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Original Article

Genetic variability of populations of the white-eared opossum, *Didelphis albiventris* Lund 1840 (Didelphimorphia; Didelphidae) in Brazil

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(With 4 figures)

Abstract

Didelphis albiventris are found throughout Northeast and Central Brazil to central-southern Uruguay and it was subject of few studies in a population level. Given this, the present study investigated the genetic variability of the species using the mitochondrial molecular marker cytochrome oxidase *c* subunit I. We analyzed samples from the different biomes within three Brazilian regions: Northeast (*Caatinga*, *Cerrado*, and Atlantic Forest), Southeast (*Cerrado*, Atlantic Forest, Cerrado/Atlantic Forest, and Cerrado/Caatinga ecotones) and South (Pampa and Atlantic Forest). Software BAPs retrieved five distinct demes: dm 1, dm 2, and dm 5 that occurs in South, Northeast and Southeast regions respectively and the dm 3 and dm 4 are wide distributed in Northeast and Southeast. Population analysis performed with AMOVA, haplotype network and Mantel test estimated the veracity of the demes. The F_{ST} shows structuring for the five demes, with dm 1 (South region) isolated from the others, however the other analysis showed the Northeast/Southeast demes (dm 2-5) united, diagnosing gene flow between them, mainly at the transitional zones, in areas as far away as areas with similar latitude interval (Southeast vs South) that was not detected gene flow. In the haplotype network, the mutational steps was conclusive in split dm1 from dm 2-5 with 15 mutational steps and the Mantel test was moderated, which is explained by genetic similarity despite the great geographic distances (Northeast/Southeast). Thus, our analysis recognized two different lineages (South and Northeast/Southeast) and indicate that the biomes were not decisive in their isolation. The sharing of demes at the transitional zones and in areas with high latitudinal intervals reflects a recent ancestral polymorphism for *D. albiventris*. The plasticity in the occupation of the space by this species contributes in its wide dispersion capability, that is, geographical distribution. Our results revealed important implications for the management of *D. albiventris* in these transitional zones areas where demes were shared.

Keywords: marsupials, COI, population diversity, geographic lineages.

Variabilidade genética de populações do gambá de orelha branca, *Didelphis albiventris* Lund 1840 (Didelphimorphia; Didelphidae) no Brasil

Resumo

Didelphis albiventris é encontrada em todo o Nordeste e região central do Brasil até o centro-sul do Uruguai e foi alvo de poucos estudos em nível populacional. Dessa forma, o presente estudo, investiga a variabilidade genética da espécie usando o marcador molecular citocromo c oxidase subunidade I. Analisou-se amostras de diferentes biomas de três regiões brasileiras: Nordeste (*Caatinga*, *Cerrado* e Floresta Atlântica), Sudeste (*Cerrado*, Floresta Atlântica, ecótonos Cerrado/Floresta Atlântica e Cerrado/Caatinga) e Sul (Pampa e Floresta Atlântica). O *software* BAPs recuperou cinco demes distintos: dm 1, dm 2 e dm 5, que ocorrem nas regiões Sul, Nordeste e Sudeste, respectivamente, e os dm 3 e dm 4, que são amplamente distribuído no Nordeste e Sudeste. Análises populacionais realizadas com AMOVA, rede de haplótipo e teste de Mantel estimaram a veracidade das demes. O F_{ST} mostrou estruturação para as cinco demes, com dm 1 (região Sul) isolada das demais, entretanto as outras análises mostraram as demes Nordeste/Sudeste (dm 2-5) unidos, diagnosticando fluxo gênico entre elas, principalmente em zonas de transição, em áreas tão distante quanto

áreas com similar intervalo de latitude (Sudeste e Sul), onde não foram detectado fluxo gênico. Na rede de haplótipo, os passos mutacionais foram conclusivos em separar dm 1 do dm 2-5 com 15 passos mutacionais, e o teste de Mantel foi moderado, o que é explicado pela similaridade genética apesar da grande distância geográfica (Nordeste/Sudeste). Assim, duas linhagens diferentes (Sul e Sudeste/Nordeste) foram encontradas, indicando que os biomas não foram decisivos em seus isolamentos. Os compartilhamentos das demes, em zonas de transição e em áreas com elevados intervalos de latitude, refletem um polimorfismo ancestral recente para *D. albiventris*. A plasticidade na ocupação do espaço por esta espécie contribui em sua ampla capacidade de dispersão, ou seja, distribuição geográfica. Nossos resultados revelam importantes implicações para o manejo de *D. albiventris* nessas áreas de zonas de transição, onde as demes são compartilhadas.

Palavras-chave: marsupiais, COI, diversidade populacional, linhagens geográficas.

1. Introduction

The marsupials of the genus *Didelphis* Linnaeus, 1758 are a small non-volant mammals that are an essential component of natural communities and good indicators of environmental changes and its degree of conservation (Conde y Vera and Rocha, 2006; Castro, 2012; Quintela et al., 2013). In particular, populations of the white-eared opossum (*Didelphis albiventris* Lund, 1840) tends to increase in density in response to environmental disturbance (Cáceres et al., 2008), with associated implications for public health given that this species is a natural reservoir for a number of human diseases such as Chagas disease and leishmaniasis (Sherlock et al., 1984; Pinho et al., 2000; De Luca et al., 2003; Bern et al., 2011; Zetun et al., 2014).

Due to its generalist habit, *D. albiventris* is highly flexible in the use of space, exploiting a variety of vertical strata and occupying large home ranges (Almeida et al., 2008; Sanches et al., 2012). The species is widely distributed in deciduous forests, occurring from northeastern and central Brazil to central and southern Uruguay, as well as in Paraguay, Argentina and Bolivia (Cerqueira and Tribe, 2008). In Brazil there are records in Caatinga, Cerrado, Pantanal, Pampas habitats and transitional zones between these biomes (Cerqueira and Tribe, 2008; Paglia et al., 2012).

Despite the genus *Didelphis* is common and ecologically successful (Durant, 2001), little is known about the relationships among of *D. albiventris* populations, with only two studies available: Sousa et al. (2012) and Rocha et al. (2015). Both works revealed some degree of genetic structuring, the former studied the mitochondrial gene cytochrome *c* oxidase subunit I (COI) of populations from southern and southeastern Brazil, and the other the mitochondrial gene cytochrome *b* from few localities in central Brazil.

Mitochondrial DNA have revealed degree of population structure for many species, being the most used molecular marker for tracing the geographic distribution of genealogical lineages (Avise et al., 1987). Males and females of the *D. albiventris* present similar patterns of dispersal (Sanches et al., 2012), and the sex of the specimen should be irrelevant to the selection of the molecular marker, consequently the matrilineal inheritance of the mitochondria should not interfere in the analyses. Notably, mitochondrial genes have been used successfully in population-level and phylogenetic studies of small mammals (Carvalho et al., 2011; Agrizzi et al., 2012; Sousa et al., 2012; Muller et al., 2013; Rocha et al., 2015). In particular, the COI gene has been used as a DNA barcode for the identification of a number of different vertebrate groups (Hebert et al., 2003), and has contributed important findings on *D. albiventris* populations (Sousa et al., 2012).

Ecological studies use population and genetic data in attempt to understand the biology of a species. With the scarcity of population genetic information throughout its distribution, especially for the inhabitants of *Cerrado* and *Caatinga*, the present work investigated how *D. albiventris* populations are distributed in Brazil using the COI as a molecular marker: 1. The species possesses populations structured in the Northeast/South axis? 2. There is more than one population per Biome? 3. If yes, they are shared between Biomes or are exclusive for one Biome? Ecotone areas has a different role in the population structure of the species? 4. Is there natural selection or are the populations selectively neutral? 5. In attempt to answer these questions we conducted genetic variability study of populations of the white-eared opossum, *D. albiventris*.

2. Material and Methods

2.1. Description of the samples

Samples of *D. albiventris* were obtained from the Northeast, Southeast and South regions of Brazil, between latitudes 04°05' S and 30º01' S (Figure 1). A total of 52 samples were obtained in the Northeast region (Paraíba/PB, Sergipe/SE, Pernambuco/PE, Ceará/CE, Piauí/PI and Maranhão/MA), from the Atlantic Forest, *Caatinga* and *Cerrado* biomes. Eighty four samples from Southeast (Minas Gerais/MG) from the Atlantic Forest and Cerrado biomes, and the Cerrado/Atlantic Forest and Cerrado/Caatinga ecotones. Six samples from South (Rio Grande do Sul/RS) corresponding to the Pampas and Atlantic Forest (Figure 1; Supplementary Material).

The samples from the Northeast were obtained in the *Universidade Estadual do Maranhão*, for which the collecting data are available in Costa et al. (2012), and from collaborations with the scientific collections of other Brazilian research institutions, such as *Universidade Federal da Paraíba* (UFPB), *Universidade Federal de Sergipe* (UFS), *Universidade Federal do Piauí* (UFPI), and *Museu Paraense Emílio Goeldi* (MPEG). All samples

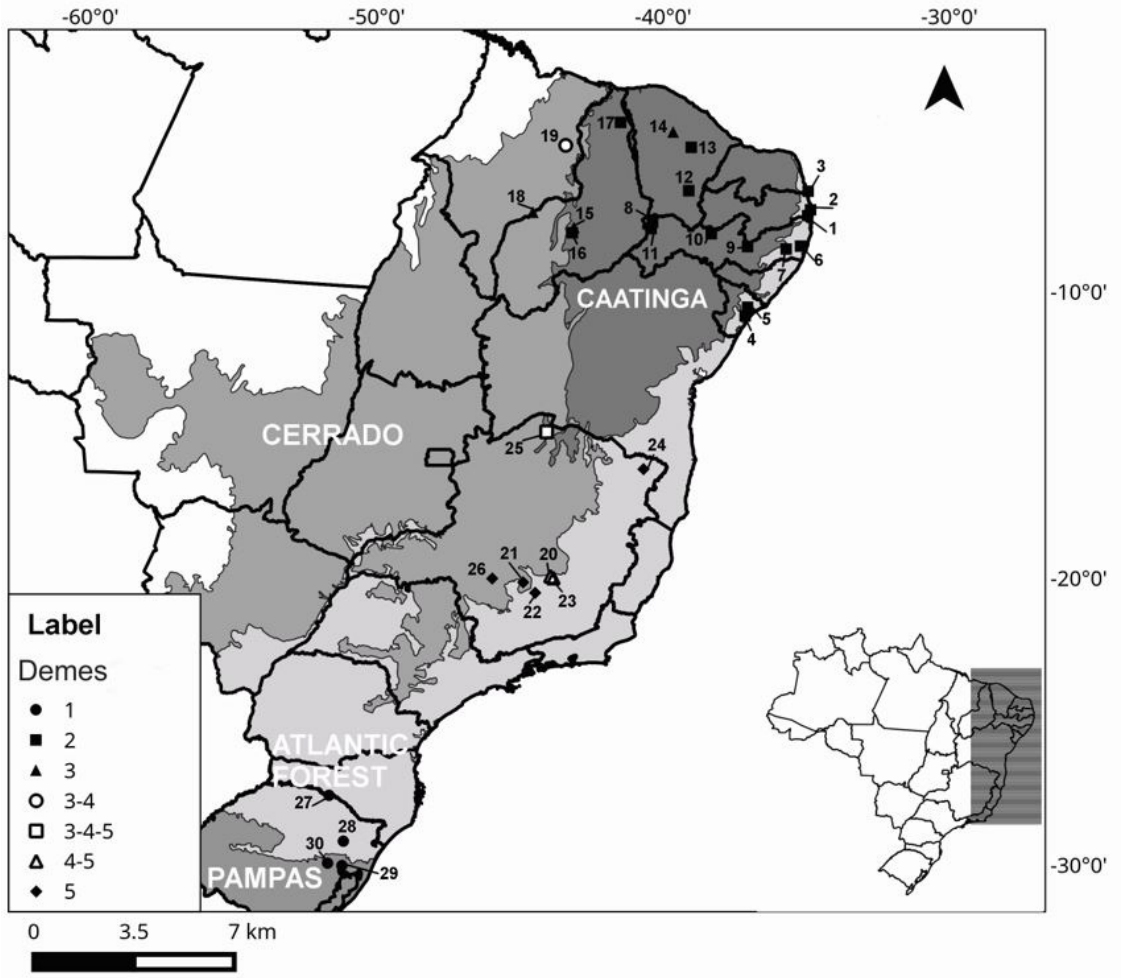

Figure 1. Localities from which the samples were obtained for the present study. The demes were defined according to the BAPs software and are represented by geometric figures with numbers (designated in Supplementary Material); Biomes are indicated in gray scale.

from Southeast and South were obtained at GenBank from Sousa et al. (2012).

2.2. DNA extraction, amplification and sequencing

DNA was extracted from muscle, liver, and ear tissue using the Phenol-Chloroform protocol of Sambrook and Russel (2001). We amplified and sequenced a fragment of the mtDNA COI gene using the primers LCO1490 and HCO2198 (Folmer et al., 1994). The COI gene was amplified in 25 µl reactions of PCR, consisting of 4.0 µl of dNTP (1.25 nM), 2.5 µl of buffer (10X), 0.5 µl of $MgCl_2$ (25 nM), 0.25 µl of each primer (200 ng/µl), 0.2 µl of Taq DNA Polymerase 10 x 250 µl (GE Healthcare), and 1 µl of template DNA (100 ng/µl), with the final volume being completed by purified water. The samples were amplified in a Veriti 96 well thermal cycler (Applied Biosystems) with an initial denaturation (94ºC for 3 min), 40 cycles of denaturation (94ºC for 45 s), annealing (48ºC for 45 s), and extension (72ºC for 1 min and 30 s), and a final extension at 72ºC for 3 min. We used the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) for sequencing reactions. The sequences were obtained using an ABI 3500 automatic DNA sequencer with associated Sequencing Analysis software (Applied Biosystems).

2.3. Data analysis

The sequences were aligned using Clustal W (Thompson et al., 1994) in BioEdit v7.0.5.2 (Hall, 1999). The analysis of the variable sites was performed in Mega v6.06 (Tamura et al., 2013). Haplotype and nucleotide diversity were estimated in DnaSP v4.9 (Rozas et al., 2003), and the groups used for the population analyses were defined using the BAPs software (Corander et al., 2008). In this method, the number of populations is treated as unknown parameter and is directly inferred from the data set without defining a prior estimate, a dendogram were generated using Unweighted Pair Group Method using Arithmetic averages - UPGMA. The populations identified by this

software were used to structure the subsequent population analyses. The haplotype networks were produced in NetWork v4.6.1.2 (Bandelt et al., 1999). An Analysis of Molecular Variance (AMOVA) was performed in Arlequin v3.5.2.1 (Excoffier and Lischer, 2010) to obtain the index of allelic fixation (F_{ST}). The neutrality tests D (Tajima, 1989) and Fu's F_S (Fu, 1997) were run in Arlequin v3.5.2.1 (Excoffier and Lischer, 2010). The Mantel test, which relates genetic distance to geographic distance, was run in the Alleles in Space software with 10,000 replications (Miller, 2005).

3. Results

3.1. Molecular characterization and neutrality tests

The study included a total of 142 sequences for the mitochondrial COI gene of *D. albiventris*, made up of a fragment of 655 bps, of which 624 bps were conserved, 31 were variable, and 21 were informative for parsimony analyses, corresponding to 19 haplotypes.

The sequences plotted in the BAPs software (Figure 2) indicated the existence of five Brazilian demes (dm), as can be seen in the haplotype network and in the map (Figure 1 e 3). The dm 1 occurs in the Pampas and Atlantic Forest biomes (South region; H1 and H2), dm 2 in the *Caatinga* and Atlantic Forest biomes (Northeast region; H3-H8) and dm 5 *Cerrado*, *Caatinga*, Atlantic Forest and ecotones (Southeast regions; H16-H19). The dm 3 (H 9-H13) and dm 4 (H14 and H15) are more widely distributed, being found simultaneously in the *Cerrado* and *Caatinga* (Northeast region) and *Cerrado*, *Caatinga*, Atlantic Forest (Southeast region). We can observe that some localities present multiple demes: Xacriabá Amerindian Reservations/MG that contains dm 3, dm 4 and dm 5; Caxias/MA dm 3 and dm 4, Belo Horizonte/MG and Nova Lima/MG dm 4 and dm 5 (Table 1).

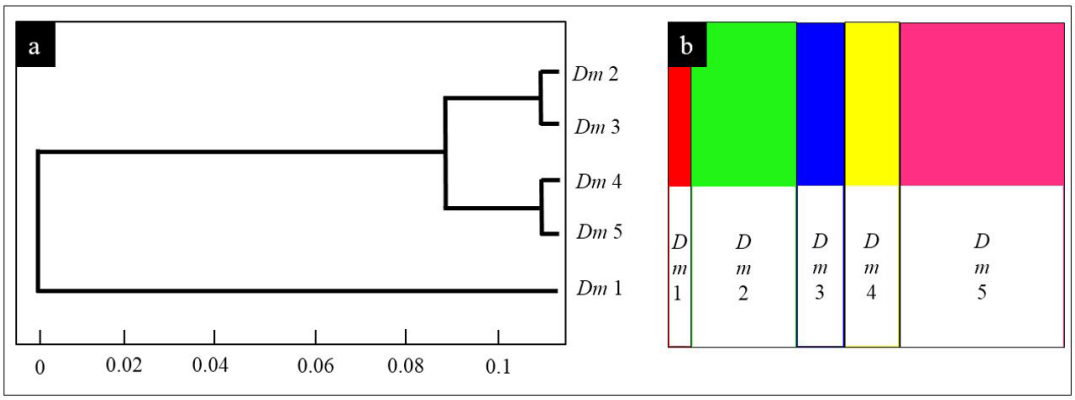

Figure 2. *Didelphis albiventris* demes defined through the BAPs software for the COI gene. (a) UPGMA tree using the Nei model. (b) Mixture among the demes obtained, the size of the color bars is proportional to the number of specimens in the populations.

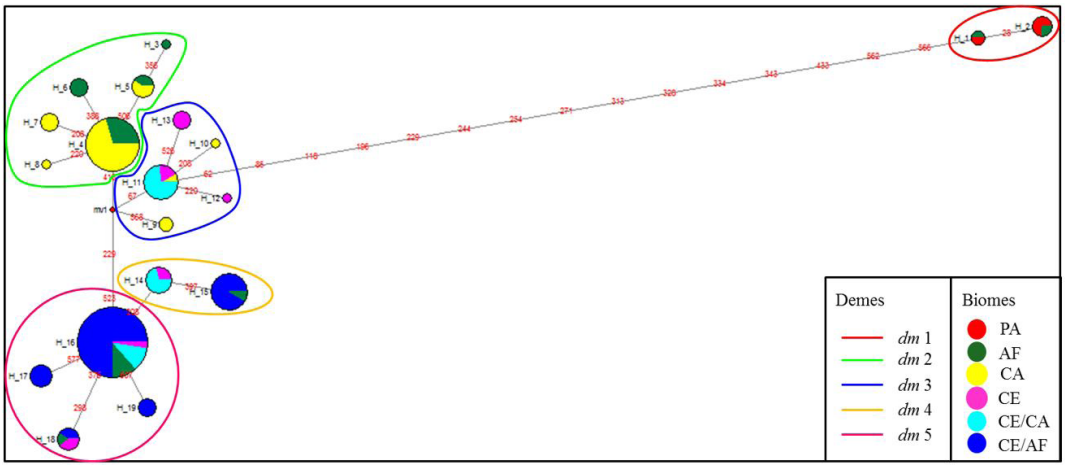

Figure 3. Haplotype network for the COI gene in the *D. albiventris* demes. The numbers represents the mutational sites between demes, while the size of the circles is proportional to the frequency of occurrence of the haplotype. Biomes: PA - Pampa; AF - Atlantic Forest; CA - Caatinga; CE - Cerrado; CE/CA - Ecotone Cerrado/Caatinga; CE/AF - Ecotone Cerrado/Atlantic Forest. The polygons delimit the haplotypes that comprises each deme.

The total haplotypic diversity (*h*) was *h* = 0.846, and varied from *h* = 0.387 for dm 5 to *h* = 0.614 for dm 3, while total nucleotide diversity (π) was π = 0.00615, and varied from π = 0.00078 for dm 4 to π = 0.00152 for dm 3 (Table 1). No significant *p* values were found in any deme for the D test (Tajima, 1989), and a significant value for Fu's F_S (Fu, 1997) was only recorded for dm 2, indicating that only this deme is influenced by demographic parameters (Table 1).

3.2. Genetic differentiation among demes

AMOVA analyses based on the five demes retrieved by the BAPs analysis (Table 1) indicated that 87.47% of the variation was due to differences among the demes, and 12.53% within the demes, with a highly significant (p = 0.00000) F_{ST} value of 0.87 (Table 2). The pairwise F_{ST} values were higher, ranging from 0.7, for dm 2 *vs.* dm 3, to 0.97 for dm 4 *vs.* dm 1 (Table 3).

The results of the Mantel test for the five demes indicated a moderate degree of correlation between genetic and geographic distances (r = 0.54; p = 0.00001; Figure 4). When dm 1 (r = -0.31; p = 0.19), dm 2 (r= -0.13; p = 0.79), and dm 5 (r= -0.04; p = 0.61) were analyzed separately the correlation were low, although dm 3 (r = 0.90; p = 0.001) and dm 4 (r = 0.89; p = 0.001) returned high correlations.

4. Discussion

Due to the ecological importance of *D. albiventris*, the investigation of the dynamics of dispersion of its populations through a genetic approach will improve the knowledge and contribute to the establishment of management and conservation measures along its geographical distribution.

Were analyzed the largest sample for this specie to date, and high levels of haplotype diversity (*h*) and nucleotide diversity (π) were found. Similar results were retrieved in Sousa et al. (2012); *h* = 0.7235; π = 0.0065) for COI and Rocha et al. (2015) for Cytochrome *b* (*h* = 0.876; π = 0.0062), reflecting the high genetic diversity of this species. According to Grant and Bower (1998) high values of *h* and π combined (above 0.5 and 0.005 respectively)

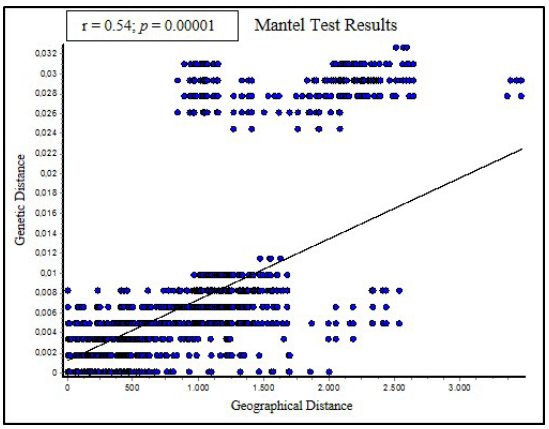

Figure 4. Results of the Mantel test for the correlation between genetic (y) and geographic (x) distances in the COI gene among the *D. albiventris* demes analyzed.

Table 1. Genetic diversity and the results of the neutrality tests for the COI gene in the *D. albiventris* demes analyzed.

Demes	Localities	N	NH	Molecular diversity		Neutrality tests	
				h	π	Test D	Test *Fs*
Dm1	27 - 30	6	2	0.533	0.00087	0.85057	0.62543
Dm2	1 - 13, 16 e 17	40	6	0.529	0.00099	-1.14587	-2.65922*
Dm3	14, 15, 18, 19 e 25	18	5	0.614	0.00152	-1.13016	-1.43002
Dm4	19, 20, 23 e 25	20	2	0.479	0.00078	1.26176	1.31130
Dm5	20-26	58	4	0.387	0.00094	-0.72085	-0.38003
Pooled demes		**142**	**19**	**0.846**	**0.00615**	**-0.17691**	**-0.50651**

N: Sample size; NH: Number of haplotypes; *h*: Haplotype diversity; π: Nucleotide diversity; (*) = p < 0.05

Table 2. Results of the AMOVA for the COI gene for the five *Didelphis albiventris* demes retrieved from BAPs.

Source of variation	Variance component	% Variation	F_{ST}	*p**
Among demes	2.18911	87.47	0.87465	0.0000
Within demes	0.31372	12.53		

*Values of *P,* calculated randomly through 1023 permutations.

Table 3. Pairwise F_{ST} values for the COI gene for five demes of *D. albiventris*.

Deme	Dm 1	Dm 2	Dm 3	Dm 4	Dm 5
Dm 1	0.00000				
Dm 2	0.96492	0.00000			
Dm 3	0.94811	0.71389	0.00000		
Dm 4	0.97321	0.87873	0.85555	0.00000	
Dm 5	0.96605	0.87873	0.81081	0.72012	0.00000

P = 0.0000.

reflects long evolutionary history. In the present study our results shows the haplotypes H11, H14 and H16 widely geographic distributed, suggesting long dispersion time and consequently long evolutionary history, corroborating Grant and Bower (1998).

By randomly treating allelic frequencies of genetic marker and genetic divergence between groups, the BAPS software retrieved five demes, which are divided between the three regions analyzed, with overlap for demes 3 and 4 in the Northeast and Southeast Brazil. The BAPS was not unanimous in considering the geographic proximity or the biomes to delimit the demes, consequently other population analyzes were carried out to verify if they correspond to different lineages.

In order to identify if there is selective neutrality, tests were performed for the five demes. The results of Tajima's D were not significant and Fu's *Fs* was only significant for dm 2, which contains haplotypes from the Northeast. Significant results for these tests indicate the presence of natural selection or demographic processes among the variants of a nucleotide sequence in a population. Dm 2 is distributed over a wide area, with different kind of biomes which could be interfering in the selective neutrality (Fu and Li, 1993; Nielsen 2001). No significant results were obtained for these same tests by Sousa et al. (2012) and Rocha et al. (2015), which indicates that this species is relatively constant. It is important to mention that these authors did not study northeastern populations.

The results of the AMOVA for the five demes indicate structuring (F_{ST} = 0.87). This structuring is evident in the case of dm 1, which has pairwise F_{ST} values of 0.95 to 0.97, indicating possible processes of allele fixation and genetic isolation. Sousa et al. (2012) also found evidence of population structuring (F_{ST}> 91%) in the Southeast and South populations.

The remaining demes analyzed in the AMOVA (dms 2, 3, 4 and 5) had also high pairwise F_{ST} values (0.71 to 0.88). Despite the high F_{ST}, the same demes are distributed in different localities, for example: dm 3 are in Caxias/MA and Xacriabá Amerindian Reservations/MG; dm 4 in Caxias/MA, Xacriabá Amerindian Reservations/MG, Belo Horizonte/MG and Nova Lima/MG; dm 5 in Xacriabá Amerindian Reservations/MG, Belo Horizonte/MG and Nova Lima/MG. We can also notice that different demes are in sympatry, for example: dm 3 and dm 4 in Caxias/MA; dm 3, dm 4 and dm 5 in Xacriabá Amerindian Reservations/MG; dm 4 and dm 5 in Belo Horizonte/MG and Nova Lima/MG.

These sympatric demes show that F_{ST} are considering different structured population in the same localities. This condition could be explained by the wide distribution of *D. albiventris* (Figure 1) and by the highly adaptable and generalist habit of the specie, since it is known that altitude and environment do not interferes in the specie distribution (Aragona and Marinho-Filho, 2009). Thus, although F_{ST} shows structuring, the connection between demes allows that Northeastern and Southeastern regions belong to the same widespread lineages.

Studies of didelphids have shown population structuring among different Brazilian biomes, for example Carvalho et al. (2011) and Caramaschi et al. (2011), found population structuring of *Monodelphis domestica* between the *Cerrado* and *Caatinga*. In the present study this relationship was not verified, as the demes are not restricted to one specific biome and are dispersed among a number of different biomes. This fact may be associated in differences in home ranges and in ecological characteristics between *M. domestica* and *D. albiventris* that could be possible directly related with body size. *Didelphis albiventris* is the largest Didelphidae weighting 500-2,750g while *M. domestica* weight 80-120g only. This size difference reflects in greater dispersion capacity (Reis et al., 2006; Wang et al., 2009) of *D. albiventris* that increases the gene flow, making population structuring more difficult. In this study, only dm 1 was isolated from the others, but this fact is not associated with biomes, since that deme occurs in Atlantic Forest and Pampa's biomes.

When comparing the mutational sites among the demes, the haplotype network also shows two lineages with different geographic distributions for *D. albiventris* in this study: one Southern lineages (dm 1) and the other comprising Northeast/Southeast (demes 2-5) showed shared haplotypes. These two principal lineages are separated by 15 mutational steps. Northeast and Southeast dm's are separated by few mutational steps (maximum of five, see Figure 3), have distribution overlapping between biomes and therefore should be considered as a single lineage.

The gene flow shown in the haplotype network represents an ancestral polymorphism, since the shared haplotypes are distributed over long geographic distances, as can be seen in dm 3 (H11) that occurs in Rio Grande do Piauí/PI (*Cerrado*) and Xacriabá Amerindian reservations/MG (*Cerrado*/*Caatinga* ecotone) (latitudes 07º 45' S - 14° 53' S). But the results of the haplotype network did not reveal connection between dm 5 (H16 and18) in Piracema/MG (Atlantic Forest) and dm 1 (H1) Machadinho/RS (Atlantic Forest) which has similar latitude interval (latitudes 20º 30' S - 27º 34' S) (Figure 1 and 3; Table S1), another indication of the isolation of these two strains.

The Mantel test indicated a moderate degree of correlation between genetic and geographic distances in the five demes. In this study, with the addition of *D. albiventris* samples from the Northeast, we obtained a reduction in the correlation obtained by Sousa et al. (2012). This reduction in the correlation reinforces the similarity found between demes 2-5, not diagnosed by these authors, and indicates that the Northeast/Southeast lineage is genetically similar although geographically distant.

With the addition of northeastern *D. albiventris* samples, were possible to conduct broader analyses encompassing a more complete distribution range of the specie. Sousa et al. (2012), found patterns of isolation between Southeast and South regions as well as ours analyses, but it represents just a small fraction of the distribution. The addition of Northeast does not interfere in the isolation of the South region from the others, but the

Northeast and Southeast are connected. It is important to mention that connection of demes occurs in the transitional areas between biomes, which strengthens the conclusions of Sousa et al. (2012) and Rocha et al. (2015) about the importance of the transition zones for research and planning. These areas are considered as hotspots of intraspecific diversity and play an important role in the genetic diversity of species and should be closely investigated (Smith, 1997; Kark et al., 2002).

Our results provide important new insights into the population dynamics of small mammals in Brazil. It has long been assumed that these kind of mammals have low vagility, with geographically structured populations and highly divergent genetic traits (Crisci et al., 2003). The extensive connection between demes of the Northeast and Southeast for *D. albiventris* may reflect the ecological plasticity of this species, resembling a medium-large mammal, with large home-range.

In the present work we diagnosed two lineages (Northeast/South axis) for the species *D. albiventris* and that the biomes do not interfere in the isolation of their demes. However, as an essential finding, the present study evidences extensive contact between demes at the transition zones, serving as reference to the conduction of important insights for the development of management actions.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. List of the D. albiventris specimens included in this study, their field numbers (BC, PR, FNI, ALN, TNM, T, PNSC, URC, LLLS, and DCN), GenBank accession number (JN), demes, haplotype number (H), locality, geographical coordinates and origin of the samples.

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