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Genetic diversity of wild populations of the grey short-tailed opossum, *Monodelphis domestica* (Didelphimorphia: Didelphidae), in Brazilian landscapes

FABIANA P. CARAMASCHI[1,2], FABRÍCIA F. NASCIMENTO[2], RUI CERQUEIRA[1] and CIBELE R. BONVICINO[2,3]*

[1]*Laboratório de Vertebrados, Departamento de Ecologia, IB, CCS, Universidade Federal do Rio de Janeiro, Rua Prof Dr Rodolpho Paulo Rocco, Caixa Postal 68020, Ilha do Fundão, 21941-901 Rio de Janeiro, RJ, Brazil*
[2]*Genetics Division, Instituto Nacional de Câncer, Rua André Cavalcante, 37, 4° andar, 20231-050 Rio de Janeiro, RJ, Brazil*
[3]*Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, Instituto Oswaldo Cruz, Fiocruz, Av. Brasil, 4365, 21045-900 Rio de Janeiro, RJ, Brazil*

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The characterization of the different taxa of the highly diverse genus *Monodelphis* is poorly understood, as is the case of their distribution. Historically, taxonomic studies of *Monodelphis* have been restricted to a few or single species, whereas molecular approaches have been used for estimating phylogenetic relationships between species. We carried out phylogenetic analyses of 14 *Monodelphis* species, including *Monodelphis domestica*, based on cytochrome *b* mitochondrial DNA sequences. Forty-five complete (1149 bp) sequences of this gene were obtained from 39 specimens of *M. domestica* collected in 23 localities of the Brazilian Cerrado, Pantanal, and Caatinga morphoclimatic domains; one of *Monodelphis umbristriata*, two of *Monodelphis americana*, and two of *Monodelphis dimidiata*. A total of 72 haplotypes were analyzed, 48 only in *M. domestica*. Analyses were carried out in conjunction with 46 other sequences retrieved from GenBank, including *M. domestica*, *Monodelphis brevicaudata*, *Monodelphis glirina*, *Monodelphis emiliae*, *Monodelphis peruviana*, *Monodelphis osgoodi*, *Monodelphis handleyi*, *Monodelphis kunsi*, *Monodelphis americana*, *Monodelphis dimidiata*, *Monodelphis iheringi*, *Monodelphis reigi*, and *Monodelphis adusta*, with six other different didelphid species used as outgroups. Maximum likelihood and Bayesian inference were similar in depicting phylogenetic relationships of different *Monodelphis* taxa. Two clades of *M. domestica* were defined on the basis of these results. Genetic distance estimates ranged from 3.2% to 6.2% between these clades of *M. domestica*. Population analyses were carried out to infer the likely demographic scenarios and the relationship between *M. domestica* haplotypes. Median-joining and spatial analyses showed two populations related to different morphoclimatic domains (Cerrado/Pantanal and Caatinga). These results indicate a population structure in *M. domestica* and the possibility that this taxon might represent a species complex.

ADDITIONAL KEYWORDS: cytochrome *b* – geographical variation – open vegetation domains – phylogeography.

INTRODUCTION

Monodelphis Burnett, 1830 is the most diverse genus of the family Didelphidae, comprising 21 recognized species of relatively small terrestrial opossums, with short tail, small ears, and variable pelage pattern (Gardner, 2005; Pine & Handley, 2008; Voss & Jansa, 2009; Solari, 2010; Vilela, Russo & Oliveira, 2010). *Monodelphis* species occur in different environments, including humid forests, and dry and wet lands, from Panama to Argentina (Costa & Patton, 2006). This genus is poorly understood, both with respect to the delimitation of taxa and species distribution (Brown, 2004; Costa & Patton, 2006). Historically, taxonomic

*Corresponding author. E-mail: cibelerb@inca.gov.br

studies of *Monodelphis* are restricted to few or single species, and descriptions have been based on the external morphology of a small number of specimens (Pine, 1975, 1976, 1977, 1979; Pine & Abravaya, 1978; Pine & Handley, 1984; Pine, Dalby & Matson, 1985; Lemos, Weksler & Bonvicino, 2000; Voss, Lunde & Simmons, 2001; Solari, 2004, 2007). Recently, molecular approaches have been used for estimating the phylogenetic relationship between species of this genus (Voss & Jansa, 2009; Lim *et al*., 2010; Solari, 2010; Vilela *et al*., 2010; Carvalho *et al*., 2011).

Monodelphis domestica (Wagner, 1842) was initially described as *Didelphys domestica* based on samples collected by Natterer in Brazil between 1817 and 1835. Wagner (1842) indicated 'Cuyaba' (currently Cuiabá, State of Mato Grosso) as the collecting locality of *M. domestica* and provided a short description of external morphology and pelage pattern. *Monodelphis* specimens with uniform greyish pelage are included in this species, distributed from the north-east of Brazil to the North of Argentina, across Paraguay and Bolivia (Emmons & Feer, 1997; Eisenberg & Redford, 1999; Brown, 2004).

Studies on reproduction and growth of *M. domestica* were carried out in specimens of the Museu Nacional – UFRJ, Brazil (Bergallo & Cerqueira, 1994), whereas genetic studies showed polymorphisms in two different captive Brazilian populations (states of Pernambuco and Paraíba), indicating the need to implement a careful strategy in captive breeding (van Oorschot, Williams-Blangero & VandeBerg, 1992). Divergence in allelic frequencies between *M. domestica* from Brazil and Bolivia have also been observed (VandeBerg & Robinson, 1997; Gouin *et al*., 2005). However, despite such evidence suggesting diversification, studies involving intraspecific variation have not been carried out to date. In this context, a phylogeographical analysis of *M. domestica* populations may contribute to an understanding of didelphid evolution since their origin in the Lower Paleocene (Oliveira & Goin, 2006).

The wide distribution of *M. domestica* in South America, as reported in different morphoclimatic domains (Amazon, Cerrado, Chaco, Pantanal, Campos, Caatinga; for definitions, see Ab'Sáber, 1977), is indicative of geographical variation. To test this hypothesis and to improve the understanding of phylogeographical patterns of *M. domestica* in Brazil, mitochondrial DNA was used to evaluate the population genetic structure of this species. mtDNA has been widely used in phylogeographical studies, providing a robust indicator of evolutionary history as a result of its rapid coalescence and fast evolution (Patton, Reis & Silva, 1996). Intra-population and spatial analyses were also performed to determine the relationships among samples from different morphoclimatic domains.

MATERIAL AND METHODS

Samples and DNA extraction

We analyzed 44 *Monodelphis* from 23 localities of the Atlantic Forest, Pantanal, Cerrado, and Caatinga, as well as transitions between these latter two morphoclimatic domains (Fig. 1, Table 1). All specimens were identified based on external and skull characters in accordance with previous standards reported in the literature (Costa & Patton, 2006; Lemos, Weksler & Bonvicino, 2000; Pine, 1976, 1979; Pine, Dalby & Matson, 1985; Pine & Handley, 2008; Voss & Jansa, 2009; Voss, Lunde & Simmons, 2001). Museum catalogue number (or collector's field number) and collecting localities were provided for vouchers and tissues (Table 1). Voucher specimens were deposited in Museu Nacional, Rio de Janeiro, Brazil (MN, MN-UFRJ, Brazil) and Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, Instituto Oswaldo Cruz – Fiocruz, Rio de Janeiro, Brazil (LBCE). Field numbers refer to the researchers: LG and CD = L. Geise (Departamento de Zoologia, Instituto de Biologia, Universidade Estadual do Rio de Janeiro-UERJ, Brazil); LMP = L. M. Pessoa (Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro – UFRJ, Brazil); JAO = J. A. Oliveira (Setor de Mamíferos, Departamento de Vertebrados, Museu Nacional–UFRJ, Brazil); CEG = C. E. Grelle (Departamento de Ecologia, Instituto de Biologia–UFRJ, Brazil); MSL = J. P. Garcia (Departamento de Zoologia, Instituto de Biologia–UFRJ, Brazil); CRB = C. R. Bonvicino (LBCE, Instituto Oswaldo Cruz – Fiocruz, Rio de Janeiro, Brazil). DNA was isolated from livers preserved in ethanol by standard procedures (Sambrook, Fritsch & Maniatis, 1989).

Amplification and sequencing

The complete mitochondrial gene cytochrome *b* (1149 bp, *mt-Cytb*) was amplified using primers L14724 (5′-CGAAGCTTGATATGAAAAACCATCGT TG-3′; Irwin, Kocher & Wilson, 1991) and CIT-REV (5′-GAATATCAGCTTTGG-3′; Casado *et al*., 2010). Amplicons were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced using the same primers plus the internal primers MVZ16 (5′-AAATAGGAARTATCATTCTGG TTTRAT-3′; Smith & Patton, 1993) and CB-in2 (5′-TGAGGACAAATATCATTYTGAG-3′; Cassens *et al*., 2000). Electropherograms were manually checked using CHROMAS, version 1.45 (MacCarthy, 1998) and CHROMAS PRO, version 1.41 (Technelysium Pty Ltd). Sequences were manually aligned in MEGA,

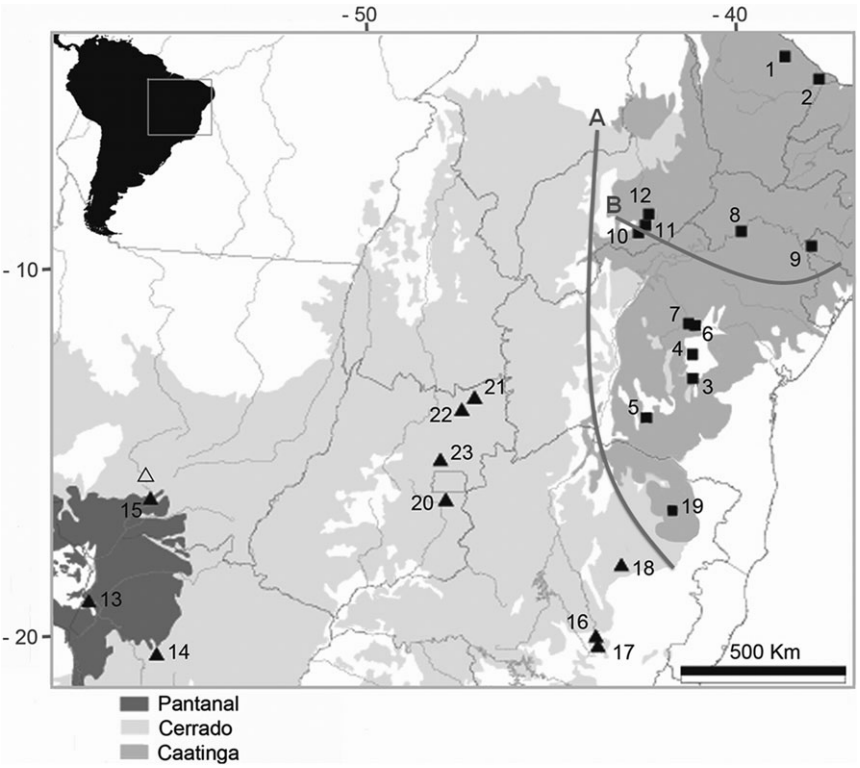

Figure 1. Map showing the localities referring to the sequenced samples of *Monodelphis domestica*. A list of the assigned localities is provided in Table 1. An open triangle indicates the type locality of the species (Cuiabá, MT state). Black triangles represent the localities referring to Clade A, black squares represents localities referring to Clade B. Line A corresponds to the first barrier and line B is the second barrier calculated by Monmonier's algorithm.

version 4 (Tamura *et al*., 2007). Haplotypes were checked using DNASP, version 5.10.01 (Librado & Rozas, 2009).

Analyses, phylogenetic reconstructions, and divergence time estimates

Forty-six GenBank sequences were included in phylogenetic analyses, 13 of which were from *Monodelphis* species and from six didelphid species used as outgroups (see Appendix, Table A1).

An index of DNA substitution saturation, I_{ss} (Xia *et al*., 2003), was calculated using DAMBE, version 5.2.5 (Xia & Xie, 2001) to identify phylogenetic signals. This analysis was performed with the complete dataset and with all outgroups and for first, second, and third codon positions. Results were compared to a critical I_{ss} value ($I_{ss.c}$) *sensu* Xia & Lemey (2009).

Pairwise genetic distances were estimated with Kimura's two-parameters and *p*-distance with MEGA, version 4, for estimating reliable distances for closely-related taxa. Because both analyses produced similar results, the Kimura's two-parameter DNA substitution model of evolution was preferred because it allowed direct comparisons with previous studies of short-tailed opossums (Patton & Costa, 2003; Solari, 2007, 2010; Carvalho *et al*., 2011). For phylogenetic reconstructions, the DNA substitution model was selected using MODELGENERATOR, version 0.85 (Keane *et al*., 2006) and the Bayesian information criterion.

A phylogeny using the maximum likelihood (ML) approach was reconstructed with PHYML, version 3.0 (Guindon & Gascuel, 2003). The tree topology space was searched using the best of nearest neighbour interchange and subtree pruning and regrafting algorithms starting from five random trees generated by BioNJ (Guindon & Gascuel, 2003; Guindon *et al*., 2010). Branch support was calculated using the approximate likelihood ratio test with Shimodaira–Hasegawa-like interpretation because it is as conservative and accurate as bootstrapping but less computationally intensive (Anisimova & Gascuel, 2006; Guindon *et al*., 2010).

Bayesian inference (BI) using the Markov chain Monte Carlo (MCMC) method was used for phylogenetic analyses of the *mt-Cytb* gene using MrBayes, version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Two chains

Table 1. Haplotype, field or museum number, locality and its number (map, Fig. 1) and GenBank access number of *Monodelphis* specimens sequenced in the present study, and/or retrieved from GenBank (HP33-48)

Haplotype	Museum/field number	Locality	Location	GenBank	Haplotype	Museum/field number	Locality	Location	GenBank
HP01	LBCE9660	Redenção, CE	1	HQ651734	HP24	MSL008	Itabirito, MG	17	HQ651764
HP02	LBCE5253	Jaguaruana, CE	2	HQ651735	HP25	LG620	Felício dos Santos, MG	18	HQ651765
HP03	LG402	Mucugê, BA	3	HQ651736	HP26	LG444	Itinga, MG	19	HQ651766
HP03	CD60	Lençóis, BA	4	HQ651737	HP27	LG415	Itinga, MG	19	HQ651768
HP03	CD129	Lençóis, BA	4	HQ651738	HP28	LBCE7517	Luziania, GO	20	HQ651769
HP04	LG334	Mucugê, BA	3	HQ651739	HP29	MN42989	Teresina de Goiás, GO	21	HQ651770
HP05	LG344	Mucugê, BA	3	HQ651740	HP30	CRB966	Cavalcante, GO	22	HQ651771
HP05	MN63365	Caetité, BA	5	HQ651741	HP31	MN46582	Cavalcante, GO	22	HQ651772
HP06	MN63408	Caetité, BA	5	HQ651742	HP32	CRB2372	Mimoso de Goiás, GO	23	HQ651773
HP07	LMP336	Lajes, BA	6	HQ651743	HP33	–	Breeding colony	–	AJ508398
HP07	LMP332	Morro do Chapéu, BA	7	HQ651744	HP34	MN36733	Minaçu, GO	–	EF154194
HP08	LMP337	Lajes, BA	6	HQ651745	HP35	MN36927	Niquelândia, GO	–	EF154196
HP09	LBCE5212	Curaça, BA	8	HQ651746	HP36	OT8047	Ipameri, GO	–	EF154205
HP10	MN42837	UHE Xingó, AL	9	HQ651747	HP37	MN36278	Colinas do Sul, GO	–	EF154210
HP11	LBCE10154	Cel José Dias, PI	10	HQ651748	HP38	UFPB2614	Caruaru, PE	–	EU750746
HP12	MN63323	Cel José Dias, PI	10	HQ651749	HP39	UFPB4068	Bezerros, PE	–	EU750751
HP13	MN65999	São Raimundo Nonato, PI	11	HQ651750	HP40	UFPB4070	Ibiapaba, PE	–	EU750749
HP14	MN63269	João Costa, PI	12	HQ651751	HP41	MVZ197457	MS	–	HM998570
HP15	LBCE1250	João Costa, PI	12	HQ651752	HP42	MSB55853	Bolivia, Chuquisaca	–	HM998571
HP16	LBCE5681	Corumbá, MS	13	HQ651753	HP43	MSB56112	Bolivia, Chuquisaca	–	HM998572
HP17	LBCE5356	Corumbá, MS	13	HQ651754	HP44	MSB67023	Bolivia, Santa Cruz	–	HM998573
HP18	LBCE4452	Aquidauana, MS	14	HQ651755	HP45	MSB(NK23288)	Bolivia, Santa Cruz	–	HM998574
HP19	LBCE4882	Aquidauana, MS	14	HQ651756	HP46	MSB(NK25144)	Bolivia, Tarija	–	HM998575
HP20	LBCE5513	Aquidauana, MS	14	HQ651757	HP47	MSB(NK23349)	Bolivia, Tarija	–	HM998576
HP21	MN64681	Barão de Melgaço, MT	15	HQ651758	HP48	MSB82534	Paraguay, Concepción	–	HM998577
HP21	MN64706	Barão de Melgaço, MT	15	HQ651759	HP49	LBCE4207	Sumidouro, RJ	–	HQ651774
HP22	CEG122	Nova Lima, MG	16	HQ651760	HP50	CEG132	Nova Lima, MG	–	HQ651776
HP22	CEG125	Nova Lima, MG	16	HQ651761	HP51	MN46570	Alto Paraíso, GO	–	HQ651775
HP22	CEG133	Nova Lima, MG	16	HQ651762	HP52	LBCE10386	Jaborá, SC	–	HQ651777
HP23	CEG126	Nova Lima, MG	16	HQ651763	HP53	CRB1920	Aratiba, RS	–	HQ651778

Museum catalogue numbers (for acronyms, see Material and Methods) are missing for vouchers housed but not yet catalogued. *Monodelphis domestica* = HP01-48; *Monodelphis americana* = HP49-50; *Monodelphis umbristriata* = HP51; *Monodelphis dimidiata* = HP52-53. AL, Alagoas; BA, Bahia; CE, Ceará; GO, Goiás; MG, Minas Gerais; MT, Mato Grosso; MS, Mato Grosso do Sul; PE, Pernambuco; PI, Piauí; RJ, Rio de Janeiro; SC, Santa Catarina; RS, Rio Grande do Sul.

were run for 8 000 000 generations and one tree per 1000 generations was collected. Convergence and mixing were evaluated using TRACER, version 1.5 (Rambaut & Drummond, 2007), and the initial 10% of runs was discarded (burn-in). A majority-rule consensus phylogram was subsequently constructed.

Intraspecific analysis

All population analyses were carried out with the complete *mt-Cytb* sequence (1149 bp). NETWORK, version 4.5.1.6 (http://www.fluxus-engineering.com) was used for reconstructing a median-joining (MJ) network (Bandelt, Forster & Rohl, 1999) using only variable sites to evaluate population structure and patterns of geographical distribution.

Analysis of mismatch distributions (distribution of pairwise differences; Slatkin & Hudson, 1991; Rogers & Harpending, 1992) were calculated with ARLEQUIN, version 3.5.1.2 (Excoffier & Lischer, 2010) to estimate demographic events such as population growth and/or range expansion. Percentile confidence intervals were based on 1000 bootstrap replicates and a level of significance at $\alpha = 0.05$ (Schneider & Excoffier, 1999). The sudden and spatial demographic expansion models were tested using bootstrap and the sum of square deviations (SSD) between the observed mismatch distribution and its simulated data as a test statistic (i.e. test of goodness-of-fit) (Schneider & Excoffier, 1999; Excoffier, 2004). ARLEQUIN was also used to calculate indices of nucleotide (π) and haplotype (h) diversity (Nei, 1987).

To distinguish models of population growth from the null hypothesis of constant population size, Fu's F_s (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) neutrality tests were calculated with ARLEQUIN and DNASP, respectively. The probability (P) of neutrality tests was calculated based on 1000 replicates. Fu's F_s and the R_2 were used because they have been shown to be the most powerful tests for detecting sudden population growth or contractions (Ramos-Onsins & Rozas, 2002; Ramirez-Soriano *et al*., 2008); with Fu's F_s being recommended for large population sizes and R_2 for small ones (Ramos-Onsins & Rozas, 2002).

Spatial analysis

All demographic analyses were carried out with the complete *mt-Cytb* sequence (1149 bp). GENELAND, version 3.2.2 (Guillot *et al*., 2005; Guillot, Mortier & Estoup, 2005) was used to analyze population genetic structure with a Bayesian model based on the MCMC computational technique. The number of clusters was determined by independently running the MCMC ten times, allowing K (the number of populations) to vary from 1 to 10. The number of MCMC interactions was set to 100 000 per run with a thinning of 100. The uncorrelated frequency model setting the uncertainty on coordinates to 0 km were used, and the maximum number of nuclei in the Poisson–Voronoi tessellation was fixed at 300 (default option). Analyses were performed using variable sites only.

BARRIER 2.2 (Manni, Guerard & Heyer, 2004) was used to compute the Monmonier's maximum-difference algorithm (Monmonier, 1973) to identify biogeographical boundaries, or zones where genetic differences between pairs of populations were largest (Manni & Guérard, 2004). The implementation of Monmonier's algorithm in BARRIER was supervised by specifying the number of biogeographical boundaries to be computed in advance. We used a similar approach to Patten & Smith-Patten (2008) in choosing a priori a maximum pairwise genetic distance allowable between haplotypes. This cut-off was determined by the mean of the maximum and minimum pairwise genetic distance between haplotypes.

RESULTS

Sequence variation, genetic diversity, and population divergence

The complete *mt-Cytb* (1149 bp) was sequenced in 44 samples of four *Monodelphis* species, totalling 39 *M. domestica*, two *Monodelphis dimidiata* (Wagner, 1847), two *Monodelphis americana* (Müller, 1776), and one *Monodelphis umbristriata* (Miranda-Ribeiro, 1936) and analyzed in conjunction with 46 GenBank sequences.

Monodelphis domestica showed 48 haplotypes with 116 variations in 106 variable sites (96 transitions and 20 transversions). Pairwise genetic distance estimates ranged from 0% to 6.2%; nucleotide and haplotype diversity are shown in Table 2. Three haplotypes were shared by specimens from different localities (Table 1). A low level of saturation was observed in the dataset containing all sequences and all outgroups, with I_{ss} significantly smaller than $I_{ss.c}$ ($P < 0.000$) for a symmetrical tree topology (Xia & Lemey, 2009). Analyses of the first, second, and third codon positions also showed a low level of saturation.

Phylogenetic reconstructions

ML and BI trees were constructed with HKY (Hasegawa, Kishino & Yano, 1985) plus a proportion of invariable sites and gamma distributed substitution rates (HKY+I + Γ) as the DNA substitution model selected by MODELGENERATOR.

ML and BI (Fig. 2) showed the same topology, with a high support for the monophyly of *Monodelphis* and for all species, except for *Monodelphis emiliae* and *M. dimidiata* species (Fig. 2). These analyses grouped *Monodelphis* in four clusters: (1) ((*Monodelphis reigi*, *Monodelphis adusta*) ((*Monodelphis handleyi*, *Mono-*

Table 2. Genetic distance estimates and number of variable sites (V), transitions (Ts), transversions (Tv), and nucleotide (π) and haplotype (h) diversity for *Monodelphis domestica* sequences

Clade	Genetic distances (minimum – maximum)	V	Ts	Tv	π	h
A	0.000–0.039	76	54	10	0.024 ± 0.001	0.991 ± 0.013
B	0.000–0.044	58	52	8	0.018 ± 0.002	0.981 ± 0.023
A versus B	0.032–0.062	NA	NA	NA	NA	NA
All	0.000–0.062	106	96	20	0.033 ± 0.001	0.994 ± 0.006

NA, not applicable.

delphis osgoodi) *Monodelphis peruviana*)); (2) (*Monodelphis americana*, *Monodelphis umbristriata*); (3) (*Monodelphis iheringi*, (*Monodelphis brevicaudata*, (*Monodelphis glirina*, *M. domestica*))); (4) (*Monodelphis brevicaudata*, (*M. glirina*, *M. domestica*)). A high branch support was observed for (*M. domestica*, *M. glirina*) (Fig. 2). ML and BI subdivided *M. domestica* in two highly supported clades designated A and B. Clade A grouped 27 haplotypes from 14 localities from the Brazilian states of Minas Gerais (MG), Mato Grosso (MT), Mato Grosso do Sul (MS), and Goiás (GO), three localities in Bolivia, and one in Paraguay. Clade B grouped 22 haplotypes from 17 localities in the Brazilian states of Minas Gerais (MG), Piauí (PI), Ceará (CE), Alagoas (AL), Bahia (BA), and Pernambuco (PE).

A MJ network (Fig. 3) recovered two well-defined groups separated by 34 nucleotide substitutions, corresponding to clades A and B that were previously observed in phylogenetic reconstructions (Fig. 2). These similarities were also found in their respective internal arrangements. In clade A, the group from MS state was geographically structured, supported by 15 nucleotide substitutions and five exclusive haplotypes. In clade B, HP33, reported in the complete genome of *M. domestica* by Mikkelsen *et al*. (2007), was connected to haplotypes from BA and AL states in agreement with ML and BI analyses, indicating a likely origin of this specimen in the north-east of Brazil.

Demographic history

Mismatch distribution in *M. domestica* showed curves with similar SSD *P*-value estimates for both sudden and spatial expansion models (only the results of the sudden expansion model are shown in Fig. 4). Clade A showed a unimodal curve and clade B showed a bimodal curve, both with a high SSD *P*-value for sudden expansion (Fig. 4).

The Fu's F_s statistical neutrality test for clade A and for clade B showed an estimate of –3.745 ($P = 0.031$) and –6.46 ($P = 0.004$), respectively, suggesting population growth for both clades. On the other hand, the R_2 statistical neutrality test showed different results for clades A and B; 0.124 ($P = 0.318$) and 0.12 ($P = 0.377$), respectively, indicating the constant size of both populations.

Using the mean of pairwise genetic distance, the cut-off for stipulating the number of boundaries estimated by the Monmonier's algorithm was set to 0.03. This allowed the identification of two barriers separating *M. domestica* haplotypes (Fig. 1). The first one separated haplotypes from the Caatinga from the remaining samples from Cerrado and Pantanal. The second barrier was internal to clade B, isolating HP01 and HP02 (CE State), HP09 (Northern BA State), and HP10 (AL State) from the others. Haplotypes from Piauí State were divided by the barrier, which coincides with the results found in the phylogenetic analyses (Fig. 2). Furthermore, all ten runs performed with GENELAND provided a K of two clusters, where all ten runs partitioned populations identically, as observed in Figure 1, and also separated haplotypes of Caatinga from Cerrado and Pantanal.

DISCUSSION

All phylogenetic analyses confirmed the monophyly of the genus *Monodelphis* and showed a straight relationship between *M. domestica*, an inhabitant of nonforested habitats, and *M. glirina* and *M. brevicaudata*, typical Amazon Forest taxons. *Monodelphis brevicaudata* showed an evident genetic structure, despite the small number of samples analyzed in the present study. Similar findings have been recently reported by Solari (2010) in the *brevicaudata* species group.

Our phylogenetic reconstructions were similar to those previously reported (Lim *et al*., 2010; Vilela *et al*., 2010; Carvalho *et al*., 2011), except for the *M. adusta* complex, comprising species from western Amazonia and the Andean region. In the present study, the *M. adusta* complex was divided into

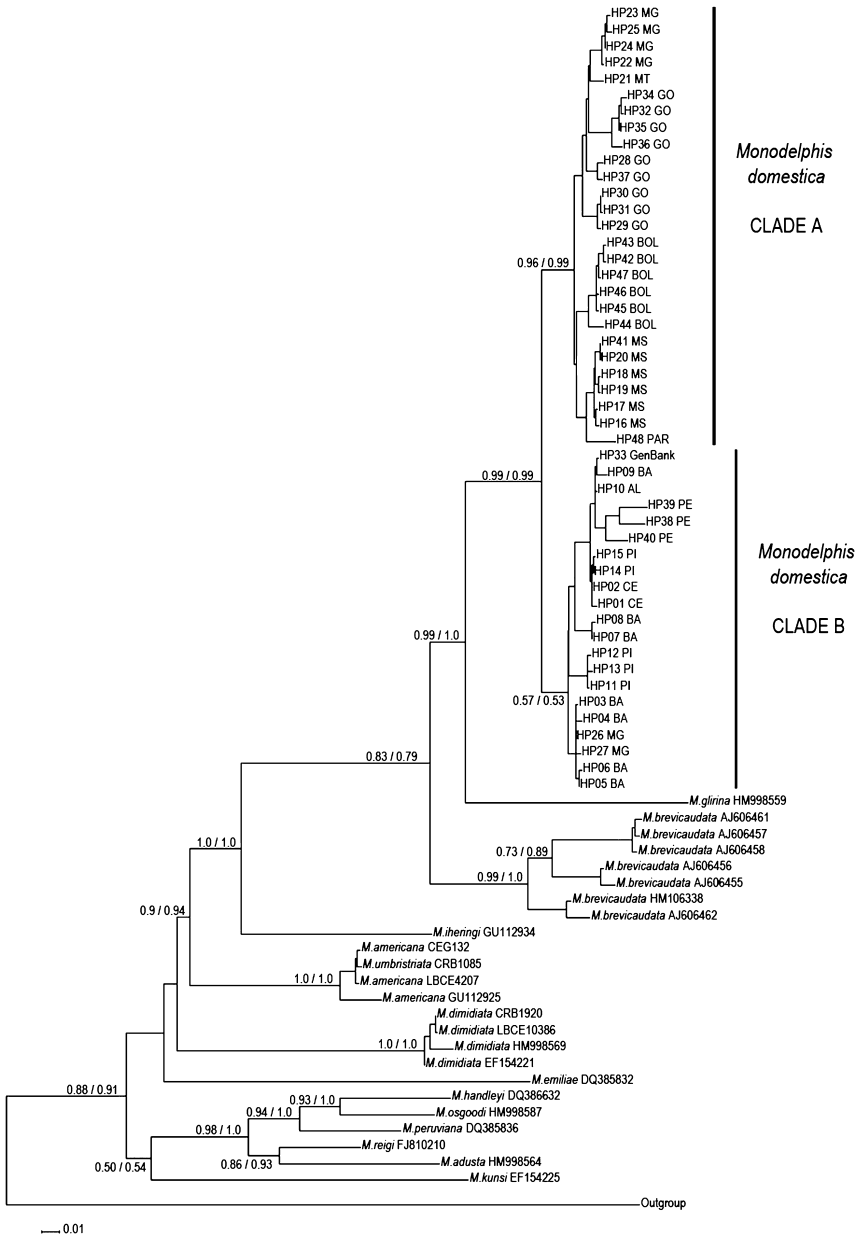

Figure 2. Maximum likelihood tree for cytochrome *b* gene of several *Monodelphis* species. Support values correspond to an approximate likelihood ratio test and Bayesian posterior probability, respectively.

two clades ((*M. adusta*, *M. reigi*) (*M. peruviana* (*M. handleyi*, *M. osgoodi*))), whereas Lim *et al*. (2010) found *M. adusta* as a sister lineage of (*M. reigi* (*M. peruviana* (*M. handleyi*, *M. osgoodi*))). These different arrangements within the *M. adusta* complex might be a consequence of the different species included in each study, as well as differences in sample size. Solari (2010) reported a different phylogenetic arrangement based on 17 of the 22 or 23 recognized species of *Monodelphis* and rejected the proposition of *Monodelphis* monophyly (Solari, 2007; Pine & Handley, 2008). The present study, although using the same molecular marker and almost the same species dataset, was based on the complete *mt-Cytb* sequence (1149 bp) in contrast to the partial sequence (810 bp) used by Solari (2010).

Monodelphis domestica was consistently subdivided into two clades (A and B) in all analyses, in agreement with the morphoclimatic patterns of their respective distributions. Brazil encloses a large variety of climates, soils, vegetations, drainage basins, rocky outcrops, and mountains, contributing

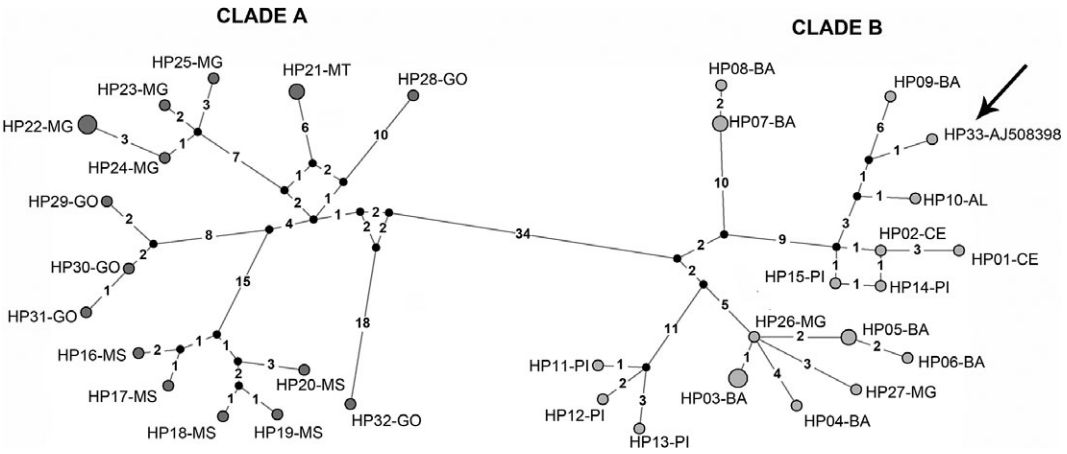

Figure 3. Median-joining of *Monodelphis domestica*. Circle sizes correspond to number of individuals carrying a given haplotype (Table 1). Haplotype (HP) numbers are followed by the state abbreviation (Table 1). Black circles are median vectors. Numbers in the middle of each connecting branch denote the number of nucleotide substitutions. Branches are not shown to scale. A black arrow indicates the GenBank sample of *M. domestica* with the complete genome.

to habit diversity and, eventually, to the dispersal and isolation of organisms. Clade A included specimens from two domains, Pantanal and Cerrado. Pantanal goes through a definite wet season when lowlands are flooded by the waters of the Paraguay river basin, whereas Cerrado is a mosaic of open woodlands, savannas (arboreal grasslands), and open grasslands that are highly variable in vegetational composition, seasonal precipitation, soil fertility, drainage, and fire exposure, showing a west boundary with the Pantanal domain (Furley, 1999; Oliveira-Filho & Ratter, 2002). Similarities between the vegetational composition of Cerrado and Pantanal (Ab'Sáber, 1988; Zeilhofer & Schessl, 2000) might explain why clade A contains *M. domestica* from these two different morphoclimatic domains.

Clade B included specimens from Caatinga, a typical semi-arid habitat with well-defined rainy and dry seasons. The Caatinga encompasses all states of the Brazilian north-eastern region, with boundaries with the Atlantic Forest in the south, the Amazon Forest in the north, and the Cerrado in the west.

These clades (Fig. 1) were well separated from one another in all analyses, suggesting a likely vicariant event, although speciation without clear barriers cannot be discarded (Aguiar *et al*., 2009). This separation was detected by GENELAND (Fig. 1), as well as by Monmonier's algorithm (barrier A; Fig. 1). The second barrier found by the Monmonier's algorithm does not exhibit a clear geographical explanation.

Neutrality tests did not show congruent results because mismatch distribution and Fu's F_s indicated population expansions in clades A and B in contrast to R_2 that recorded a stable population size in both clades. This latter possibility is more likely because the sample size of each clade was small (15 haplotypes in A and 18 haplotypes in B), and because the R_2 test provides a better assessment for small rather than large samples (Ramos-Onsins & Rozas, 2002). This scenario is in agreement with the MJ pattern (Fig. 3) that indicated a clear distinction between clades A and B but lacked a star-like pattern characteristic of a recent diversification.

Our data showed that these populations belong to two evolutionary lineages. The median genetic distance between clades (5%) suggested that, during this time span, these lineages became independent evolutionary units. Solari (2007) found a genetic distance estimate of 9% between *M. osgoodi* and *M. handleyi*, which are two sister species to the evolutionary lineages of *M. domestica*.

The influence of past climatic oscillations in the current distribution of several species and their genetic diversity has been well documented for the Quaternary (Novaes *et al*., 2010). Nevertheless, the geomorphological status of current, open landscapes of Brazil during the period involving the Late Miocene and Pliocene is unclear (Hoorn *et al*., 2010). In the Middle Pliocene, the warm equable climates were replaced by recurring ice ages (Molnar & Cane, 2002), and it is likely that, during the Pliocene, these steady climates went through wet periods, leading to the isolation of *M. domestica* populations in different regions.

Other studies found the same pattern of population structure of *M. domestica* (Solari, 2010; Carvalho *et al*., 2011), although the results were inconclusive with respect to considering this taxon as a species complex. Speciation involves disparate and discordant points of view (Coyne & Orr, 2004), mainly when one

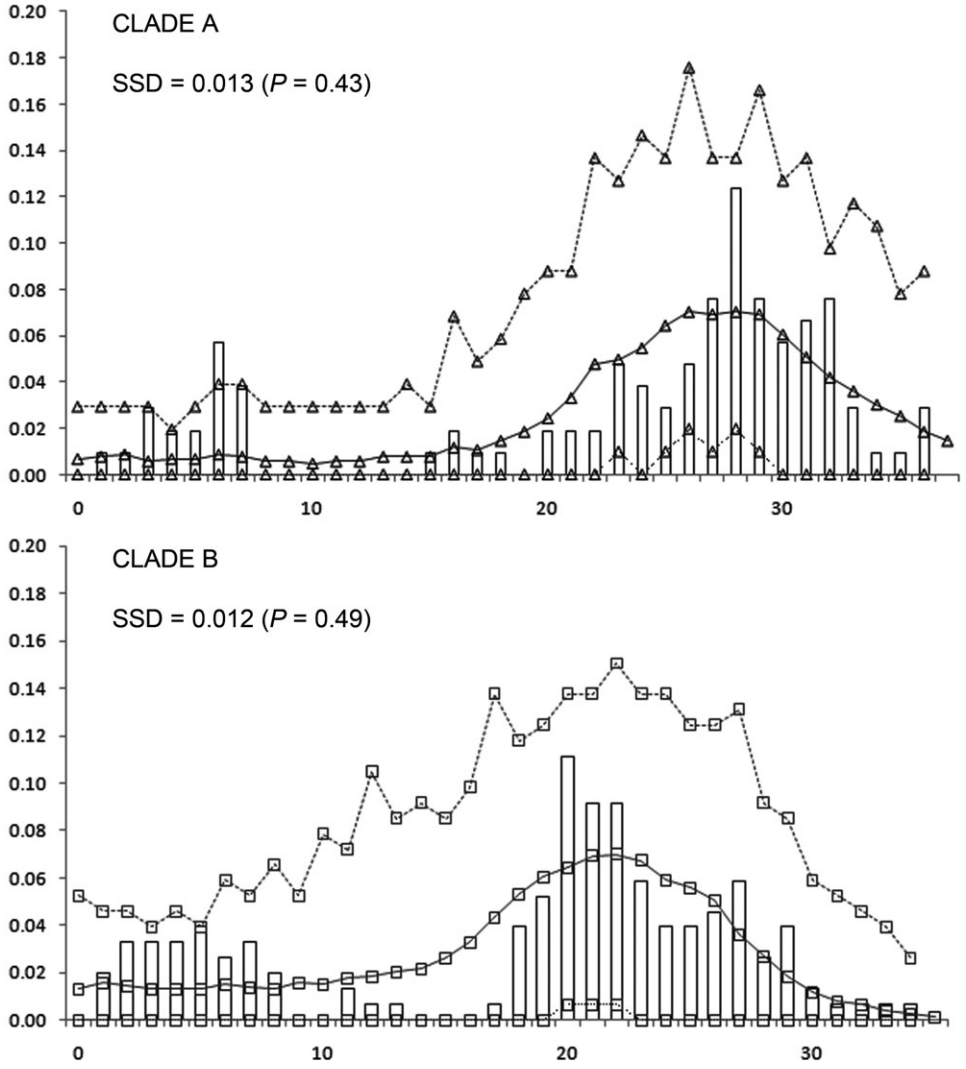

Figure 4. Mismatch distribution for clade A (above) and clade B (below). White bars, observed distribution; solid line, simulated distribution under the demographic expansion model; dashed lines, 95% and 5% percentiles; *x*-axis, number of pairwise differences; *y*-axis, frequency of observed and simulated curves; SSD, sum of square deviations under the sudden demographic expansion models.

or few markers are used for decision-making. We agree with Solari (2010) that the *mt-Cytb* gene is not the best marker for inferring distant phylogenetic relationships; however, we consider it valid for intrageneric analyses.

The results obtained in the present study indicated that *mt-Cytb* was evolutionary conserved, with low saturation, suggesting a potentially useful marker for population analyses of didelphids. It is likely that, in these marsupials, *mt-Cytb* evolution has been slower than in Eutherian mammals.

The separation of the two populations of *M. domestica* must be taken into account in studies involving this species. Because it is widely used as an animal model, its genetic background should not be ignored. Captive bred colonies available in several countries are likely to contain descendants of specimens from one or both lineages, a possibility that should not be overlooked. Additional studies, using other molecular markers and morphological traits, are needed to validate our proposition of two different evolutionary lineages.

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APPENDIX

Table 1. List of specimens analyzed, including species name, GenBank accession, size of the *mt-Cytb* gene (bp), and literature reference of each of the 41 sequences obtained from GenBank

Taxon	GenBank	Size (bp)	Reference
Monodelphis domestica			
	AJ508398	1149	Nilsson *et al*., 2003
	EU750746	640	Carvalho *et al*. (2011)
	EU750749	640	Carvalho *et al*. (2011)
	EU750751	640	Carvalho *et al*. (2011)
	EF154194	728	Carvalho *et al*. (2011)
	EF154196	770	Carvalho *et al*. (2011)
	EF154205	1149	Carvalho *et al*. (2011)
	EF154210	753	Carvalho *et al*. (2011)
	HM998570-77	801	Solari, 2010
Monodelphis brevicaudata			
	AJ606455-58	800	Steiner & Catzeflis (2004)
	AJ606461-62	800	Steiner & Catzeflis (2004)
	HM106338	1146	Gutierrez *et al.* (2010)
Monodelphis dimidiata			
	EF154221	1122	Carvalho *et al*. (2011)
	HM998569	822	Solari (2010)
Monodelphis emiliae			
	DQ385832	830	Solari (2007)
Monodelphis americana			
	GU112925	801	Agrizzi *et al.* (unpubl. data)
Monodelphis iheringi			
	GU112934	801	Agrizzi *et al.* (unpubl. data)
Monodelphis glirina			
	HM998559	830	Solari (2010)
Monodelphis peruviana			
	DQ385836	772	Solari (2007)
Monodelphis osgoodi			
	HM998587	807	Solari (2010)
Monodelphis handleyi			
	DQ386632	1149	Solari (2007)
Monodelphis reigi			
	FJ810210	1149	Lim *et al*. (2010)
Monodelphis adusta			
	HM998564	801	Solari (2010)
Monodelphis kunsi			
	EF154225	737	Carvalho *et al*. (2011)
Didelphis virginiana			
	NC001610	1149	Janke *et al*. (1994)
Thylamys elegans			
	AJ508401	1149	Nilsson *et al*. (2003)
Marmosa lepida			
	U34668	1149	Patton *et al*. (1996)
Marmosa (*Micoureus*) *demerarae*			
	U34673	1149	Patton *et al*. (1996)
Marmosa (*Micoureus*) *regina*			
	U34675	1149	Patton *et al*. (1996)
Caluromys philander			
	AJ628362	828	Steiner *et al*. (2005)