M. demerarae* and *M. paraguayanus* still needs to be more clear and more samples of *M. paraguayanus* need to be morphologicaly identified as such. We report genetic evidence that the species *M. paraguayanus* (Tate 1931) is distributed in the Atlantic Forest from southeastern Brazil, in the Minas Gerais state, to Paraguay and Argentina. All five samples from the southern region of the Bahia State investigated in this study were grouped with *M. demerarae* in our phylogenetic analyses (though each had been classified as 'unknown' on the basis of morphology) and not to *M. paraguayanus* as previously suggested by Costa (2003), Wilson and Reeder (2005), and Gardner (2007). On the basis of our results, we concur with Brito and Fonseca (2006) and suggest that *M. paraguayanus* should be included in the Endangered Species Brazilian Red List, as the species of the same genus are already included in the IUCN—Red List, category near threat (NT). We have also presented DNA sequence diagnostics for both *M. demerarae* and *M. paraguayanus* that will assist conservation biologists and taxonomists in identifying new specimens of this taxon in the field with broader sampling.

Acknowledgments We would like to thank our colleagues from the Sackler Institute of Comparative Genomics, especially Matt Leslie; colleagues Heitor Cunha and Raquel Moura from the Mammalogy Lab of the General Biology Department of ICB-UFMG, Tudy Camara from the Museum of Natural Sciences of PUC-MG, and Cibele R. Bonvincio from INCa, Rio de Janeiro for kindly providing samples. This study is funded by the CNPq Project PELD-PERD, the CAPES Foundation (PhD. student fellowship) and the Sackler Institute for Comparative Genomics—AMNH. In addition, the AMNH authors on this paper thank the Louis and Dorothy Cullman Program in Molecular Systematics at the AMNH.

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