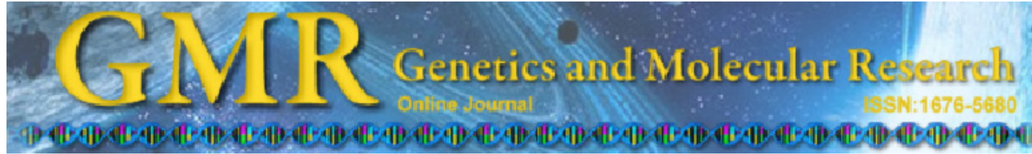

Molecular characterization of an opossum *Didelphis albiventris* (Marsupialia: Didelphidae) population in an urban fragment of the Brazilian Atlantic Rainforest and support to species barcode identification

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ABSTRACT. We made a molecular study of 40 opossums, *Didelphis albiventris*, from an urban fragment of the Atlantic Rainforest in southeastern Brazil, analyzing a 653-bp sequence of cytochrome c oxidase, subunit I. We found three close connected haplotypes, with low nucleotide diversity and a haplotype diversity of 59.1% and confirmed sympatry between *D. albiventris* and *D. aurita* in this region. The clear phylogenetic separation shows the appropriateness of DNA barcode identification methodology for effectively discriminating between these opossum species.

Key words: *Didelphis*; Molecular characterization; Sympatry; Barcode; Cytochrome oxidase I

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INTRODUCTION

The white-eared opossum *Didelphis albiventris* Lund, 1840 has a wide distribution (Figure 1), occurring in Brazil, Paraguay, Uruguay, Argentina, Bolivia (Costa et al., 2008; Gardner, 2008), Ecuador, Peru, and Colombia (Wilson and Reeder, 2005). The Brazilian common opossum *D. aurita* Wied-Neuwied, 1826 also has widespread distribution and occurs in Brazil, Argentina, and Paraguay (Wilson and Reeder, 2005; Gardner, 2008) (Figure 1). Both *D. albiventris* and *D. aurita* are listed as "Least Concern" in the Red List Categories (Costa et al., 2008).

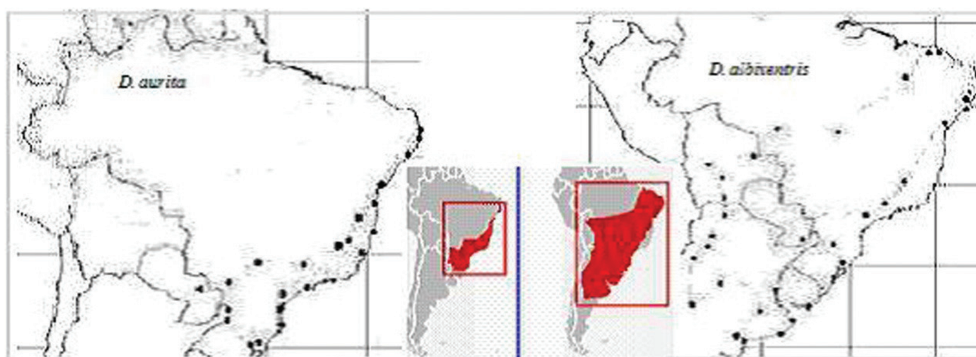

Figure 1. Map showing the distribution of *Didelphis aurita* and *D. albiventris*.

D. albiventris tolerates cultivated lands and their neighborhoods, deforested zones, and other disturbed places (Costa et al., 2008) and is found in many urban habitats, including major cities, revealing an ability to coexist with the disturbance caused by human exploitation of natural spaces.

Atlantic Forest is included in the biodiversity hotspot list (Mittermeier et al., 1998), and its biological uniqueness justifies and makes imperative the conservation of this biome (Chiarello, 2000). At present, the original forest territory includes enormous agricultural areas; urban and industrial centers, including the major Brazilian cities; and a vast populated contingent (about 110 million people). This has resulted in the reduction of the Atlantic Forest to less than 8% of its original area. Extensive mammalian diversity is observed, with 261 mammal species being found in this biome, of which 55 are endemic (Fundação SOS Mata Atlântica, 2011).

The distribution of *D. albiventris* overlaps with that of *D. aurita* one (Brito et al., 2008; Costa et al., 2008; Gardner, 2008), although sympatry records are not common. Sympatry between *D. albiventris* and *D. aurita* seems rare (Cerqueira, 1985; Gardner, 2008) but was found in disturbed areas (Varejão and Valle, 1982; Gardner, 2008). The distinction between *D. aurita* and *D. albiventris* opossum specimens is generally made on the basis of morphological characters such as the color of the ear and observed dentition patterns.

Exclusive maternal inheritance and high rates of nucleotide substitution contributed to the use of mtDNA as a molecular marker to trace the geographic distribution of genealogical lineages, even at the intra-specific level (Avise et al., 1987). Being useful for the accurate identification of specimens, the mtDNA conservative protein-coding gene cytochrome oxidase I (COI) has been selected as the marker for species discrimination by the Barcode of Life Database,

which aims at sequencing a section of cytochrome oxidase I (COI) in all living species on Earth and generating a base for species identification (Folmer et al., 1994; Wilson-Wilde et al., 2010).

Remaining forest habitats persist as archipelagos of small forest fragments (Silva and Tabarelli, 2000), and the abundance and distribution of mammals decrease as the fragment becomes smaller (Chiarello, 2000; Pontes et al., 2007). Small mammalian species involved in the dispersion process are important for the maintenance of diverse forest ecosystems. Opossums can defecate viable seeds (Cáceres, 2002; Cantor et al., 2010) and play an important role as dispersors; this is especially true in urban forest fragments, where vegetation needs to be restored and the specialist frugivores are frequently absent (Cantor et al., 2010). Knowledge about *Didelphis* population biology is important for further understanding the involved ecosystems. Our objectives are to molecularly characterize the *D. albiventris* population of the analyzed Atlantic Forest urban fragment and to further molecularly support the previous morphological register of species sympatry between *D. albiventris* and *D. aurita* in Belo Horizonte Metropolitan Region (BHMR).

MATERIAL AND METHODS

Sampling

This study was developed under license for scientific purposes granted by IBAMA/SISBIO, number 20170-2, which was renewed on February 2011. The institutions that collaborated with donations also have their own scientific licenses.

The analyzed area (Figure 2) is the capital of Minas Gerais state and part of its boundaries. Belo Horizonte is the sixth most populous Brazilian city, with 2,375,444 habitants and a demographic density of 7,167 hab/km^2 (Fundação SOS Mata Atlântica, 2011; IBGE, 2011).

Five localities in BHMR (Figure 2) were unequally sampled. *D. albiventris* samples were from Capitão Eduardo (32 specimens), Coração Eucarístico (3 specimens), Instituto Agronômico (2 specimens), Mangabeiras (1 specimen), and Jardim Canadá (2 specimens). A unique *D. aurita* specimen was collected from the Capitão Eduardo area. The approximate distances between the studied areas ranged from 6.5 to 26 km.

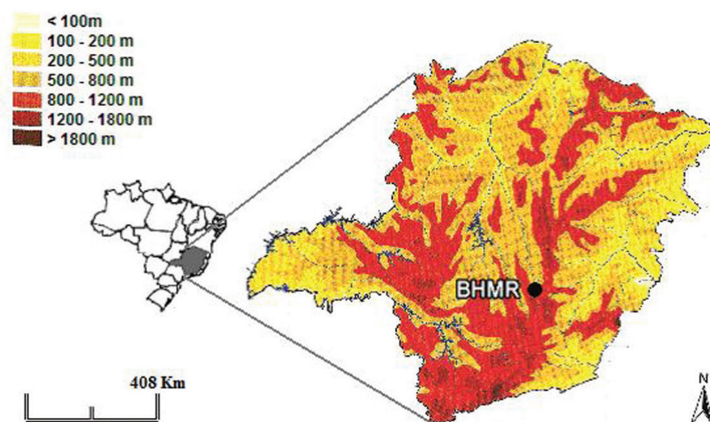

Figure 2. Minas Gerais Brazilian State, with a detach to Belo Horizonte Metropolitan Region (BHMR).

DNA samples from these 41 BHMR opossums were analyzed, starting with the morphologically identified 40 *D. albiventris* and 1 *D. aurita* specimens. To determine the sympatry between the specimens, we used the barcode methodology. In this case, for comparison purposes, DNA samples from other areas were used: 3 *D. albiventris* individuals from Porto Alegre and Triunfo, Rio Grande do Sul (RS) State and from Xacriabá Reserve in Minas Gerais (MG) State; and 7 *D. aurita* individuals from Cotia and Ribeirão Grande in São Paulo (SP) State, Macaé in Rio de Janeiro (RJ) State, Conceição da Barra in Espírito Santo (ES) State, Serra do Ibituruna, Viçosa, and Piracema in Minas Gerais State. Linear geographic distances between BHMR and aforementioned areas range between 548 and 1364 km for *D. albiventris* and between 94 and 657 km for *D. aurita.* Distance data and collection point coordinates are shown in Table 1. Most samples were kindly donated by research institutions (Table 1). All studied sequences were developed in this study.

Table 1. Studied samples (*Didelphis*), collection sites, distance to Belo Horizonte Metropolitan Region (BHMR), origin and geographic coordinates.

Species	State	Locality/City	Distance to BHMR	N	Origin	South	West
D. albiventris	MG	BHMR - Capitão Eduardo	-	32	CPqRR	19°49'57"	43°52'08"
D. albiventris	MG	BHMR - Instituto Agronômico	-	2	Road Killed	19°52'49"	43°55'19"
D. albiventris	MG	BHMR - Coração Eucarístico	-	3	MHN-PUC	19°55'27"	43°59'29"
D. albiventris	MG	BHMR - Mangabeiras	-	1	Road Killed	19°57'25"	43°55'02"
D. albiventris	MG	BHMR - Jardim Canadá	-	2	Road Killed	20°03'04"	43°58'10"
D. albiventris	RS	Porto Alegre	1343 km	1	FZB-RS	29°56'34"	51°43'13"
D. albiventris	RS	Triunfo	1364 km	1	FZB-RS	30°01'41"	51°13'43"
D. albiventris	MG	Xacriabá Reserve	548 km	1	CPqRR	14°53'29"	44°04'42"
D. aurita	MG	BHMR - Capitão Eduardo	-	1	CPqRR	19°49'57"	43°52'08"
D. aurita	SP	Cotia	519 km	1	USP	46°59'56"	23°45'46"
D. aurita	SP	Ribeirão Grande	657 km	1	USP	24°05'52"	48°22'17"
D. aurita	RJ	Macaé	354 km	1	UFRJ	22°22'18"	41°47'08"
D. aurita	ES	Conceição da Barra	228 km	1	MBML	18°35'29"	39°44'06"
D. aurita	MG	Serra do Ibituruna	230 km	1	CPqRR	18°44'56"	42°13'00"
D. aurita	MG	Viçosa	145 km	1	UFV	20°45'15"	42°52'56"
D. aurita	MG	Piracema	94 km	1	CPqRR	20°30'28"	44°22'57"

DNA extraction, DNA amplification, and sequencing

We mostly used liver tissues and, in few cases, spleen and muscle tissues were used. For the road-killed animals, ear tissue fragments were collected. Tissue samples were preserved in 95% ethanol and stored in a freezer.

DNA from macerated tissue fragments was extracted following the standard phenol-chloroform protocols, as described by Sambrook and Russel (2001).

DNA sequences of the mitochondrial COI gene were amplified using the universal primers LCO 1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO 2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer et al., 1994). Each PCR were performed in a 20 µL final volume containing 50 ng genomic DNA, 10X buffer III B (Phoneutria®; 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 100 mM Tris-HCl, pH 8.4, 1% Triton-X, 15 mM $MgCl_2$), 0.8 µM dNTPs, 0.5 µM of each primer, 1% bovine serum albumin (BSA), and 1 U *Taq* DNA polymerase (Phoneutria®). After an initial denaturing step of 3 min at 94°C, the PCR conditions for the *COI* fragments followed a standard 3-step protocol, with 30 cycles of 1) denaturing for 1 min at 94°C, 2) annealing for 45 s at 47°C, and 3) extension for 30 s at 72°C, followed by a final

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extension step for 5 min at 72°C. Satisfactory amplifications were visualized on 6% polyacrylamide gels. Amplified DNA products were cleaned using 20% polyethylene-glycol (PEG 8000) and 2.5 M NaCl, according to the protocol reported by Sambrook and Russel (2001).

PCR products were sequenced in both directions by using the same primers: LCO 1490 or HCO 2198 (Folmer et al., 1994) on ABI3100® automated sequencer with the Applied Biosystems BigDye® Terminator Kit v3. Alternatively, some sequences were obtained on a MegaBACE automated capillary sequencer by using the GE Healthcare ET® dye terminator kit.

Statistical data analyses

All mtDNA sequences were "base called" by using the Phred v.0.20425 software (Ewing et al., 1998; Ewing, 1998), checked for quality using the Phrap v.0.990319 software (Green, 1994), and the assembled chromatograms were verified and edited in Consed 12.0 (Gordon et al., 1998). Consensus was conferred with visual verification of chromatogram peaks. A sequence set with consensus was aligned using the Clustal W algorithm implemented in MEGA 4.1 (Kumar et al., 2007); a 653-bp fragment showed high levels of sequence quality for all individuals. All sequences were deposited in GenBank (accession Nos. JN638891-JN 638922; JN638976-JN638991).

MEGA 4.1 (Kumar et al., 2007), DNAsp v.5 (Librado and Rozas, 2009), and Arlequin v.3.1 (Schneider et al., 2000) was used to analyze intrapopulation genetic diversity and identify standard indices of genetic variation as haplotype diversity (h) and nucleotide diversity (π). MEGA 4.1 (Kumar et al., 2007) was used to visualize nucleotide variation and verify polymorphism coherence by using the translating approach.

Haplotype network was constructed on the basis of statistical parsimony by using TCS 1.21 (Clement et al., 2000). This method estimates the evolutionary relationships between haplotypes, connecting related ones and representing substitution steps between them.

Mismatch distribution and neutrality tests to verify excess of recent mutations as evidence of recent population expansion for BHMR *D. albiventris* population were performed in Arlequin v.3.1 (Schneider et al., 2000).

Phylogenetic inference was estimated using maximum parsimony, minimum evolution, Bayesian analyses, and neighbor joining models and analyzed using PAUP 4.0 (Swofford, 2002), Phy ML 3.0 (Guindon and Gascuel, 2003), MR BAYES (Huelsenbeck and Ronquist, 2001), and MEGA 4.1 (Kumar et al., 2007). The best evolutionary model was determined using Modeltest 3.7 (Posada and Crandall, 1998). The aim was to discriminate between *D. albiventris* and *D. aurita* specimens. In this context, barcode methodology was used to test the morphological register of sympatry and verify whether the molecular data support grounded the constatation of sympatry. Inter-specific variability was also studied.

For barcode discrimination, sequences for other localities (Table 1) were added to molecularly confirm the sympatry register.

RESULTS

For *D. albiventris* BHMR population, specimens from Capitão Eduardo (sub-area 1), Instituto Agronômico (sub-area 2), Coração Eucarístico (sub-area 3), Mangabeiras (sub-area 4), and Jardim Canadá (sub-area 5) were studied, with a maximum linear distance of about 26

km. The great majority, 32 specimens, were from Capitão Eduardo sub-area. Three different COI haplotypes were observed for BHMR *D. albiventris* population: haplotype 1 in sub-areas 1, 3, 4, and 5; haplotype 2 in sub-areas 1, 2, and 5; and haplotype 3 in specimens from sub-areas 1 and 2.

BHMR *D. albiventris* database revealed 3 polymorphic sites, all corresponding to synonymous substitutions. Haplotype 1, the most frequent, was observed in 22 samples; haplotype 2, in 13 individuals; and haplotype 3, in 5 specimens. Haplotype diversity (*h*) of 0.591 (± 0.051), nucleotide diversity (π) of 0.00185, and an average number of nucleotide differences (*k*) of 1.124 were found for BHMR population. Haplotype network showed a maximum of 3 steps between haplotypes within the *D. albiventris* group (Figure 3). Mismatch distribution and neutrality tests for verifying excess of recent mutations as evidence of recent population expansion showed non-significant P values for BHMR *D. albiventris* data.

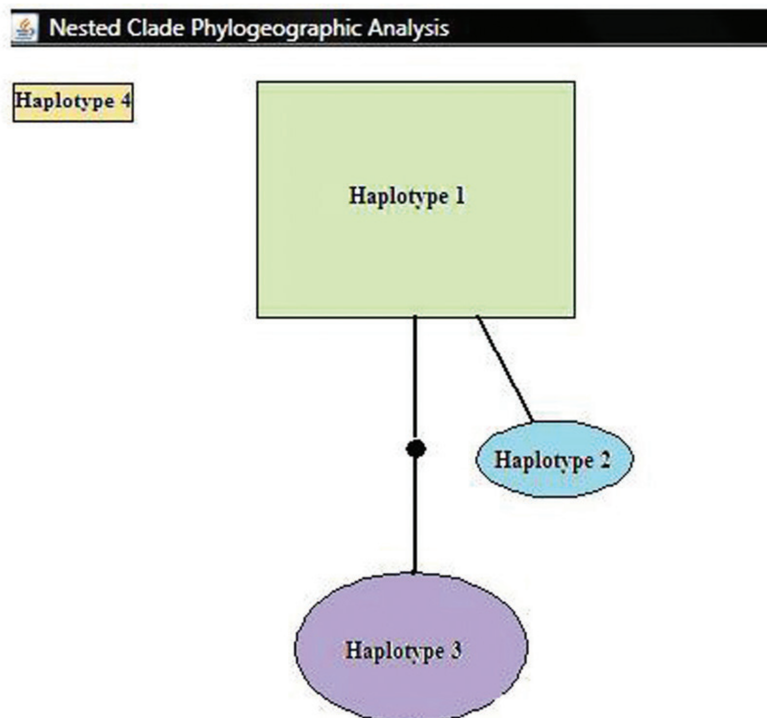

Figure 3. Haplotype network for 41 opossums collected in Belo Horizonte Metropolitan Region urban fragment.

When the same 653-bp COI fragment of the unique BHMR *D. aurita* sample was studied, a different haplotype was observed (haplotype 4). Adding a *D. aurita* specimen to the 40 specimens of *D. albiventris* from BHMR in an inter-specific analyses, 36 polymorphic sites were found, all corresponding to synonymous changes and 32 showing fixed differences. There was an average number of 33.25 nucleotide differences and a nucleotide divergence of 0.05439 between the species.

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BHMR opossum haplotype network (Figure 3) exhibited 4 haplotypes, 2 of which were identified as probable ancestral (*D. albiventris* haplotype 1 and *D. aurita* haplotype 4).

The HKY 85 evolutionary model of nucleotide substitution was the most appropriate for the analyzed dataset, as revealed by the Akaike informative criterion in Modeltest 3.7 (Posada and Crandall, 1998) analyses.

Phylogenetic relationships revealed by tree topologies of BHMR opossums were concordant to morphological identification. *D. albiventris* haplotype 1 was separated from *D. aurita* haplotype 4 by a large connection length of 35.64 (Table 2) compared to the largest connection length for *D. albiventris* intra-specific haplotypes, which was 2.01. The results from our sequence analysis are unambiguous.

Table 2. Connection length between 4 Belo Horizonte Metropolitan Region opossum haplotypes.

Haplotype comparison	Connection length
Haplotype 1 x Haplotype 2	2.01
Haplotype 1 x Haplotype 3	1.00
Haplotype 1 x Haplotype 4	35.64

Three *D. albiventris* and 07 *D. aurita* (Table 1) specimens from other localities were included in our initial database of 40 BHMR *D. albiventris* and 01 BHMR *D. aurita* for comparison to test the sympatry register with the barcode methodology (Figure 4). For this dataset, 8 haplotypes, nucleotide diversity of 0.01773, and haplotype diversity of 0.74 were observed. Inter-specific analyses revealed 46 polymorphic sites, all of which were synonymous substitutions; 24 fixed inter-species differences; and no shared substitutions. A nucleotide diversity (π total) of 0.00444, an average number of nucleotide differences of 32.881 and a nucleotide divergence of 0.05399, was observed.

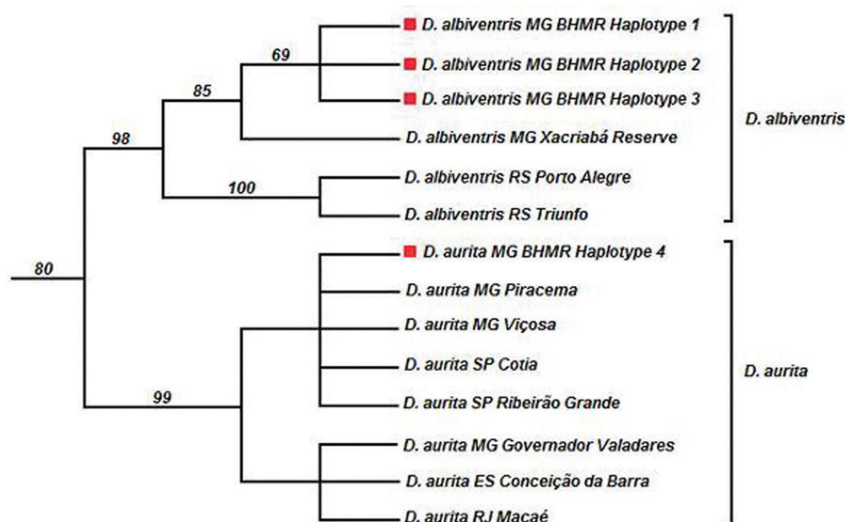

Figure 4. Maximum parsimony tree with *Didelphis albiventris* and *D. aurita* specimens from different geographic localities.

Phylogeny inference was estimated using different evolutive models (neighbor joining, maximum likelihood, maximum parsimony, and Bayesian) returned similar results. The proposal topologies showed a clear separation between the studied species, as expected, suggesting that it is possible to discriminate *D. albiventris* and *D. aurita* specimens by using the studied mtDNA COI fragment. Phylogeny revealed 2 genetic clusters, each of which corresponded to a different opossum species, being completely concordant with previous morphological identification. BHMR *D. aurita* grouped with *D. aurita* from other localities, showing a perfect separation between opossum species and confirming local BHMR sympatry.

DISCUSSION

Opossums are habitat generalists and can move easily between forest fragments (high vagility) (Chiarello, 2000; Pires et al., 2002), showing a greater movement rate compared to other small mammals (Pires et al., 2002). The coverage of their distribution includes agricultural and urban places. Therefore, *Didelphis* seems to be an unique population when different fragments on a continuous area are analyzed, which is because of the ecological characteristics of opossums, such as the ability to move long distances (Gentile and Cerqueira, 1995) and the generalist habitat (Paglia et al., 1995; Passamani, 1995; Emmons and Feer, 1997). Radiotelemetry revealed a mean home range size of 122.7 ha for males and 12 ha for females of *D. marsupialis* (closely related to *D. aurita*) in Venezuela (Sunquist et al., 1987). The present data possibly represent a comprehensive geographic context, which can be tested in future studies in other geographic areas. Haplotype frequencies were 55% for haplotype 1, 32.5% for haplotype 2, and 12.5% for haplotype 3; hence, no rare haplotype was observed, and there was no evidence of population expansion or contraction. Mismatch distribution showed non-significant P values for BHMR *D. albiventris* data.

The haplotype distribution, with the same form (haplotype 1) shared by different BHMR areas, indicates that even with streets characterized by intense urban traffic and consequent high rates of road-killed animals, about 26 km of linear distance seems to be a small area when opossum ecological characteristics are considered, suggesting that it was necessary to analyze 40 BHMR *D. albiventris* specimens as a single population. Hence, haplotype distribution and occurrence indicates population unity. The BHMR *D. albiventris* population returned a haplotype network with 3 closely connected haplotypes, with a maximum of 3 steps of separation between haplotypes (Figure 3).

The molecular diagnosis results are in accordance with morphological identification, as expected, confirming *D. albiventris* and *D. aurita* sympatry in the urban Atlantic forest fragment. DNA barcode method was successfully used previously with opossums to effectively identify *D. marsupialis* and *D. virginiana* species from areas of sympatry in Mexico (Cervantes et al., 2010). Haplotype network (Figure 2) constructed using BHMR opossums showed 4 haplotypes, 2 of which were identified as probably ancestral (haplotype 1 and haplotype 4). Ancestral haplotypes were not linked to each other; such a linkage would require many steps and hence highly improbable. The occurrence of 2 ancestral forms in the haplotype network indicates the presence of 2 isolated genetic clusters. Absence of haplotype connection between haplotype 4, morphologically identified as *D. aurita*, and the others, clearly indicated a genetic distance between them. This indicated absence or near absence of gene flow.

Sympatry between opossums in Brazil was previously reported between *D. albiven-*

tris and *D. marsupialis* in Curitiba (Cáceres and Monteiro-Filho, 1999). *D. aurita* was considered as a disjunct population of *D. marsupialis* until Cerqueira (1985) proposed species separation (Corbet and Hill, 1991). BHMR *D. aurita* grouped with *D. aurita* from other localities and was clearly separated from *D. albiventris* individuals, confirming the sympatry between these opossum species in BHMR and highlighting the potential of COI barcode analysis to discriminate specimens.

Thus, molecular characterization of BHMR *D. albiventris* population produced a haplotype network with 3 closely connected haplotypes, and haplotype distribution and occurrence indicated population unity. Our findings confirm sympatry between *D. albiventris* and *D. aurita* in the studied area. A clear separation between species enabled doubtless barcode identification for phylogenetic analyses with COI sequences, indicating the usefulness of barcode methodology to effectively discriminate opossums in the Atlantic forest region.

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