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TESE DE DOUTORADO

VARIABILIDADE GENÉTICA, ESTRUTURA POPULACIONAL E IDENTIDADE MOLECULAR DOS MARSUPIAIS *Didelphis albiventris* E *Marmosops incanus* NO BRASIL

ORIENTADA: Luciene Cássia Corrêa de Sousa

ORIENTADORA: Profª Drª Cleusa Graça da Fonseca

BELO HORIZONTE
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Luciene Cássia Corrêa de Sousa

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Tese apresentada ao Programa de Pós-Graduação em Genética do Departamento de Biologia Geral do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de doutor em Genética.

Orientadora: Profª Drª Cleusa Graça da Fonseca

BELO HORIZONTE

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“Não sei por que você se foi
Quantas saudades eu senti
E de tristezas vou viver
E aquele adeus, não pude dar
Você marcou a minha vida,
Viveu, morreu na minha história
Chego a ter medo do futuro,
E da solidão, que em minha porta bate.
E eu” ... Vou te amar sempre Fran

Tim Maia

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Abordagens Iniciadas e Resultados Preliminares de Linhas de Pesquisa Relacionadas

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LISTA DE ABREVIATURAS, SIGLAS E UNIDADES

$(NH_4)_2SO_4$	Sulfato de Amônio
~	Aproximadamente
®	Marca registrada
°C	Graus centígrados
ABI	*Applied Biosystems* ®
Alm	Almenara
AMOVA	Análise de variância molecular
Bam	Bambuí
BH	Belo Horizonte
BHMR	Região Metropolitana de Belo Horizonte
BI	Inferência Bayesiana
BOLD	*Barcode of life database*
BP; pb	Pares de base
BSA	*Bovine serum albumin* (Albumina de soro bovino)
COI	Citocromo oxidase C
CPqRR	Centro de Pesquisas René Rachou
Cyt b	Citocromo B
D.	*Didelphis*
Div	Divinópolis
DNA	Ácido desoxirribonucléico
dNTPs	Desoxirribonucleotídeos trifosfatados
ES	Espírito Santo
FAPEMIG	Fundação de Amparo à Pesquisa do Estado de Minas Gerais
Fig.	Figura
FioCruz	Fundação Oswaldo Cruz
FNR	Floresta Nacional de Ritápolis (Flona de Ritápolis)
F_{ST}	Índice de Fixação
FZB-RS	Fundação Zoo Botânica do Rio Grande do Sul
G	Gramas
GE	*General Eletric* ®
GMB	*Genetics and Molecular Biology*

GMR	*Genetics and Molecular Research*
GTR	*General time reversible model*
H	Número de haplótipos
Ha	Hectares
Hab/Km^2	Habitantes por quilômetros quadrados
Hap	Haplótipo
HCl	Ácido Clorídrico
Hd; h	Diversidade haplotípica
HKY 85	*Hasegawa, Kishino and Yano (1985) evolutionary model*
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
IBGE	Instituto Brasileiro de Geografia e Estatística
ICB	Instituto de Ciências Biológicas
ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
ID	Identidade
IRBP	*interphotoreceptor retinoid - binding protein*
IUCN	*International Union for Conservation of Nature*
K	*Average number of nucleotide differences* (número médio de diferenças nucleotídicas)
K2P	Modelo Kimura 2 parâmetros
KCl	Cloreto de Potássio
Km	Quilômetros
M	Metros
M	Molar
M.	*Marmosops*
MBML	Museu de Biologia Professor Mello Leitão
MG	Minas Gerais
$MgCl_2$	Cloreto de magnésio
MCN-PUC	Museu de Ciências Naturais da PUC MG
min	Minutos
ML	*Maximum likelihood* (máxima verossimilhança)
mM	Milimolar
MP	Máxima parsimônia

mtDNA	DNA mitocondrial
Mya	*Million years ago* (Milhões de anos atrás)
N	Tamanho amostral
N	Número haplóide
NaCl	Cloreto de Sódio
NCA	*Nested clade analysis*
nDNA	DNA nuclear
Ng	Nanogramas
NJM	Regiões Norte e Jequitinhonha/ Mucuri
PCR	Reação em cadeia da polimerase
PEG	Polietileno Glicol
PELD	Programa de Ecologia de Longa Duração
PESB	Parque Estadual da Serra do Brigadeiro
PESRM	Parque Estadual da Serra do Rola Moça
Pir	Piracema
Prof/ Prof[a]	Professor (a)
PUC	Pontifícia Universidade Católica
P-values; p	Valores de probabilidade
R	Correlação entre as distâncias geográfica e genética
RIX	Reserva Indígena Xacriabá
RJ	Estado do Rio de Janeiro
RPPNC	Reserva do Particular Patrimônio Natural do Caraça
RS	Rio Grande do Sul
S	Segundos
S	Sul
S	Número de sítios polimórficos
SCP/DEPLAN	Secretaria do Planejamento do Governo do Rio Grande do Sul
SP	Estado de São Paulo
spp.	Várias espécies
U	Unidades
UFMG	Universidade Federal de Minas Gerais
UFRJ	Universidade Federal do Rio de Janeiro
UFSC	Universidade Federal de Santa Catarina

UFV	Universidade Federal de Viçosa
UHI	Usina Hidrelétrica de Irapé
USP	Universidade de São Paulo
v.	Versão
W	Oeste
µL	Microlitros
µM	Micromolar
Π	Diversidade nucleotídica

RESUMO

Foram estudados aspectos genético-populacionais de duas espécies da família Didelphidae e testada a adequação da metodologia de DNA Barcode para fins de identificação. O estudo molecular de 40 gambás da espécie *Didelphis albiventris* que habitam um fragmento urbano da Mata Atlântica brasileira foi realizado a partir da análise de seqüências de 653 pb da subunidade I do gene da citocromo oxidase c. Foram observados três haplótipos proximamente ligados, baixa diversidade nucleotídica e diversidade haplotípica de 59,1%. A simpatria entre *D. albiventris* e *D. aurita* foi molecularmente confirmada para a capital do estado de Minas Gerais (BHMR) e também para a região de Piracema, distantes 94 Km, através da utilização da metodologia de *DNA barcode*, demonstrando que a técnica é apropriada para efetivamente discriminar essas espécies de gambás. Expandindo os trabalhos populacionais com *D. albiventris*, utilizamos o mesmo fragmento da COI para realizar a análise de 93 amostras biológicas provenientes de sete localidades brasileiras, com distâncias lineares que variam entre 58 e cerca de 1800 km, com a finalidade de analisar o efeito da distância geográfica sobre a variabilidade e sobre a diferenciação genética. A rede haplotípica resultante exibe nove haplótipos distribuídos em dois grupos genéticos totalmente compatíveis com as duas distantes áreas geográficas estudadas, o estado de Minas Gerais, localizado no sudeste brasileiro, e o estado do Rio Grande do Sul, no extremo sul do país; dentro de cada grupo, observamos baixa diversidade nucleotídica e diversidade haplotípica elevada, sugerindo que suas populações são compostas por haplótipos proximamente relacionados. Índices de moderados a altos de diferenciação genética (F_{ST}) e sinal filogeográfico muito fraco caracterizam as comparações entre os demes dentro do estado de Minas Gerais, e estão correlacionados com a presença de haplótipos exclusivos (privativos). Em maior escala geográfica, as comparações entre Minas Gerais e Rio Grande do Sul produziram valores de

F_{ST} altos e padrão filogeográfico forte. Como as taxas de dispersão são reconhecidamente maiores nos machos de *Didelphis*, estes provavelmente contribuem mais para o fluxo gênico, e trabalhando com um marcador genético de herança materna, não podemos argumentar sobre a diversidade de *D. albiventris* de modo completo. Nossos resultados informam sobre a história mutacional das linhagens genealógicas maternas. O cenário observado é inesperado e sugere fortemente que o fluxo de genes mitocondriais não foi suficiente para manter a coesão populacional.

Como esperado, *Marmosops incanus* mostra resultados completamente diferentes dos exibidos por *D. albiventris*. *M. incanus* demonstra significativa preferência de habitat, tendo sido apontado como espécie indicadora, por exibir elevada sensibilidade à fragmentação (Rocha *et al*., 2011), exibe também características ecológicas, como baixa mobilidade e semelparidade (Lorini *et al.* 1994; Loretto & Vieira, 2008), que podem comprometer a conexão entre populações de diferentes demes. Estudamos um fragmento de 509 pb do gene da COI, de indivíduos coletados em 17 localidades brasileiras e observamos a influência da distância geográfica na diferenciação genética entre as populações. As distâncias genéticas entre os haplótipos de diferentes demes e o grande número de formas privativas produziram uma rede, caracterizada pelo grande número de passos entre alguns dos haplótipos estudados. Valores de F_{ST} elevados, indicando forte estruturação genética, altos valores para o percentual de variação observado entre filogrupos e elevada correlação entre as distâncias genéticas e as geográficas caracterizam as análises interpopulacionais; a rede haplotípica e as árvores filogeográficas demonstram claramente a compatibilidade quase absoluta entre os haplogrupos moleculares e o agrupamento geográfico. O padrão observado de distribuição geográfica das linhagens genealógicas é caracterizado pelo sinal filogeográfico forte; nossos resultados, especialmente os elevados valores de divergência entre os demes, sugerem que, mais do que o efeito da fragmentação de habitats, a história de vida de *M. incanus* é a chave central para explicá-los. A estrutura genética forte e a grande variação entre filogrupos estão

relacionadas a processos antigos que influenciaram a distribuição geográfica das linhagens, refletindo eventos de fragmentação que antecedem a destruição do habitat natural governada pela ocupação humana. Características ecológicas de *M. incanus*, como baixa mobilidade e alta sensibilidade à fragmentação do habitat, são desfavoráveis ao fluxo gênico, concorrendo para a manutenção dos altos níveis de diferenciação genética.

ABSTRACT

We studied population genetics aspects of two species of the family Didelphidae and the suitability of the DNA barcode methodology to effectively identify them. A molecular study of a 653 bp sequence of cytochrome c oxidase, subunit I from 40 *Didelphis albiventris* from an urban fragment of the Brazilian Atlantic Forest, showed three closely connected haplotypes, low nucleotide diversity and a haplotype diversity of 59.1%. We confirmed the sympatry between *D. albiventris* and *D. aurita* for the BHMR region, located on the capital of Minas Gerais state, and also for the 94 Km distant Piracema region, using DNA barcode methodology, which demonstrates the suitability of these techniques to effectively discriminate between these opossum species. Expanding the population surveys of *D. albiventris*, we used the COI fragment to 93 biological samples from seven Brazilian localities with linear distances ranging between 58 and about 1800 km, to study the effect of geographic distances on variability and genetic differentiation. The haplotype network exhibits nine haplotypes distributed in two genetic clusters completely compatible with the two distant geographic areas of Minas Gerais, Southeastern Brazil, and Rio Grande do Sul, South Brazil. Within each cluster we observed low nucleotide diversity and high haplotype diversity, suggesting that their populations are composed of closely related haplotypes. Moderate to high F_{ST} differentiation values were observed and a very weak phylogeographic signal characterized interdemes comparisons within Minas Gerais, which were correlated with the presence of closely and exclusive haplotypes. In a larger geographic scale, comparisons between Minas Gerais and Rio Grande do Sul produced a high F_{ST} values and a strong phylogeographic pattern. As *Didelphis* dispersion rates are recognized to be greater in males, they probably contribute more for gene flow. Working with a maternal inherited genetic marker, we can not argument about complete *D. albiventris* diversity. Our results informed

only on the mutational history that corresponds to maternal lineages genealogical information. The observed scenario was unexpected and strongly suggests that mtDNA gene flow was not enough to maintain population cohesion. As expected, *Marmosops incanus* showed completely different results. *M. incanus* shows significant habitat preference and has been pointed out as an indicator species, being sensitive to habitat fragmentation (Rocha *et al*., 2011). It also exhibits ecological characteristics like low mobility and semelparity (Lorini *et al.* 1994; Loretto & Vieira, 2008), which can compromise interdemes connection. We studied a 509 bp fragment of COI obtained from specimens collected in 17 Brazilian localities and observed the influence of geographic distance in genetic differentiation among populations. The genetic distances between demes haplotypes and the great number of forms produced a network with a large number of steps between some studied haplotypes. Large F_{ST}, indicating strong genetic structure, great values for phylogroups variation and elevated correlation between genetic and geographical distances characterized interpopulational analyses. Haplotype network and phylogeographic trees clearly showed molecular haplogroups mostly compatible with geographic clustering. The observed pattern of geographic distribution of genealogic lineages showed strong phylogeographic signal; our findings, specially the great divergence values between demes, suggest that, more than one effect of habitats fragmentation, *M. incanus* life history is central to explain the observed results. The strong genetic structure and the great variation among phylogroups are related to ancient processes that influenced lineages geographic distribution reflecting fragmentation events that predate human driven habitat destruction. *M. incanus* ecological characteristics, like low mobility and high sensibility to habitat fragmentation, are not suitable to gene flow, allowing the maintenance of high levels of genetic differentiation.

PREFÁCIO

A presente tese de doutorado está organizada em cinco partes, consistindo em uma introdução à pesquisa e em quatro capítulos que correspondem aos quatro artigos científicos que foram gerados a partir dos resultados obtidos. Ao final do trabalho, é apresentada uma seção incluindo as conclusões gerais e, na sequência, referências bibliográficas adicionais (citadas em tópicos externos aos capítulos). Na sequência, comentamos brevemente perspectivas e alguns resultados preliminares que obtivemos com a região controle do DNA mitocondrial e com microssatélites, incluímos a autorização para atividades com finalidade científica (nº 20170-1) e sua renovação e a correspondência entre números de acesso no Genbank e dados amostrais. Adicionalmente, optamos por incluir ao final da tese as versões nos formatos publicados dos artigos descritos nos capítulos I e III.

A introdução aborda aspectos importantes para permitir a melhor compreensão do trabalho e aproximá-lo do público não especialista na literatura específica da área de pesquisa em foco. Neste tópico foram incluídos também os objetivos da pesquisa.

O capítulo I consiste no artigo intitulado “Molecular characterization of an opossum *Didelphis albiventris* (Marsupialia: Didelphidae) population in an urban fragment of Brazilian Atlantic Rainforest and support to species barcode identification”, efetivamente publicado no periódico *Genetics and Molecular Research*. O artigo aborda o estudo molecular de amostras biológicas de 41 gambás coletados em um fragmento urbano da Mata Atlântica do sudeste do Brasil. Os indivíduos foram caracterizados para um fragmento de 653 pb da subunidade I da citocromo oxidase c, com o propósito de realizar análises genético-populacionais e verificar molecularmente o registro local de simpatria entre *Didelphis albiventris* e *Didelphis aurita*, testando assim a viabilidade deste fragmento da COI para facultar a discriminação entre estas

duas espécies de gambás a partir da metodologia de *barcode* genético.

O capítulo II inclui o artigo intitulado “DNA Barcode Methodology confirms sympatry of opossum species in an Atlantic Forest fragment of southeastern Brazil”, que será submetido ao periódico “Mitochondrial DNA”. O trabalho consiste na confirmação molecular do registro de simpatria entre *Didelphis albiventris* e *Didelphis aurita* na Mata Atlântica, em uma região com características rurais no município de Piracema (MG), utilizando a metodologia de barcode genético.

O capítulo III inclui o artigo intitulado “Mitochondrial genetic variability of *Didelphis albiventris* (Marsupialia: Didelphidae) in Brazilian localities”, efetivamente publicado pelo periódico *Genetics and Molecular Biology*. O artigo aborda aspectos genético-populacionais do marsupial *Didelphis albiventris*, uma espécie de gambá comum em diversos ecossistemas brasileiros. Foram geradas e analisadas seqüências de 653 pares de bases do gene COI, provenientes de 93 espécimes coletados em sete localidades brasileiras separadas por distâncias lineares entre 58 e aproximadamente 1800 km, com o objetivo principal de analisar o efeito da distância geográfica sobre a variabilidade e sobre a diferenciação genética. As localidades estudadas correspondem a áreas em dois agrupamentos geográficos mais amplos: o estado de Minas Gerais, no sudeste brasileiro, e o estado do Rio Grande do Sul, no extremo sul do país.

O capítulo IV inclui o artigo intitulado “Genetic Diversity and Phylogeography of *Marmosops incanus* (Marsupialia: Didelphidae) in southeastern Brazil”, que será submetido ao periódico PLOS ONE e que trata de aspectos genético-populacionais e filogeográficos de uma espécie de cuíca típica da Mata Atlântica brasileira, mas com registros de ocorrência no Cerrado e na Caatinga. A espécie exibe várias características ecológicas (semelparidade, alta sensibilidade à fragmentação ambiental, baixa mobilidade) que podem dificultar o fluxo gênico entre as populações.

Os artigos foram escritos com o propósito principal de contribuir para a melhor compreensão de aspectos da Genética de Populações das duas espécies marsupiais estudadas, produzindo e disponibilizando informações que permitem um melhor conhecimento sobre a biologia de *Didelphis albiventris* e de *Marmosops incanus*. Aspectos gerais sobre o padrão filogeográfico por elas exibidos também foram contemplados. Adicionalmente, analisamos aspectos relacionados à identificação molecular, através de *barcode* genético, e confirmamos a adequação desta técnica à identificação de gambás em áreas em que duas espécies de *Didelphis* ocorrem em simpatria.

INTRODUÇÃO

1. Revisão Bibliográfica

Os pequenos mamíferos, grupo composto por marsupiais e roedores de pequeno porte (até 5 kg) são conhecidos por sua grande importância para a manutenção dos ecossistemas naturais, com papel relevante na dispersão de sementes e conseqüentemente no processo de recuperação e reflorestamento de áreas impactadas (Cáceres, 2002; Machado *et al*., 2008; Cantor *et al*., 2010). Várias espécies são florestais, muitas arborícolas, o que resulta em fragilidade e maior susceptibilidade aos efeitos do desflorestamento e da fragmentação de habitats (Machado *et al*., 2008). O Brasil reúne em seu território uma grande diversidade natural, distribuída em seis biomas: a Amazônia, o Cerrado, a Mata Atlântica, a Caatinga, o Pampa e o Pantanal (IBGE, 2011). Dois deles, Mata Atlântica e Cerrado, constam na lista dos *hotspots* da biodiversidade como áreas prioritárias para a conservação, e são caracterizados pelo grande número de espécies que abrigam e pelo elevado grau de endemismo (Brooks *et al*., 2006; Mittermeier *et al*., 1998). Algumas espécies de pequenos mamíferos, como as que hospedam agentes etiológicos de zoonoses e exoparasitas, estão implicadas no que tange aspectos de saúde pública (Machado *et al*., 2008).

Os marsupiais são ao nascimento organismos semelhantes a embriões, que completarão o seu desenvolvimento anexos às tetas maternas, o que pode ou não ocorrer em um marsúpio (Jones, 2003). Sua origem constitui assunto polêmico. As duas hipóteses principais são a de surgimento na Ásia, embasada pelo fóssil de *Asiatherium* do fim do Cretáceo, até o momento o mais antigo deste grupo (Trofomov & Szalay, 1994), e a de surgimento na América do Norte (Simpson, 1980). A distribuição dos marsupiais, que no passado incluiu todos os continentes, encontra-se atualmente restrita às Américas e à Australasia (Jones *et al.*, 2003).

Existem mais do que 330 espécies recentes de marsupiais (Wilson & Reeder, 2005; Meredith *et al*., 2008). Os didelfídeos são originários da América do Norte, onde ocorriam abundantemente no final do Cretáceo; na atualidade são muito mais abundantes e diversificados na América do Sul (Simpson, 1980). Com base no tamanho corporal, os integrantes deste grupo podem ser incluídos em três categorias: didelfídeos de grande porte (*Chironectes, Didelphis, Lutreolina e Philander*), de médio porte (*Metachirus*) e de pequeno porte (*Lestodelphys, Monodelphis, Marmosa, Micoureus, Marmosops, Thylamys* e *Gracilinanus*) (Jones *et al*., 2003).

No presente trabalho analisamos aspectos genético-populacionais e aspectos filogeográficos gerais dos didelfídeos *Didelphis albiventris* e *Marmosops incanus*. Adicionalmente, testamos a eficiência do método de *barcode* genético para discriminar as espécies *D. albiventris* e *D. aurita* em locais nos quais a simpatria havia sido morfologicamente constatada.

1.1 Características gerais de *Didelphis albiventris* (Lund, 1840)

O registro fóssil de *Didelphis* (Linnaeus, 1758) na América do Sul data do meio do Pleistoceno (Gardner, 2008). Os representantes sulamericanos do gênero *Didelphis* estão reunidos em dois grupos: gambás de orelhas pretas (*D. marsupialis* e *D. aurita*) e gambás de orelhas brancas (*D. albiventris, D. pernigra* e *D. imperfecta*). O termo gambá originou-se do tupi-guarani em que “gã’ ba” ou “guaambá” significa seio oco (Ferreira, 1986). De modo geral, gambás se reproduzem em média três vezes por ano, apresentam ninhadas de aproximadamente sete filhotes e proporção sexual de 1:1 na prole (Moraes *et al.*, 2000). Após curto período gestacional, em que processos básicos de morfogênese crânio-facial ainda estão em curso, o didelfídeo está apto a respirar, sugar e segurar as tetas maternas para completar,

no marsúpio, o seu desenvolvimento (Moraes *et al.*, 2000). O marsúpio das fêmeas de *Didelphis* é bem desenvolvido (Figura 1) (Gardner, 2008).

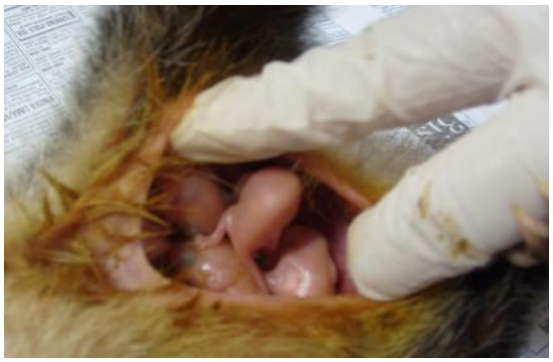

Figura 1. Marsúpio do gambá *Didelphis albiventris* com filhotes

O gambá de orelha branca *Didelphis albiventris* está classificado na categoria de baixo risco na lista vermelha da IUCN (Costa *et al*., 2008). Terrestre com hábito escansorial, é onívoro, com dieta composta principalmente por invertebrados, frutas e pequenos vertebrados, exibe pêlos longos, orelhas grandes e cauda longa e preênsil (Moraes *et al*., 2000; Cáceres, 2002; Gardner, 2008). O tempo de vida aproximado é de dois anos para fêmeas e de três a cinco anos para machos (comunicação pessoal, Dr. Maurício Graipel, UFSC).

D. albiventris tem ampla distribuição, ocorrendo no Brasil, Paraguai, Uruguai, Argentina, Bolívia, Equador, Peru e Colômbia (Wilson & Reeder, 2005; Costa *et al*., 2008; Gardner, 2008). A espécie está presente em diversos ambientes florestais preservados, sendo também encontrada em ambientes agrícolas e urbanos, incluindo algumas das principais cidades, demonstrando capacidade de coexistir com o impacto causado pela exploração humana nos espaços naturais. *Didelphis* motiva ações de controle sanitário por ser reservatório em potencial de agentes etiológicos relacionados a diversas zoonoses (Schallig *et al.*, 2007) e devido ao hábito de revirar lixo em busca de alimento. Dentre as doenças potencialmente relacionadas com os gambás como vetor, reservatório ou hospedeiro estão a raiva (Peixoto, 1998), a leishmaniose (Schallig *et al.*, 2007), algumas tripanossomíases (Mangia, 1999),

parasitoses relacionadas a nematóides (Gomes, 2003), doenças causadas por fungos (Taylor, 1962) e uma variedade de ectoparasitoses (Muller, 2005).

Uma das importantes contribuições dos gambás aos ecossistemas em que estes ocorrem consiste na sua atuação como dispersores de sementes e como espécie envolvida no processo de recuperação de ambientes florestais (Cáceres, 2002; Cantor *et al*., 2010). Sementes de diversas espécies permanecem viáveis após passar pelo sistema digestivo de *D. albiventris*. No estado do Paraná, constatou-se que a maior parte dos frutos consumidos por *D. albiventris* provém de espécies pioneiras. Esta característica dos gambás encontra relevância também em fragmentos florestais urbanos, em que a necessária restauração da vegetação é dificultada pela ausência de muitas das espécies frugívoras, que geralmente atuam como dispersoras de sementes em seus locais de ocorrência (Cáceres, 2002; Cantor *et al*., 2010). Algumas espécies, principalmente as generalistas, como os gambás, são capazes de se mover entre fragmentos florestais (Chiarello, 2000), exibindo taxas de deslocamento e de movimentação entre fragmentos florestais elevadas quando comparadas a outras espécies de pequenos mamíferos (Pires *et al*., 2002).

Os machos de *Didelphis* são poligâmicos (Loretto & Vieira, 2005). No que diz respeito à área de alcance de seu hábitat, as fêmeas tendem a ser mais estáveis e os machos tendem a migrar mais, o que resulta em uso diferente do espaço entre os sexos (Loretto & Vieira, 2005; Loretto & Vieira, 2008). A área de vida observada na Venezuela para machos de *D. marsupialis* (122,7 ha) é cerca de 10 vezes maior do que a verificada para fêmeas (12 ha) (Sunquist *et al*., 1987).

Os cariótipos de *Didelphis* têm 2n=22 (Svartman & Vianna-Morgante, 1999).

1.2- Características Gerais de *Marmosops incanus* (Lund, 1840)

O registro fóssil mais antigo do gênero *Marmosops* data do Pleistoceno, da região de Lagoa Santa (MG), onde foram encontrados também fósseis recentes (Gardner, 2008).

O gênero *Marmosops* inclui 15 espécies, das quais 14 ocorrem na América do Sul (a exceção é *M. invictus*, endêmica do Panamá). No Brasil constam registros de nove espécies, a maioria delas restrita ao bioma Amazônico, mas duas, *M. incanus* e *M. paulensis*, ocorrem fora deste bioma, inclusive em simpatria (Gardner, 2008).

A cuíca *M. incanus* (Figura 2) ocorre na Mata Atlântica, no Cerrado e na Caatinga dos estados da Bahia, Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo e Paraná (Câmara *et al*., 2003; Gardner, 2008; Lange & Jablonski, 1998). Apresenta hábitos escansoriais (semi-arbóreos) e noturnos. A dieta é composta por insetos e frutas (Emmons & Feer, 1990; D'Elia, 1999). Apresenta nítido dimorfismo sexual, com padrões de pelagem diferenciados entre os sexos (nos indivíduos adultos) e com maior tamanho corporal dos machos (Gardner, 2008). As fêmeas de *M. incanus* não possuem marsúpio, os filhotes permanecem no abrigo escolhido pela mãe, enquanto esta forrageia (Papi *et al*., 2007).

Figura 2. A cuíca *Marmosops incanus* (UFRJ, 2010)

Aproximadamente 67% de seu movimento ocorre no solo. Machos e fêmeas usam os extratos florestais de modo similar e preferem os extratos inferiores para construir ninhos e refúgios temporários (Loretto & Vieira, 2008). *M. incanus* resiste a alguns tipos de ambientes impactados, tendo sido observada como a espécie de mamífero mais abundante no interior de

plantações de eucalipto que mantiveram sub-bosque nativo (Fonseca, 1997). No entanto, é apontada como espécie indicadora, classificada como a mais sensível ao desequilíbrio ambiental e à fragmentação do habitat, quando comparada a 14 outras espécies de pequenos mamíferos (Rocha *et al*., 2011). A baixa mobilidade exibida por *M. incanus* e os hábitos arbóreos (Loretto & Vieira, 2008) podem resultar em diferenciação interpopulacional.

A semelparidade, estratégia evolutiva em que indivíduos se reproduzem apenas uma vez, está presente em *M. incanus* (D'Elia, 1999; Emmons & Feer, 1997; Lorini *et al*., 1994; Zangrandi *et al.,* 2007). O período reprodutivo principal é seguido pela morte dos machos adultos e, aproximadamente seis meses depois, as fêmeas adultas também morrem (D'Elia, 1999; Zangrandi *et al.,* 2007). Tal período reprodutivo está associado à estação chuvosa, e diferenças no mês de ocorrência são esperadas, para diferentes regiões geográficas (D'Elia, 1999; Zangrandi *et al.*, 2007).

Elevados níveis de diferenciação e de divergência genética (até 17,9%) em comparações entre biomas caracterizam o gênero *Marmosops* (cyt b) (Silva & Patton, 1998; Costa, 2003). A divergência entre *M. incanus* e quatro outras espécies do gênero é muito antiga tendo, de acordo com Costa (2003), ocorrido há aproximadamente oito milhões de anos.

Os cariótipos de *Marmosops incanus* têm 2n=14 (Svartman & Vianna-Morgante, 1999).

1.3 Registros de Simpatria entre Espécies do mesmo Gênero e Identificação por DNA *Barcode*

O gambá de orelha branca *Didelphis albiventris* (Lund, 1840) tem ampla distribuição e ocorre no Brasil, Argentina, Paraguai, Uruguai, Bolívia, Equador, Peru e Colômbia (Wilson & Reeder, 2005; Costa *et al*., 2008; Gardner, 2008). O gambá de orelha preta *D. aurita* (Wied-Neuwied, 1826), também amplamente distribuído, ocorre no Brasil, Argentina e Paraguai

(Wilson & Reeder, 2005; Gardner, 2008). A distribuição de *D. albiventris* se sobrepõe à de *D. aurita* (Figura 3) (Brito *et al*., 2008; Costa *et al*., 2008; Gardner, 2008). A simpatria entre *D. albiventris* e *D. aurita* é rara (Cerqueira, 1985; Gardner, 2008), exceto em áreas impactadas (Varejão & Valle, 1982; Gardner, 2008).

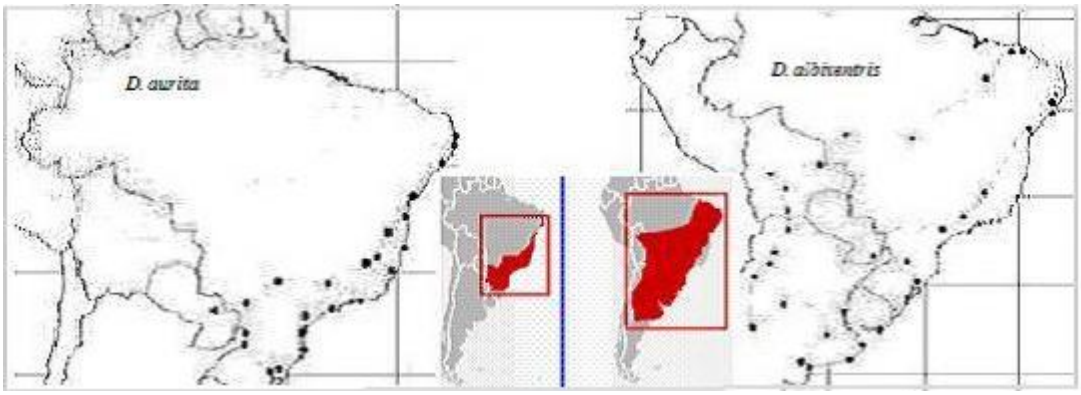

Figura 3. Distribuição de *D. aurita* e de *D. albiventris* (Brito *et al*., 2008; Costa *et al*., 2008; Gardner, 2008)

As espécies *M. incanus* e *M. paulensis* são endêmicas do Brasil (Gardner, 2008). *M. incanus* ocorre nos estados da Bahia, Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo e Paraná (Lange & Jablonski, 1998; Câmara *et al*., 2003; Gardner, 2008), *Marmosops paulensis* é encontrada nos estados de Minas Gerais, Rio de Janeiro, São Paulo e Paraná (Wilson & Reeder, 2005; Gardner, 2008) (Figura 4). Há relatos de simpatria entre *M. incanus* e *M. paulensis* (Gardner, 2008).

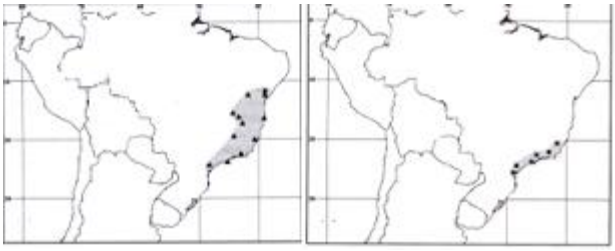

Figura 4. Distribuição de *M. incanus* e de *M. paulensis* (Gardner, 2008)

Tanto nos gambás (*D. albiventris* x *D. aurita*) como nas cuícas (*M. incanus* x *M. paulensis*) a distinção entre as espécies simpátricas do mesmo gênero é realizada com facilidade por ecólogos e zoólogos com prática na área da Mastozoologia. Entretanto, a identificação

morfológica requer prática em campo e conhecimentos detalhados da espécie, e poucos biólogos estão aptos a realizá-la adequadamente. Neste contexto, é comum encontrar um considerável número de espécimes em Museus à espera de profissional qualificado para realizar a identificação. Também é comum encontrarmos amostras com identificação incompleta, em que consta apenas o nome do gênero, sem que o epíteto específico tenha sido definido. Em alguns casos, o conhecimento da área de coleta é utilizado para completar a identificação, mas esta prática tem grande potencial para conduzir a erros, e não contempla, por exemplo, a distinção das espécies em locais onde ocorre simpatria.

Em *Didelphis*, a identificação morfológica baseia-se na cor da orelha e no padrão de dentição observado (o 3º pré-molar é maior em *D. aurita*). Este último critério é essencial à identificação, sobretudo em jovens, cuja cor da orelha pode ser diferente da característica da espécie (comunicação pessoal, Dr. Maurício Graipel, UFSC). A identificação molecular de gambás através da metodologia de DNA barcode foi utilizada para discriminar as espécies *Didelphis marsupialis* e *D. virginiana* em áreas de simpatria no México (Cervantes *et al*., 2010).

A identificação morfológica permite a distinção entre *M. incanus* e *M. paulensis*. O forame incisivo é curto em *M. incanus* e longo em *M. paulensis*; a fenestra palatina, ausente em *M. paulensis*, está presente em *M. incanus* (Gardner, 2008). Adicionalmente, em *M. incanus* uma linha de pêlos de base cinza separa a pelagem ventral da dorsal; padrão ausente em *M. paulensis* (Gardner, 2008).

A confirmação molecular dos registros morfológicos de simpatria entre espécies do mesmo gênero é viabilizada pela metodologia do DNA *barcode*. O gene da COI tem sido usado para discriminar espécies, permitindo a identificação precisa de espécimes. Códigos de barras de DNA podem auxiliar taxonomistas na determinação de grupos de espécies, facilitando o reconhecimento das unidades e escalas apropriadas para o planejamento da conservação

(Francis *et al*., 2010). Este marcador mitocondrial corresponde a uma região codificante bastante conservada, e consiste na opção utilizada e padronizada pelo BOLD (*barcode of life database*), que pretende reunir sequências de um fragmento específico do gene da COI de representantes de todas as espécies atuais, gerando uma base completa para a identificação. Esta ferramenta permite que a identificação seja feita de modo dinâmico pela simples comparação da amostra a ser identificada com outras amostras depositadas no Genbank (Folmer *et al*., 1994; Wilson-Wilde *et al*., 2010).

1.4. Status do Conhecimento Genético-populacional e Filogeográfico

Cada estruturação estatisticamente significativa tem o seu significado e a sua história, e reflete um nível diferente de separação evolutiva. O reconhecimento de unidades populacionais em Biologia da Conservação envolve a distinção entre dois tipos de estruturação genética: a recente (ou contemporânea) e a profunda (ou antiga).

A fragmentação do hábitat constitui relevante evento causador da estruturação recente. Neste contexto, características da espécie, sobretudo as ecológicas, influenciam fortemente parâmetros genético-populacionais. Distribuição geográfica, capacidade de dispersão, antropofilia, hábitos semi-arbóreos, características inerentes ao ciclo reprodutivo (como semelparidade), número efetivo populacional, dentre outros, podem direta ou indiretamente governar o fluxo gênico, a seleção e a deriva, ocasionando efeitos sobre a variabilidade e a diferenciação genética.

A ocorrência de fluxo gênico é extremamente relevante para a aproximação genética entre as populações e, ainda que eventual ou esporádica, pode dificultar a visualização ou até mesmo apagar os sinais de uma estruturação genética histórica quebrada recentemente. O estabelecimento de barreiras ao fluxo gênico não necessariamente ocorre abruptamente,

podendo constituir um processo gradual, ao qual as espécies respondem de acordo com sua ecologia (Carnaval, 2002). Ditchfield (2000) encontrou valores de divergência para análises em grandes escalas geográficas frequentemente entre 1 e 2,5 % para espécies de morcegos usando cyt b. Com o mesmo marcador e, ainda, em trabalho com quirópteros, Hoffmann e Baker (2003) encontraram valores referentes à contribuição da variação entre grupos em relação à variação total entre 59% e 84% na AMOVA. Baixa diferenciação genética entre populações distantes até 740 km do roedor semi aquático *Nectomys squamipes* estão provavelmente associadas à ocorrência de elevado fluxo gênico (Almeida *et al*., 2005). Almeida e colaboradores (2005) utilizaram *loci* microssatélites para estudar o roedor *Nectomys squamipes* e relataram ausência de correlação positiva entre as distâncias geográficas e as genéticas.

Os trabalhos realizados com Genética de Populações de pequenos mamíferos, principalmente roedores, comumente utilizam marcadores mitocondriais, muitas vezes permitindo a integração entre estudos genéticos e evolutivos, especialmente estudos filogeográficos intra-específicos concatenados com comparações entre *taxa* distintos. Estudando cinco gêneros de equimídeos arbóreos, Silva e Patton (1993) verificaram índices de divergência entre sequências (cyt b) variando entre menores do que 1 % até maiores do que 20% entre unidades geográficas dentro dos gêneros. Utilizando a região controle para o estudo de duas espécies de esquilos voadores, Oshida e colaboradores (2004) demonstraram a existência de três filogrupos em *Hylopetes fimbriatus* e de quatro filogrupos em *Petaurista petaurista albiventer*, e associaram a maior ocorrência de transversões em *H. fimbriatus* a uma história evolutiva mais longa.

Mora *et al*. (2010) detectaram subestruturação populacional analisando oito locos microssatélites para o roedor *Ctenomys australis* em escalas geográficas muito reduzidas (~4 Km de distância entre as localidades) e associaram os resultados à dispersão maior em

machos. Ainda considerando análises realizadas em escalas geográficas reduzidas (fina escala), Abdelkrim e colaboradores (2010), utilizaram oito locos microssatélites em suas análises e observaram índices de estruturação genética muito baixos em *Rattus rattus* na Nova Zelândia, Gonçalves e colaboradores (2009) verificaram a ausência de estruturação genética (F_{ST}<1,5% em análises da região controle) ao compararem populações dos sigmodontíneos *Oligoryzomys nigripes* e *Euryoryzomys russatus* em 58 Km de um gradiente altitudinal na Mata Atlântica do sudeste brasileiro.

De acordo com Sherwin e colaboradores (1989), a diferenciação genética entre subpopulações, subespécies e espécies de marsupiais parece ser um pouco menor do que a observada para eutérios (Sherwin *et al.*, 1989). Kovacic e colaboradores (1979) analisaram 31 *loci* gênicos de 85 espécimes de *Didephis virginiana* com o objetivo de comparar a população do leste com a do oeste dos Estados Unidos, e não observaram diferenciação genética significativa.

Estudos populacionais podem contribuir para a compreensão da história evolutiva das espécies e para a elaboração de hipóteses. Miranda e colaboradores (2009) analisaram cyt b e IRBP e concluíram que o gênero Oligoryzomys é composto por dois grupos de espécies: o do Amazonas-Cerrado e o do Pampas-Andes, e que o padrão geográfico norte / sul apoia a hipótese de que o gênero surgiu no norte dos Andes, ocupou a Amazônia e o Cerrado, e depois dispersou-se para as regiões mais ao sul (Miranda *et al.*, 2009).

Elevados níveis de divergência entre populações podem refletir eventos de fragmentação que antecedem a destruição de habitats causada pela ação humana (Carnaval, 2002).

A filogeografia intraespecífica consiste no estudo das relações entre a genealogia e a geografia. Avise e colaboradores (1987) citam três hipóteses filogeográficas relevantes: (1): Muitas espécies são constituídas por populações geográficas cujos membros ocupam ramos

distintos de uma árvore filogenética intraespecífica; (2): Uma estrutura filogeográfica populacional limitada caracteriza espécies com histórias de vida associadas à dispersão e à ausência de limitações relevantes ao fluxo gênico entre os locais que compõem sua área de distribuição; (3) grupos monofiléticos que diferem por grandes intervalos filogenéticos surgem em decorrência de barreiras extrínsecas antigas ao fluxo gênico. A partir destas hipóteses básicas são derivadas muitas outras, como a hipótese de história vicariante comum, em que a distribuição geográfica concordante dos intervalos filogeográficos para diferentes *taxa* identifica possíveis limites biogeográficos e eventos históricos comuns (Avise *et al*., 1987; Silva & Patton, 1993, Silva & Patton, 1998; Costa, 2003).

Nos marsupiais didelfídeos *Micoureus demerarae*, *Metachirus nudicaudatus* e *Marmosa murina*, em comparações envolvendo localidades da Amazônia e da Mata Atlântica, Costa (2003) encontrou distâncias genéticas (K2p) maiores entre localidades dentro de domínios do que entre os domínios.

De acordo com Bermingham & Moritz (1998), análises filogeográficas comparativas podem contribuir para estudos mais amplos de Ecologia e Evolução em muitos aspectos. Estudando 15 gêneros (35 espécies) de mamíferos amazônicos (cinco marsupiais e dez roedores), Silva e Patton (1998) obtiveram para alguns *taxa* valores de diferenciação (cyt b) superiores a 10%, com a maioria dos índices obtidos superior a 6%, o que os autores classificaram como sugestivos de tempo elevado de divergência. Para *Didelphis spp*. foram observados índices de divergência de 3,1% em compararações entre a Mata Atlântica e a Amazônia, e de 2,31% como valor máximo observado entre duas regiões dentro da Amazônia (Silva e Patton, 1998). Para *Marmosops spp.* (excluindo *M. parvidens*) foram observados percentuais de divergência de 17,9% entre os dois biomas e de 12,21% entre duas regiões dentro da Amazônia (Silva e Patton, 1998; Mustrangi & Patton, 1997).

A enorme diferença entre os índices obtidos para *Didelphis* e *Marmosops* provavelmente reflete o somatório ponderado de duas realidades distintas, resultantes do acúmulo dos efeitos de vários fatores. A primeira refere-se a eventos recentes (contemporâneos) e a características ecológicas. A segunda refere-se a eventos mais antigos, já que substanciais níveis de divergência entre clados podem refletir uma história evolutiva profunda envolvendo seus tempos de diferenciação (Silva & Patton, 1998; Patton & Silva, 1997).

A especiação neotropical não pode ser explicada por um único modelo de vicariância ou mudanças climáticas (Costa, 2003). Alguns gêneros ou grupos de espécies exibem uma forte estrutura hierárquica em toda a área de distribuição amostrada; outros, ainda que geograficamente estruturados, exibem pouca resolução no que diz respeito às relações entre espécies ou clados geográficos dentro das espécies. Várias separações ocorreram consideravelmente antes do Pleistoceno, e, portanto, não podem ser interpretadas à luz da hipótese dos refúgios pleistocênicos, comumente citada para explicar a diversidade neotropical (Costa, 2003). Neste sentido, Carnaval & Moritz (2008) observaram maior diversidade em populações de *M. incanus* de regiões classificadas como externas aos refúgios do que as exibidas pelas populações localizadas dentro de um refúgio hipotético, contrariando as previsões do modelo de estabilidade.

A divergência mais antiga dentre os *taxa* estudados por Costa (2003) ocorreu há aproximadamente oito milhões de anos e separou *M. incanus* das outras espécies do gênero *Marmosops* (Costa, 2003). Em análises filogeográficas com cyt b, o gênero *Marmosops* exibiu altos níveis de diferenciação em comparações entre biomas e tempo estimado de divergência elevado. Adicionalmente, há importantes questionamentos taxonômicos, grande parte relacionados a *M. parviden*s, envolvendo este gênero marsupial (Silva e Patton, 1998; Costa, 2003).

2. Objetivos

Este trabalho teve como objetivo geral estudar a distribuição da variabilidade genética em *D. albiventris* e em *M. incanus* em algumas de suas áreas de ocorrência, verificando e discutindo o efeito da distância genética sobre a diferenciação interpopulacional.

Com o intuito de alcançar o objetivo geral traçamos os seguintes objetivos específicos:

1- Gerar sequências do gene mitocondrial da Citocromo Oxidase c subunidade I e depositá-las no Genbank, tornando a informação acessível;

2- Avaliar a utilidade do gene da COI em estudos de diversidade molecular e análises da variabilidade genética intra e interpopulacional em *D. albiventris*;

3- Avaliar a utilidade do gene da COI em estudos de diversidade molecular e análises da variabilidade genética intra e interpopulacional em *M. incanus*;

4- Estudar o padrão de distribuição geográfica das linhagens genealógicas de *D. albiventris* e de *M. incanus*, classificando a intensidade do sinal filogeográfico exibido pela espécie na área de distribuição considerada;

5- Testar a utilidade do marcador COI e da metodologia de *barcode* genético para a identificação de duas espécies do gênero *Didelphis* cujas distribuições se sobrepõem;

6- Testar molecularmente registros morfológicos de simpatria entre *D. albiventris* e *D. aurita* utilizando a subunidade I do gene da COI e a metodologia do *barcode* genético.

CAPÍTULO I

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

Running title: Molecular diversity of an urban opossum population

L. C. C. Sousa[1], C. M. F. Gontijo[2], G. A. Lacorte[1], S. N. Meireles[1], A. P. Silva[1], C. G. Fonseca[1]

[1]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

[2]Laboratório de Leishmanioses, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG, Brasil

Corresponding author address: L.C.C. Sousa

E-mail: lucienecsousa@gmail.com

Abstract. We made a molecular study of 40 opossums, *Didelphis albiventris* from an urban fragment of 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.

Keywords: *Didelphis*, Molecular Characterization, Sympatry, Barcode, COI

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).

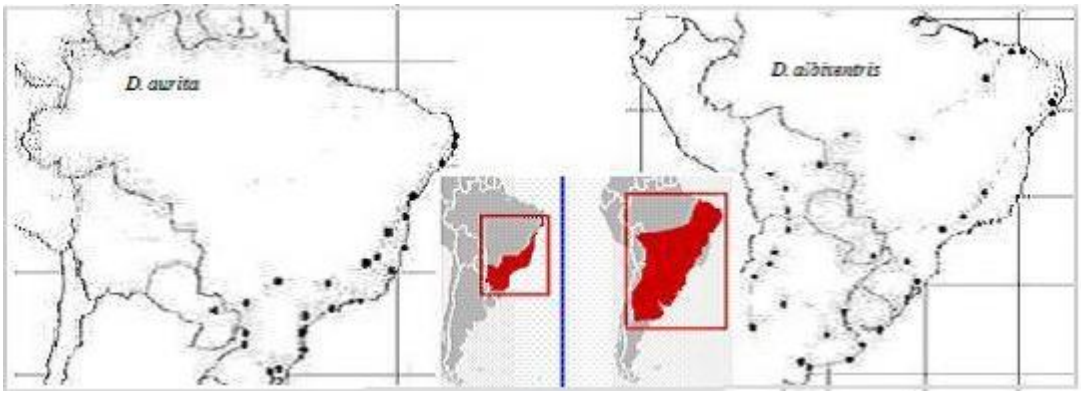

Figure 1. Map showing the distribution of *D. aurita* and *D. albiventris* (Brito *et al*., 2008; Costa *et al*., 2008; Gardner, 2008)

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 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).

MATERIALS 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).

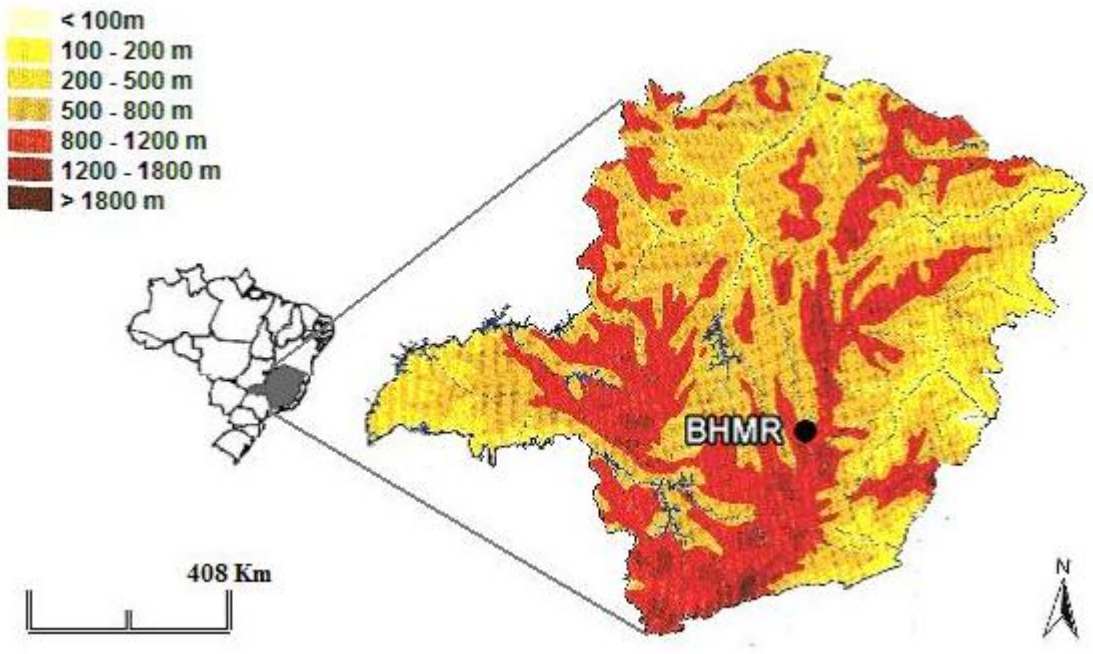

Figure 2. Minas Gerais Brazilian state, with a detach to Belo Horizonte Metropolitan Region (BHMR)

Five localities in BHMR (Figure 2) were unequally sampled. *D. albiventris* samples were from Capitão Eduardo (32 specimens), Coração Eucarístico (03 specimens), Instituto Agronômico (02 specimens), Mangabeiras (01 specimen), and Jardim Canadá (02 specimens). A unique *D. aurita* specimen was collected from the Capitão Eduardo area. The approximate distances between the studied areas ranged from 6.5 km to 26 km.

DNA samples from these 41 BHMR opossums were analyzed, starting with the morphologically identified 40 *D. albiventris* and 01 *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: 03 *D. albiventris* individuals from Porto Alegre and Triunfo, Rio Grande do Sul (RS) state and from Xacriabá Reserve in Minas Gerais (MG) state; and 07 *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: Collection sites, distance to 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	MCN-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 *et al*. (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, 10× 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 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 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 *et al*. (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 Applied Biosystems BigDye® Terminator Kit v3. Alternatively, some sequences were obtained on a MegaBACE automated capillary sequencer by using GE Healthcare ET® dye terminator kit.

Statistical data analyses

All mtDNA sequences were “base called” by using software Phred v.0.20425 (Ewing *et al*., 1998; Ewing and Green, 1998), checked for quality using 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 numbers: 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 (Hd) 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.

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.

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).

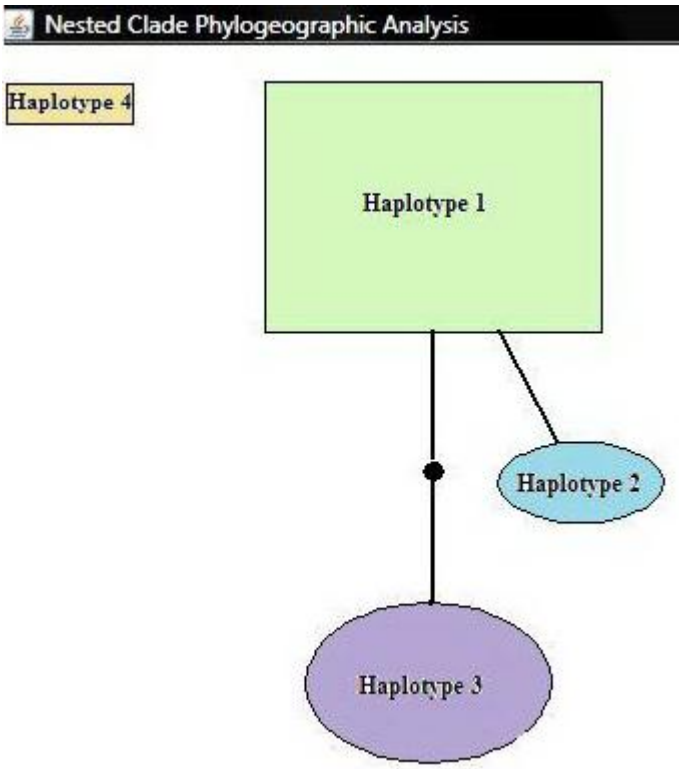

Figure 3. Haplotype network for 41 opossums collected in BHMR urban fragment

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 BHMR opossums 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.

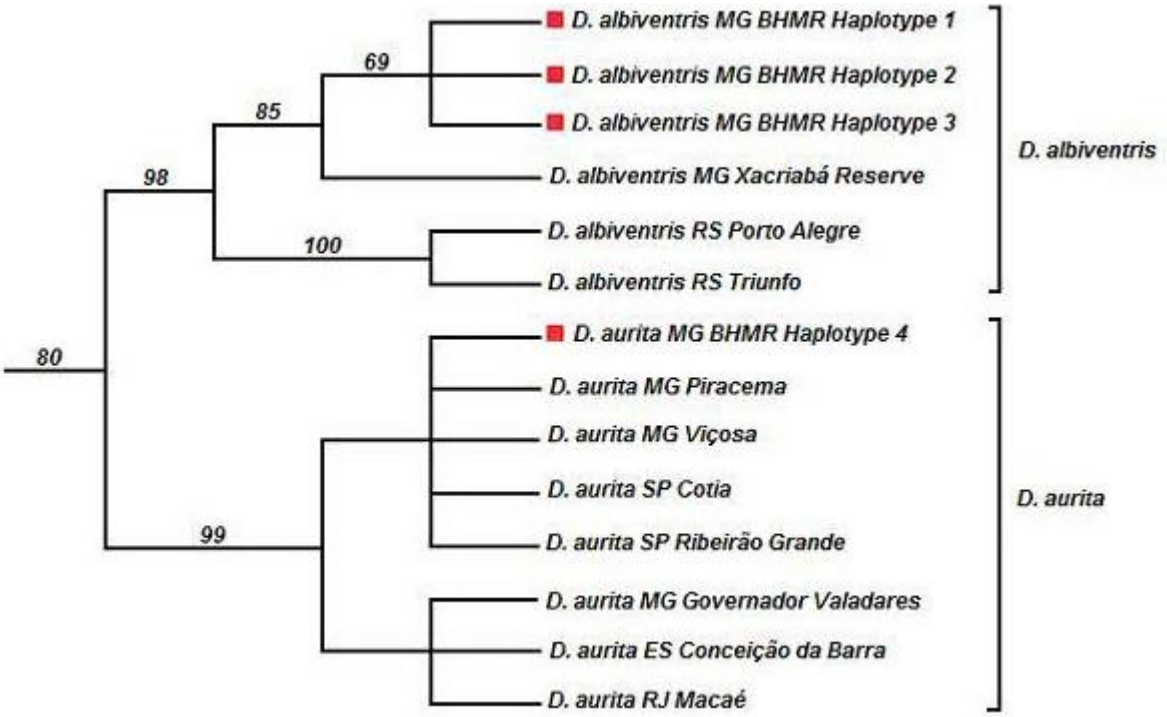

Figure 4. Maximum Parsimony tree with *D. albiventris* and *D. aurita* specimens from different geographic localities

Phylogeny inference was estimated using different evolutionary 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. albiventris* 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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CAPÍTULO II

O artigo será submetido ao periódico *"Mitochondrial DNA"*.

DNA Barcode Methodology confirms sympatry of opossum species in an Atlantic Forest fragment of southeastern Brazil

Luciene CC Sousa[1*], José E Santos Jr[2], Célia MF Gontijo[3], Leonardo S Meneguini[1], João Carlos Pinto Dias[2], Liléia Diotaiuti[2], Cleusa G Fonseca[1]

[1]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

[2]Laboratório de Triatomíneos e Epidemiologia da Doença de Chagas, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG, Brasil

[3]Laboratório de Leishmanioses, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG, Brasil

* Corresponding author address: L.C.C. Sousa; e-mail: lucienecsousa@gmail.com

Abstract

DNA barcode methodology was used to test the morphologically based record of sympatry between *D. albiventris* and *D. aurita* in the Piracema Atlantic Forest fragment. Sequences of cytochrome c oxidase obtained for three *D. albiventris* and three *D. aurita* collected in Piracema were compared. *D. aurita* and *D. albiventris* COI sequences from other Brazilian localities were included to validate the comparison. Our results showed a clear phylogenetic separation and divergence between the *D. albiventris* and *D. aurita* sequences and effectively confirmed the local sympatry between these species, supporting the appropriateness of DNA barcode for species identification. Additionally, our data provided evidence that the mtDNA diversity tends to be greater in *D. albiventris* than in *D. aurita.*

Keywords: DNA barcode, opossum, sympatry, *D. albiventris*, *D. aurita*, Atlantic Forest

Introduction

Opossums of the genus *Didelphis* have an important ecological role to the maintenance of diverse ecosystems, acting as seed dispersers (Cantor *et al*. 2010). They tolerate disturbed areas and are present in different habitats, including large cities and other urban areas, revealing an ability to coexist with the environmental impact caused by human exploitation. Widespread and able to move easily between forest fragments (Chiarello 2000), this generalist group of species exhibits parasitological importance as a reservoir of etiological agents, such as *Trypanosoma cruzi* and *Leishmania braziliensis* (Moreno & Carcavallo 1999; Schallig *et al.* 2007).

Sympatry records between *D. albiventris* and *D. pernigra* in Bolivia and between *D. marsupialis* and *D. virginiana* in Mexico have been previously recorded (Anderson 1997; Lemos & Cerqueira 2002; Cervantes *et al*., 2010). It was also reported in Curitiba Brazilian city (Cáceres & Monteiro-Filho 1999). The distribution of *D. albiventris* and *D. aurita* overlap (Brito *et al*., 2008; Costa *et al*., 2008; Gardner, 2008). Sympatry between these two species seems rare, but it was reported in human disturbed areas (Varejão & Valle 1982; Gardner 2008), as the Belo Horizonte Metropolitan Region, an urban area 94 Km distant to Piracema, where sympatry between these species was confirmed by barcode technology (Sousa *et al.* 2012b).

In a wide biological perspective, morphological identification of species has limitations: phenotypic plasticity and characters genetic variability may lead to incorrect identifications; morphological keys are often effective only for a particular life stage or gender; and the use of keys often demands such a high level of expertise that misdiagnoses are common (Hebert *et al.* 2003). The distinction between *D. aurita* and *D. albiventris* is generally made on the basis of morphological characters such as dentition patterns and ear color, the latter being the most identification character, although easily applied, it may lead to misdiagnoses when used to identify young individuals. Limitations inherent to morphology-based identification systems and the dwindling pool of taxonomists signal the need for a new approach to taxon recognition, and in this context, barcode methodology appears as an effective tool and as a real perspective to ensure a correct identification when the morphologically based identification is not enough (Hebert *et al.* 2003).

COI, the marker of choice for species discrimination by the Barcode of Life Database (BOLD), is useful for species identification and to study differentiation in large scale structure studies (Borisenko *et al*. 2008, Wilson-Wilde *et al*. 2010, Sousa *et al*. 2012a). This study aims

to test opossum sympatry in Piracema, using DNA barcoding with sequences from samples collected in diverse Brazilian localities to base the approach.

Materials and Methods

Study area and sampling

The sample included *D. albiventris* and *D. aurita* from 14 Brazilian localities and one sequence of *D. pernigra* from Suriname (accession number EU095421.1; Borisenko *et al*. 2008). Specimens from the Pampa, Caatinga, and especially Atlantic Forest and Cerrado biodiversity hotspot biomes (Mittermeier *et al.* 1998) were studied. The barcode methodology was used to test the morphologically based record of sympatry between *D. albiventris* and *D. aurita* in Piracema, Minas Gerais state, Brazil. Interspecific nucleotide divergence and diversity patterns were also analyzed.

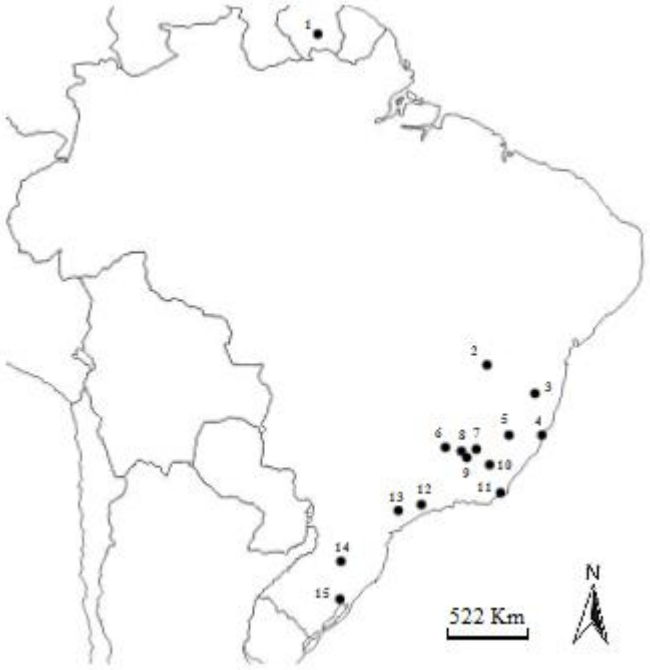

Figure 1. Studied localities: 1: Suriname, 2: Reserva Indígena Xacriabá, 3: Almenara, 4: Conceição da Barra, 5: Serra do Ibituruna, 6: Bambuí, 7: Belo Horizonte, 8: Divinópolis, 9: Piracema, 10: Viçosa, 11: Macaé, 12: Cotia, 13: Ribeirão Grande, 14: Machadinho, 15: Porto Alegre.

This work was performed under the license ICMBIO/SISBIO, number 20170-2, which was renewed on February 2011. The institutions that donated material had their own licenses.

DNA sequences from six opossums collected in Piracema, three of them morphologically identified as *D. albiventris* and three as *D. aurita*, were produced. The COI sequences obtained were phylogenetically compared with sequences of 15 opossum specimens, 14 of them produced by our group:

[1] seven *D. albiventris*, five from Minas Gerais (MG), localities of Almenara, Belo Horizonte, Reserva Indígena Xacriabá (RIX), Divinópolis and Bambuí, and two from Rio Grande do Sul (RS) Brazilian extreme south state, localities of Porto Alegre and Machadinho; [2] seven *D. aurita*, representing all Brazilian southeast states, being three from Minas Gerais state, locations of Belo Horizonte, Serra do Ibituruna and Viçosa, one from Espírito Santo (ES) state, locality of Conceição da Barra, one from Rio de Janeiro (RJ) state, locality of Macaé, and two from São Paulo (SP) state, localities of Cotia and Ribeirão Grande. The COI sequence of one *D. pernigra* from Suriname (accession number EU095421.1; Borisenko *et al*. 2008), was used as outgroup in phylogenetics analysis. Linear geographic distances between Piracema and the aforementioned areas range between 58 and 1250 km for *D. albiventris* and between 94 and 561 km for *D. aurita*.

DNA extraction, DNA amplification, and sequencing

DNA from macerated tissue fragments and from blood samples was extracted following the standard phenol-chloroform protocols, as described by Sambrook *et al.* (2001). COI gene studied fragment DNA 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, 10× 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 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 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 *et al*. (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 Applied Biosystems BigDye® Terminator Kit v3. Alternatively, some sequences were obtained on a MegaBACE automated capillary sequencer by using GE Healthcare ET® dye terminator kit.

Computational statistics analysis

All mtDNA sequences were "base called" by using software Phred v.0.20425 (Ewing *et al*. 1998; Ewing and Green 1998), checked for quality using Phrap v.0.990319 (Green 1994) software, 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. Studied sequences were developed in this study or in previous studies realized by the first author research group and deposited in Genbank (accession numbers JN638891; JN638931; JN638941; JN638959; JN638962: JN638964; JN638968; JN638970; JN638974; JN638984; JN638985; JN638987: JN638991; JN638986, JQ738373; JQ738374). The

exception is *D. pernigra* from Suriname, whose sequence was extracted from the Genbank (accession number EU095421.1; Borisenko *et al.* 2008).

Mega 4.1 (Kumar *et al.* 2007), DNAsp v.5 (Librado and Rozas 2009), and Arlequin v.3.1 (Schneider *et al*. 2000) were used to analyze patterns of genetic diversity and nucleotide divergence between species. Mega 4.1 (Kumar *et al*. 2007) was used to visualize nucleotide variation and verify polymorphism coherence by using the translating approach. 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).

Results

A preliminary diversity analysis within groups of ten *D. albiventris* and ten *D. aurita*, without any population study purpose, was performed and for *D. albiventris*, we observed five haplotypes, 20 polymorphic sites corresponding to 20 substitutions, an average number of nucleotide differences of 6.8, haplotype diversity (Hd) of 0.7556, and nucleotide diversity (π) of 0.0114. For *D. aurita*, we found two haplotypes, one polymorphic site corresponding to a single substitution, an average number of nucleotide differences of 0.467, haplotype diversity of 0.4667, and nucleotide diversity of 0.00077.

A total of 45 polymorphic sites and 46 mutations were observed in the 20 specimens analyzed. Interspecific analysis showed 25 fixed differences, 20 substitutions polymorphic only in *D. albiventris*, and one substitution polymorphic only in *D. aurita*; all substitutions were synonymous. No shared substitution was observed. The average number of nucleotide

differences between *D. albiventris* and *D. aurita* was 33.5 and the observed nucleotide divergence between these species was 0.0574.

Euclidean square distance matrix, constructed with the Tamura & Nei distance method, evidenced great distances between species, with *D. albiventris* haplotypes clearly separated from those of *D. aurita* by a large connection length, which ranged between 33.97 and 38.56.

The Hasegawa, Kishino and Yano (1985) evolutionary model (HKY 85) of nucleotide substitution was the most appropriate for the analyzed dataset, as revealed by the Akaike informative criterion in Modeltest 3.7 analysis (Posada and Crandall, 1998).

Phylogeny inference was estimated using different evolutionary models (neighbor joining, maximum likelihood, maximum parsimony, and Bayesian) and provided similar results. The proposed topologies showed a clear separation between the studied species, as expected, supporting the barcode methodology and the mtDNA COI fragment appropriateness to effectively identify *D. albiventris* and *D. aurita* specimens. In the resulting phylogeny, two genetic clusters were observed, each corresponding to a different species showing a perfect separation between them (Figure 2).

Phylogenetic relationships revealed by the tree topologies were concordant with the morphological identification. The results from our sequence analysis were unambiguous.

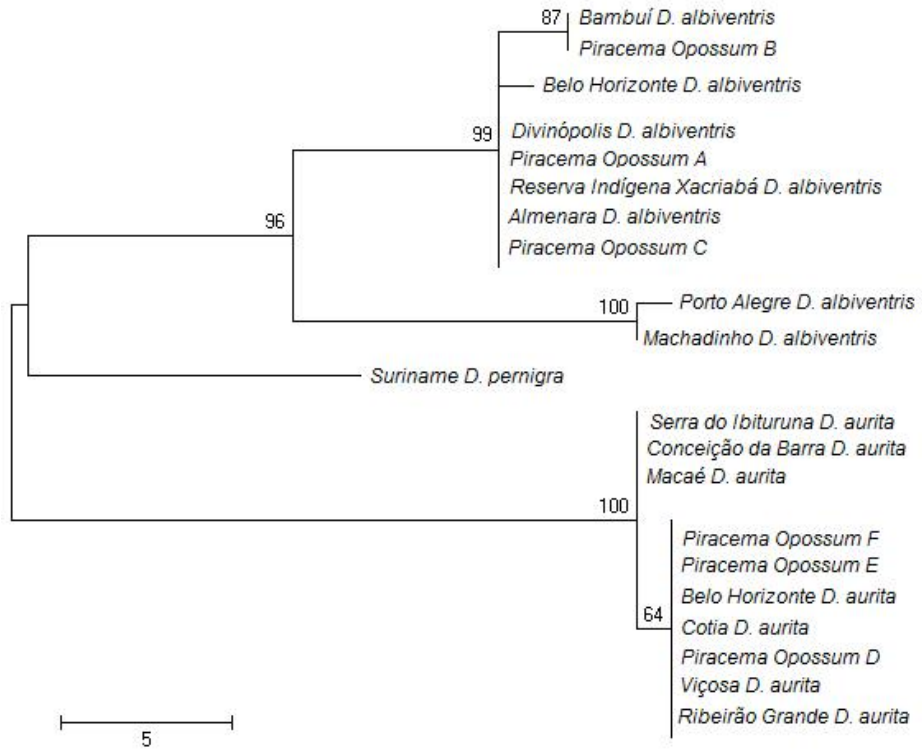

Figure 2. Maximum Parsimony tree constructed using *D. albiventris* and *D. aurita* sequences from different geographic localities. Each sample was identified by its collection locality and species identification: *D. albiventris*, *D. aurita* or only opossum (for the six Piracema sympatric specimens, under barcode identification) followed by its field identification (A to F). Bootstrap test of inferred phylogeny, with 10,000 replications and random seed of 24,054. Total length is 46.

Discussion

Preliminary intragroup diversity analysis of *D. albiventris* and *D. aurita* were performed. Each group was composed from opossums collected in different and in most cases, distant geographic localities in the widespread distribution of the species. This analysis showed a considerably greater diversity in *D. albiventris*, which exhibited larger values for nucleotide and haplotype diversity, number of haplotypes, and number of polymorphic sites, than those observed for *D. aurita*. These results suggest that *D. albiventris* is genetically more diverse than *D. aurita*, but this is not doubtless, as different geographic distances and contexts may represent a bias for these variability comparisons. Opossum's genetic variability is related to selection process. The hability to answer to ecological changes or variations depends on the

existence of a rich genetic pool, so the larger molecular diversity observed in *D. albiventris* may represent an advantage for this species when compared to *D. aurita*. At present, we have evidence that the mtDNA diversity is larger in *D. albiventris* than in *D. aurita*, but additional studies are necessary to verify if this scenario is real in other geographic areas and different genomic regions.

The large connection length between haplotypes from the studied *taxa* indicates the mtDNA genetic distance between them. The clear separation between species enabled doubtless barcode identification in phylogenetic analyses with COI sequences and, as observed in the literature for many other species, demonstrates the viability of COI as a marker for species identification.

Observed results were in accordance with morphological identification and confirm the sympatry between *D. albiventris* and *D. aurita* in Piracema, supporting the appropriateness of the DNA barcode methodology to effectively discriminate between Atlantic Forest opossum species.

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Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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CAPÍTULO III

Status: publicado no periódico *"Genetics and Molecular Biology"*. O artigo no formato da publicação está incluso após os anexos.

Genet Mol Biol 35 (2): 522-529

Mitochondrial Genetic Variability of *Didelphis albiventris* (Didelphimorphia, Didelphidae) in Brazilian localities

Luciene CC Sousa[1], Célia MF Gontijo[2], Helbert A Botelho[2] and Cleusa G Fonseca[1]

[1]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.

[2]Laboratório de Leishmanioses, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG, Brazil.

Running title: Genetic diversity of *D. albiventris*

Keywords *Didelphis albiventris*, marsupial, variability, COI, genetic differentiation

Send correspondence to Luciene C.C. Sousa. Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil. E-mail: lucienecsousa@gmail.com

Abstract *Didelphis albiventris* is a well- known and common marsupial. Due to its high adaptability, this very widespread generalist species occurs under various environmental conditions, this even including protected regions and disturbed urban areas. We studied a 653 bp fragment of cytochrome oxidase c (COI) from 93 biological samples from seven Brazilian localities, with linear distances ranging between 58 and about 1800 km to analyze the effects of geographic distances on variability and genetic differentiation. The haplotype network presented nine haplotypes and two genetic clusters compatible with the two most distant geographic areas of the states of Minas Gerais, in the southeast, and Rio Grande do Sul, in the extreme south. As each cluster was characterized by low nucleotide and high haplotype diversities, their populations were obviously composed of closely related haplotypes. Surprisingly, moderate to high F_{ST} differentiation values and a very weak phylogeographic signal characterizes interpopulation comparisons within Minas Gerais interdemes, these being correlated with the presence of privative haplotypes. On a larger geographic scale, a comparison between demes from Minas Gerais and Rio Grande do Sul presented high F_{ST} values and a robust phylogeographic pattern. This unexpected scenario implies that mtDNA gene flow was insufficient to maintain population cohesion, reflected by the observed high differentiation.

Introduction

The white-eared opossum *Didelphis albiventris* Lund, 1840 (Didelphimorphia, Didelphidae) is widely distributed throughout Brazil, Paraguay, Uruguay, Argentina, Bolivia (Gardner, 2008; Costa *et al*., 2008), Ecuador, Peru and Colombia (Wilson and Reeder, 2005). The species is listed as "Least Concern" in the IUCN Red List Category (Costa *et al*., 2008). Through their presence in a wide variety of habitats, and adaptability to disturbed areas, such as large towns and other urban habitats, *D. albiventris* manifests the capacity of coexisting

with environmental impacts caused by human exploitation of natural spaces. Another characteristic is their importance as parasite reservoirs, highly relevant in populated urban areas (Schallig *et al.*, 2007).

One of the most important factors affecting mammals in small fragments is the lack of food resources. Little is known on Neotropical forest mammal movement between forest-patches. Even so, anecdotal evidence indicates facile mobility in some species, especially habitat generalists, as opossums (Chiarello, 2000). This was apparent in a southeastern Brazilian Atlantic Forest region, where *D. aurita* manifested interfragment movement in 19.4% of recaptures, the highest, when compared to seven other small mammals (Pires *et al*., 2002). *Didelphis* are polygynous, the females presenting more stable home ranges and the males migrating more, hence the differences among sexes in the use of space (Loretto and Vieira, 2005), as verified for *D. marsupialis* in Venezuela, where, on using radiotelemetry methodology, a mean home range 10 times greater for males (122.7 ha) than for females (12 ha) was observed (Sunquist *et al*., 1987). In this context, white-eared opossums, as seed dispersers, make an important contribution to the maintenance of diverse ecosystems, mainly where specialist frugivores are frequently absent, as in urban forest fragments (Cantor *et al*., 2010).

In the present survey, four Brazilian biomes in the wide *D. albiventris* distribution were sampled, viz., Atlantic Forest, Cerrado, Caatinga and Pampa. The Atlantic Forest and Cerrado appear on the biodiversity hotspots list, which highlights 24 priority conservation areas (Mittermeier *et al*., 1998). Biodiversity hotspots, occupying only 1.4% of the Earth's surface, concentrate more than 60% of terrestrial species (Mesquita, 2004), and mainly consist of heavily exploited and often highly fragmented ecosystems, greatly reduced in extent, and with less than 25% of the original vegetation remaining (Mittermeier *et al*., 1998). Several vegetal formations are observed in the Brazilian Atlantic Forest,, such as the Seasonal Forest (semi-

deciduous and deciduous, the latter occurring on a reduced scale) and the Rain Forest (dense and moist). The seasonal semi-deciduous forest is under extreme risk, formerly caused by sugar cane and coffee plantations, and currently by growing urbanization, especially around the major cities (IBGE, 2011, Fundação SOS Mata Atlântica, 2011). The Araucaria Moist Forest, an endangered ecosystem (only 12.6 % remaining) of the Atlantic Rain Forest, is mostly distributed among small fragments surrounded by anthropogenic habitats, such as cattle pasture, farming and exotic-tree monoculture (Ribeiro *et al.*, 2009; Emer and Fonseca, 2011). The Cerrado, Caatinga and Pampa biomes are characterized by open grassland vegetation. The Cerrado biome, with savanna vegetation, predominates in central Brazil, the Caatinga, with savanna-steppe vegetation, is typical of the semiarid northeast, and the Pampa, restricted to the extreme south, is characterized by steppe vegetation (IBGE, 2011).

Mitochondrial DNA, the most used molecular marker for tracing the geographic distribution of genealogical lineages, even at the intraspecific level, has been consolidated by such characteristics as maternal inheritance and high rates of nucleotide substitution (Avise *et al*., 1987). Molecular DNA techniques, besides forming the basic tool in population genetics studies for defining taxonomic units (Wilson-Wilde *et al*., 2010), have also been widely used in mammal diversity surveys when analyzing variability characteristics and distribution, as in the genetic structure analyses of the Atlantic Forest sigmodontine rodents *Oligoryzomys nigripes* and *Euryoryzomys russatus* (Gonçalves *et al.*, 2009) and the short-tailed bats *Carollia brevicauda*, *C. perspicillata*, *C. sowelli* and *C. castanea* (Hoffmann *et al*., 2003). COI, the marker of choice for species discrimination by the Barcode of Life Database (BOLD), is useful for species identification, and the study of differentiation in large-scale structure assaying (Wilson-Wilde *et al*., 2010; Sousa *et al*., 2012).

Knowledge on species population genetics is important for a better understanding of species biology, including ecological correlations. The aim here was to study *Didelphis albiventris*

population genetic patterns, by focusing on the geographic distance effect on both variability and genetic differentiation among demes.

Materials and Methods

Sampling

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

Didelphis albiventris samples from two distant geographic areas in Brazil, herein considered as two geographic clusters, were studied, viz., Minas Gerais (MG), a southeastern state, and Rio Grande do Sul (RS), the southernmost. Linear distances between the studied localities range from 58 to 1795 km (Table 1). In MG, six localities were sampled (Figure 1). Piracema (Pir) and Almenara (Alm), both in the Atlantic Forest, and Bambuí (Bam), in the Cerrado, were poorly sampled (Figure 1). As the Belo Horizonte Metropolitan Region (BH, 40 samples), Divinópolis (Div, 18 samples) and the Reserva Indígena Xacriabá (RIX, 18 samples) were well-sampled, the hypothesis of separate demes was tested here. Although BH and Div are geographically situated in transitional regions between the Cerrado and Atlantic Forest, both sampled areas present characteristics of the Atlantic Forest biome. RIX, a transitional area connecting the Cerrado and Caatinga biomes, presents ecotone characteristics. The RS geographic cluster (Figure 1) comprised samples collected in two localities in the Araucaria Moist-Rain Forest, a domain of the Atlantic Forest (Machadinho and Caxias do Sul), four collected in the Pampa biome (Porto Alegre and Triunfo) and three from road killed animals from RS, but without exact locality information. For Rio Grande do Sul, distances correspond to the average among known state collection localities (Table 1).

Table 1. Matrix with linear geographic distances (Km) between sampling areas. The meanings of abbreviations are cited in the topic 'sampling' in "Material and Methods"

Demes	BH	Div	RIX	Bam	Pir	Alm	RS
BH							
Div	111						
RIX	548	590					
Bam	221	115	600				
Pir	94	58	621	164			
Alm	523	622	393	700	620		
RS	1256	1180	1725	1130	1170	1795	

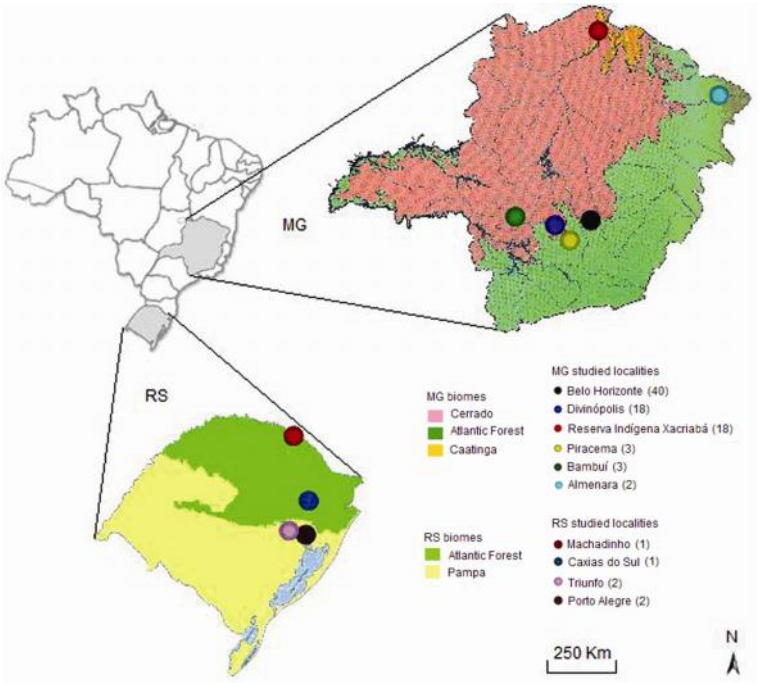

Figure 1 - Minas Gerais, a southeastern state (Drummond *et al*., 2005), and Rio Grande do Sul, the southernmost state of Brazil (SCP/DEPLAN, 2007), with approximate collection locations, sample numbers (in parentheses) and biome correspondence. Three samples from road killed animals from RS, but without exact information on locality, were not represented

In the entire analysis, and due to the small size of the samples obtained from each location, as a whole, the RS sample group was treated as one single study area.

DNA extraction, amplification and sequencing

The tissue samples used were mostly obtained from the liver, and in a few cases, the spleen, muscle and blood. Ear-tissue fragments were collected from road killed animals. Tissue samples were preserved in 95% ethanol and stored at -20 ^{o}C. Most of the samples were kindly

donated by researchers from the Centro de Pesquisa René Rachou/FIOCRUZ, Museu de Ciências Naturais da Pontifícia Universidade Católica de Minas Gerais, Fundação Zoo-Botânica do Rio Grande do Sul, and the Pontifícia Universidade Católica do Rio Grande do Sul. DNA from macerated tissue fragments was extracted according to standard phenol-chloroform protocols, as described by Sambrook *et al.*, (2001).

DNA sequences of the mitochondrial cytochrome oxidase I gene (COI) 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 was carried out in a 20 µl final volume, containing 50 ng of 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 $MgCl_2$ 10x), 0.8 µM of dNTPs, 0.5 µM of each primer, 1% bovine serum albumin (BSA), and 1 unit of *Taq* DNA polymerase (Phoneutria). After an initial denaturing step of 3 min at 94 °C, the PCR conditions followed a standard three-step protocol, with 30 cycles of 1 min at 94 °C, 45 s at 47 °C 30 s at 72 °C, followed by a final extension step for 5 min at 72 °C. Satisfactory amplifications were visualized in 6% polyacrylamide gels. Amplified DNA products were purified using 20% polyethylene-glycol (PEG 8000) and 2.5M NaCl, according to Sambrook *et al*., (2001).

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

Statistical Data analysis

Sequences were base-called with Phred software (Ewing *et al*., 1998; Ewing and Green, 1998), and checked for quality with Phrap software (Green, 1994), whereas the assembled

chromatograms were checked and edited in Consed (Gordon *et al*., 1998). Chromatogram peaks for each sequence were visually verified to ensure consensus fidelity. Sequence groups were aligned using the Clustal W algorithm implemented in MEGA 4.1 (Tamura *et al*., 2007), with a 653 bp fragment showing high levels of sequence quality for all individuals. The studied sequences were deposited in GenBank (accession numbers JN638891 to JN 638983).

MEGA 4.1 (Tamura *et al*., 2007), DNAsp v.5 (Librado and Rozas, 2009) and Arlequin v.3.1 (Excoffier *et al*., 2005) were used for analyzing intrapopulation genetic diversity and estimating standard indices of genetic variation, such as haplotype (Hd) and nucleotide (π) diversities. Arlequin v.3.1 (Excoffier *et al*., 2005) was also used for calculating differentiation indices and analyzing molecular variance (AMOVA), with the Tamura & Nei distance method and 10,100 permutations. This software was also used for calculating Mismatch Distribution, Tajima's D and Fu's Fs tests of neutrality, thereby assaying demographic expansion, and whether mutations were neutral or under the influence of selection.

The haplotype network was constructed based on statistical parsimony. The maximum number of steps parsimoniously connecting two haplotypes was informed by TCS v.1.21, which estimates genealogical relationships among sequences (Clement *et al*., 2000).

Alleles in Space (AIS) (Miller, 2005) was used for analyzing the relationship between inter-individual spatial and genetic information, and Mantel testing and spatial autocorrelation analysis for predicting patterns, such as correlations between genetic and geographical distances.

The best evolutionary model was determined with Modeltest v.3.7 (Posada and Crandall, 1998). Phylogeographic inference using Maximum Parsimony, Maximum Likelihood and Bayesian analyses were carried out with PAUP* 4.0 (Swofford, 2002), PHY ML 3.0 (Guindon and Gascuel, 2003) and MrBayes (Huelsenbeck and Ronquist, 2001), respectively.

Results

Molecular characterization of mtDNA COI fragments

The analysis of 93 mtDNA COI sequences revealed 24 polymorphic sites, all of which corresponding to synonymous transitions. Nucleotide composition was 34.1% thymine, 22.4% cytosine, 28.2% adenine and 15.3% guanine. Nine haplotypes (H=9), all with three or more recordings of occurrence, were observed. Haplotype 1 occurred throughout all the areas studied in MG, whereas seven were private to just one analyzed area, viz., haplotypes 2 and 3 to Belo Horizonte (BH), 4 and 5 to Reserva Indígena Xacriabá (RIX), 7 to Divinópolis (Div), and 9 and 10 to Rio Grande do Sul State (RS) (Table 2).

Table 2. Haplotype (Hap) occurrence in populations. The meanings of abbreviations are cited in the topic 'sampling' in "Material and Methods"

Locality	Hap1	Hap2	Hap3	Hap4	Hap5	Hap6	Hap7	Hap8	Hap9	Total
BH	22	13	5	0	0	0	0	0	0	40
Divinópolis	13	0	0	0	0	2	3	0	0	18
RIX	6	0	0	7	5	0	0	0	0	18
Bambuí	1	0	0	0	0	2	0	0	0	3
Piracema	2	0	0	0	0	1	0	0	0	3
Almenara	2	0	0	0	0	0	0	0	0	2
RS	0	0	0	0	0	0	0	4	5	9
Total	46	13	5	7	5	5	3	4	5	93

Haplotype diversity (Hd) of 0.7235 (with a standard deviation of 0.044) and nucleotide diversity (π) of 0.0065, characterized the analyzed data set (Table 3). Hd and π for each population can be seen in Table 3.

Table 3. Intrapopulation and total diversities. Number of samples (N), number of haplotypes (H), number of polymorphic sites (S), nucleotide diversity (π), haplotype diversity (Hd). The meanings of locality abbreviations are cited in the topic 'sampling' in "Material and Methods"

Locality	N	H	S	% π	% Hd
BH	40	3	3	0.17	59.10
Divinópolis	18	3	3	0.11	46.41
RIX	18	3	4	0.3	69.94
Bambuí	3	2	2	0.20	66.67
Piracema	3	2	2	0.20	66.67
Almenara	2	1	0	0	0
RS	9	2	1	0.09	55.56
Total	93	9	24	0.65	72.35

When RS sequences were excluded, and only the six MG localities analyzed, nine polymorphic sites were found, these corresponding to seven haplotypes (H=7), haplotype diversity (Hd) of 66.52%, and nucleotide diversity (π) of 0.23%.

Genetic differentiation among populations

The haplotype network produced with the 93 *D. albiventris* specimens using statistical parsimony, and with a 91% connection limit, showed two distant genetic clusters compatible with the MG and RS geographic clusters (Figure 2).

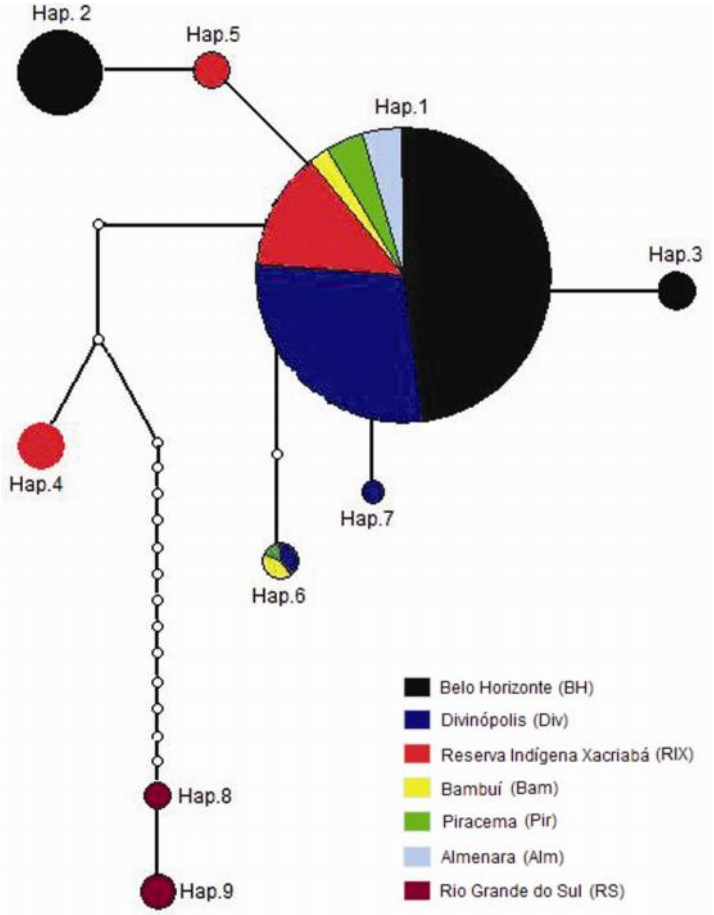

Figure 2 - Haplotype network for *D. albiventris* using statistical parsimony. Numbered circles represent haplotypes (Hap.), with the circle size corresponding to haplotype frequency. Colors represent the sampling sites. Small open circles indicate missing haplotypes

Highly significant population pairwise differentiation values were observed (Table 4). On comparing demes between geographic clusters (RS x MG), obtained values proved to be higher than 91%, the smallest F_{ST} being observed in the comparison RIX x RS (the meanings of abbreviations are cited in "Materials and Methods").

Table 4. Population pairwise F_{ST} calculated using the Tamura & Nei distance method. The meanings of abbreviations are cited in "Material and Methods"

Comparison	F_{ST}	P values
BH x RIX	0.2774	0.0000 ± 0.0000
BH x Div	0.1935	0.0029 ± 0.0016
RIX x Div	0.2790	0.0000 ± 0.0000
BH x RS	0.9431	0.0000 ± 0.0000
Div x RS	0.9622	0.0000 ± 0.0000
RIX x RS	0.9127	0.0000 ± 0.0000

AMOVA indicated genetic structuring (F_{ST} = 93.6%, P=0.000). According to the Tamura & Nei distance method, and on comparing the two geographic clusters (MG and RS), intergroup differences contributed with 91.3% of the total genetic variance (Table 5), whereas interpopulation variance, within the groups was 2.26%, and within the populations themselves, 6.44%. All the results were significant.

Table 5. AMOVA using the Tamura & Nei distance method, considering MG and RS as groups, and collection localities as populations

Source of variation	Percentage of variation
Among groups	91.30
Among populations within groups	2.26
Within populations	6.44
FST=0.9356; p= 0.00 ± 0.00	

By way of analysis using the Tamura & Nei distance method, and with BH and Divinópolis as a first group and RIX as a second, variation partitioning revealed 17.04% of intergroup variance, 13.21% of interpopulation within groups, and 69.74% of intrapopulation, with F_{ST} = 30.26% (P=0.000) (Table 6).

Table 6. AMOVA using the Tamura & Nei distance method, considering BH, Div and RIX as populations. The meanings of abbreviations' are cited in "Material and Methods"

Source of variation	Percentage of variation
Among groups	17.04
Among populations within groups	13.21
Within populations	69.74
FST= 0.3026; p= 0.00 ± 0.00	

Two groups were formed, the first comprising BH and Divinópolis samples and the second RIX (the meanings of abbreviations are cited in "Material and Methods").

Tajima's D (P > 0.35) and Fu's F_S statistics (P > 0.51) neutrality tests were non- significant. As a test of recent population expansion, applied mismatch distribution analysis indicated non-significant bimodal distribution (Figure 3).

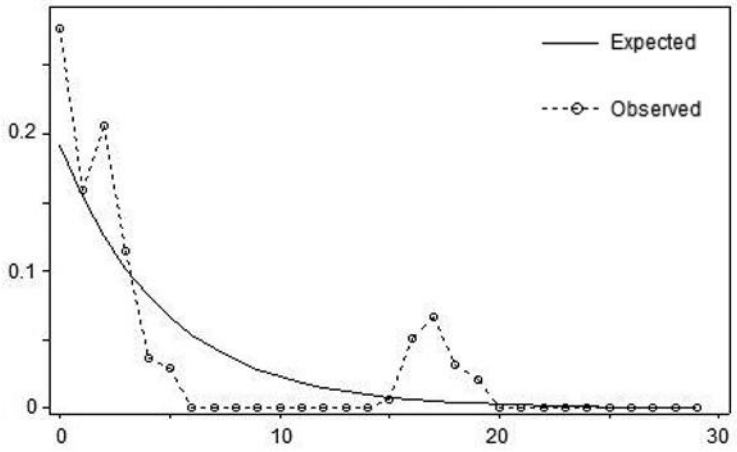

Figure 3 - Mismatch distribution analysis showing bimodal distribution (non-significant)

On compiling a complete dataset, Mantel test analysis revealed two geographical clusters corresponding to genetic clusters (Figure 4). Although, on analyzing MG and RS populations, genetic and geographical distances were highly correlated (r = 0.8901; P of a correlation greater than or equal to that observed = 0.001), they were considerably less so (r = 0.2216; P of a correlation greater than or equal to that observed = 0.002), when analyzing only MG.

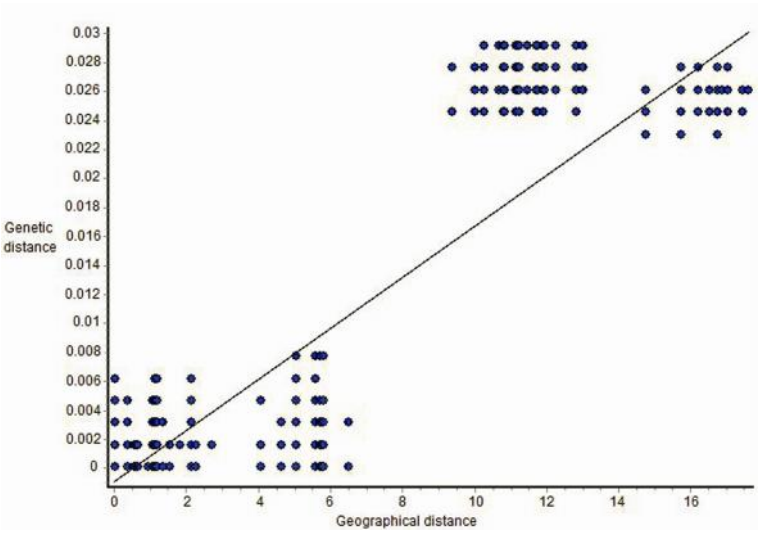

Figure 4 - Mantel Test Results showing correlations between genetic and geographical distances

The HKY 85 evolutionary model of nucleotide substitution, together with the Akaike informative criterion in Modeltest 3.7 (Posada and Crandall, 1998), was found to be the most appropriate for dataset analysis. As a whole, phylogeographic analysis with Maximum Parsimony, Maximum Likelihood and Bayesian analysis revealed a weak phylogeographic pattern for *D. albiventris*, except for MG and RS, where there was a clear differentiation into two distinct haplogroups (data not shown).

Discussion

As expected when working with a conserved functional gene, all the 24 polymorphic sites in the COI fragment analyzed were synonymous transitions. This situation influences diversity indices, which tend to be considerably lower than for non-coding regions.

Four biomes and several natural conditions were sampled in this survey. In the case of the Atlantic Forest, this involved various threatened ecosystems. In the haplotype network (Figure 2), a large number of steps were observed between the haplotypes of the RS Araucaria Atlantic Rain Forest and those of the MG Seasonal Semi-Deciduous Forest, which illustrates the great mtDNA genetic distance between *D. albiventris* haplotypes from both of these ecosystems. As similarly great distances were also observed in all the other biome pairwise comparisons that involved locations from different geographic clusters (MG x RS), an association between geographic and genetic distance is strongly implied. The small genetic distances observed between haplotypes from distinct biomes within each geographic cluster reinforce this argument. As an example, the Rio Grande do Sul haplotypes are cited: the forms from the Araucaria Rain Forest are the same as those occurring in the Pampa biome.

The number of polymorphic sites was considerably greater in the analysis involving all the studied areas (24 variable sites) than in that excluding RS state (only nine). Hence, nucleotide diversity (π) was nearly three times greater in the first situation. On analyzing only MG-state samples, the low nucleotide diversity (π) (0.227%) and haplotype diversity of 66.52%, indicate the presence of haplotypes with few nucleotide differences, thus coherent with the observed haplotype network (Figure 2).

On comparing intrapopulation diversity indices (*π, Hd)*, it was observed that the highest values were attributed to Reserva Indígena Xacriabá (RIX) in MG state. In accordance, the smallest pairwise F_{ST} value obtained between geographic clusters (MG and RS) was when comparing RIX x RS (Table 4). Although unexpected, when considering the effect of distance, this is understandable, when thinking of the higher diversity indices exhibited for this ecotone area, located in a transitional area between the Cerrado and Caatinga biomes. Ecotones may be a source of evolutionary novelty (Smith *et al*., 1997), playing an important role in the maintenance of genetic diversity, in divergence, and in the speciation process (Kark *et al.*, 2002). Greater attention and higher priority in conservation research and planning should be dedicated to transitional zones which potentially serve as within-species diversity hotspots (Smith *et al*., 1997; Kark *et al.*, 2002).

By applying statistical parsimony to the haplotype network for 93 *D. albiventris* specimens two separate genetic clusters, clearly compatible with the two major geographic clusters (MG and RS), could be discerned. The connection between both required a large number of steps, and could only be observed by reducing the connection limit to 91%, this corresponding to a minimum nucleotide distance of 15 steps between haplotypes from the two areas. The observed genetic differentiation was probably the result of distance effect, and is consistent with low mtDNA gene flow, insufficient for maintaining population unity. Haplotypes were genetically close to each other within each geographic cluster.

The high F_{ST} values between MG and RS genetic clusters, in all the population pairwise comparisons, were significant (P = 0.00 ± 0.00) and extremely high (FST > 91%), clearly reflecting population structure, with 91.3% of intergroup contribution. On a more restricted scale, when comparing demes in the MG geographic cluster alone, a considerable part of variation (69.74%) was intrapopulation (Table 6), with F_{ST} lower than 28% (P ≤ 0.0029 ± 0.0016).

According to Edelaar and Björklund (2011), considering F_{ST} as a measurement of population differentiation is a misunderstanding, as it actually measures the fixation of alleles. In fact, the observed F_{ST} values were surprisingly high, and seemed to much more reflect the presence of deme privative haplotypes than molecular distances between haplotypes.

On comparing only MG zones, phylogeographic analysis revealed a very weak phylogeographic pattern, with a complete shuffling of samples of different origins in all the constructed phylogenies, whereby a pattern with haplotype admixture between localities. The resultant tree showed no separation between localities, even when linearly 700 Km apart. The observed pattern is consistent with those observed in species with a limited or narrow phylogeographic population structure, and life histories conductive to dispersal, occupying ranges without long-term impediments to gene flow (Avise, 2000). Although apparently incompatible with the high to moderate F_{ST} values observed, assuredly phylogeographic analysis, although less sensitive to evidence of population differentiation, reflects two important factors, the presence of haplotype 1 in all the MG localities studied, thereby connecting them, and the low number of polymorphic sites separating haplotypes.

In contrast, MG and RS specimen phylogenies confirmed genetic separation between these geographic clusters, thus giving evidence of two spatially circumscribed haplogroups, genetically relatively far apart. This pattern seems to distinguish deep allopatric lineages in a gene tree, probably explained by long-term extrinsic barriers to gene flow (Avise, 2000)

Nevertheless, this cannot be interpreted as major phylogeographic discontinuity, since there is a significantly wide sample gap. Thus, this clear separation between specimens from MG and RS in the gene tree, appears to be a result of considerable geographic separation (about 1800 Km of linear distance). If this gap area were studied, a pattern with a weak phylogeographic signal characterizing *D. albiventris* lineage spatial distribution would possibly be found.

Tests of neutrality (the Tajima D and Fu F_S statistics) to check excess of rare mutations, as evidence of recent population expansion, were non-significant. Although involved p-values were non-significant, mismatch distribution analysis for testing demographic expansion presented a graph with bimodal distribution, thus consistent with allopatric divergence followed by population growth. This could represent a possible hypothesis for the present study-case. The Mantel test and spatial autocorrelation analysis confirmed the strong correlation (0.8901) between geographic and genetic distances, when analyzing the MG and RS clusters. Differentiation probably reflects both the great distance between localities, and the existence of barriers in the wide range of species distribution. As only gene flow can genetically connect populations, the maintenance of COI gene differentiation implies the presence of barriers to mtDNA gene flow, although other important factors seem to be closely related to the observed differentiation results, viz., the bridge between a methodology based on a haplotypic mtDNA system, and ecological characteristics, especially *D. albiventris* sex-biased dispersion.

Due to its wide distribution, generalist habits, high adaptability, capacity to move long distances (Gentile and Cerqueira, 1995; Chiarello, 2000; Pires *et al*., 2002), and outstanding mobility, when compared to other small mammals (Pires *et al*., 2002), *D. albiventris* populations are presumed to be genetically connected. The unexpectedly high differentiation among MG demes seems to be totally unaligned with the above cited ecological characteristics, whence the importance of considering an alternative. A mean home range ten

times greater for males (122.7 ha) than for females (12 ha) has been observed for *D. marsupialis* (Sunquist *et al*., 1987). Although dispersion competence is relevant to promoting genetic approximation between populations, it is in no way a guarantee of gene flow. Even so, as *Didelphis* dispersion is recognizably greater in males, this probably does indeed contribute more. Working with a maternally inherited genetic marker, it was impossible to discuss complete *D. albiventris* diversity history, since mtDNA analysis told nothing about the male's effective contribution to connecting demes. Hence, our results furnished data only on the mutational history corresponding to maternal lineage genealogical information. The observed mtDNA COI genetic differentiation was consistent with mtDNA gene flow insufficiency in maintaining population unity, or to effectively approximate separated demes in the large geographic scenario studied. Additional research with nuclear markers (microsatellites and/or sequences) could complete our findings, thereby providing a better understanding of species population genetics. As to the female contribution to *D. albiventris* population structure, the haplotype network and differentiation values strongly suggest that female gene flow is insufficient in connecting and effectively approximating the populations under study. The contact with this widespread and important species emphasized the need for additional surveys towards a better understanding of its interesting biology.

Acknowledgments

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Internet Resources

Arlequin Software, http://cmpg.unibe.ch/software/arlequin3/ (July 31, 2011)

DNAsp Software, http://www.ub.edu/dnasp/ (July 31, 2011)

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MEGA 4 Software, http://www.megasoftware.net/ (July 31, 2011)

MrBayes Software, http://mrbayes.sourceforge.net/download.php (July 31, 2011)

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CAPÍTULO IV

O artigo será submetido ao periódico *"PLOS ONE"*.

Genetic Diversity and Phylogeography of *Marmosops incanus* (Marsupialia: Didelphidae) in southeastern Brazil

Luciene C.C. Sousa[1]*, Edeltrudes M.V.C. Câmara[2,3], Cleusa G. Fonseca[1]

1 Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, 31270-901, Belo Horizonte, MG, Brazil, **2** Museu de Ciências Naturais, Pontifícia Universidade Católica de Minas Gerais, Av. Dom José Gaspar, 500, Coração Eucarístico, 30535-610, Belo Horizonte, MG, Brazil, **3** Bicho do Mato Instituto de Pesquisa, Rua Perdigão Malheiros, 222, Luxemburgo, 30380-234, Belo Horizonte, MG, Brazil.

*Corresponding author. E-mail: lucienecsousa@gmail.com

Running title: Genetic Variability of *Marmosops incanus*

Abstract

Background: *Marmosops incanus* is typical from the Atlantic Rain Forest, with occurrence records in the Cerrado and Caatinga biomes. This didelphid shows significant habitat preference and was pointed out as an indicator species, being the most sensitive to disturbance and to the landscape fragmentation among other small mammal species [34]. It also exhibits ecological characteristics like low mobility and semelparity, which can influence interdemes population connection.

Methodology/Principal Findings: We studied a 509 pb fragment of cytochrome oxidase c, subunit 1 for 17 Brazilian localities and observed the influence of geographic distance in the genetic differentiation among populations. The genetic distances between demes haplotypes and the great number of exclusive forms produced an interesting haplotype network, with a large number of steps between some studied haplotypes. A large and significant $F_{ST,}$ indicating strong genetic structure, large values of interphylogroups variation and elevated correlation between genetic and geographical distances characterized interpopulational analyses. The haplotype network and phylogeographic trees clearly showed molecular haplogroups mostly compatible to geographic clustering. The observed pattern of geographic distribution of genealogic lineages showed a strong phylogeographic signal.

Conclusions/Significance: The species life history is the central to explain our results, specially the great interdemes divergence. The strong genetic structure and the great variation among phylogroups are related to ancient processes that influenced the lineages geographic distribution, reflecting fragmentation events that predate human driven habitat destruction. Currently, genetic drift and insufficient gene flow allow the maintenance of high levels of genetic differentiation for this indicator species.

INTRODUCTION

The genus *Marmosops* includes 15 species, 14 of them occurring in South America (the exception is *M. invictus*, endemic to Panama) and nine in Brazil, mostly restricted to the Amazon [14]. The gray slender opossum, *Marmosops incanus* (Lund 1840) is endemic to Brazil, occurring in the Atlantic Forest, Cerrado and Caatinga of the states of Bahia, Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo and Paraná [3, 14, 25].

M. incanus presents nocturnal scansorial (semi arboreal) habits, with a diet consisting mostly of insects and fruits [7, 8], average adult weights around 64g and presents a clear sexual dimorphism and low mobility [7, 8, 28, 43]. Females have no pouch and the offspring remains in a shelter while the mother forages [32]. Males appear to be more sedentary than females [13], although locomotory behavior of this marsupial is still poorly known [28]. *M. incanus* has, on average, 67 % of its movements on the ground and prefers lower forest strata to build nests and temporary refuges [28]. *M. incanus* showed significant habitat preference and was pointed out as the most sensitive species to disturbance and landscape fragmentation among 15 small mammal species in Minas Gerais, Brazil, being thus suggested an indicator species [34]. Biological features like reproductive pattern, low mobility and semi arboreal habits [28], may impact interpopulation differentiation.

M. incanus has an interesting reproductive cycle. Among its features, the semelparity, which is an evolutionary strategy where individuals reproduce only once (type I strategy) that has also been reported in several dasyurid marsupials, in the neotropical didelphid *Marmosops paulensis* [26] and in *M. incanus* [7, 8, 29, 43]. The main reproductive period is followed by the death of adult males, and about six months later, adult females also die [7, 43]. The species reproductive period is generally associated with the rainy season, and geographic variation in the month of occurrence is expected [7, 43].

High levels of differentiation and cytochrome b divergence in interbiomes comparisons characterizes the genus *Marmosops* [6, 37]. *Marmosops spp.* divergence between biomes was 17.9%, and 12.2% between two regions in Amazon (excluding *M. parvidens*, because of taxonomic uncertainties) [37]. The divergence between *M. incanus* and other four species of the genus was estimated to have occurred about eight million years ago, very ancient when compared to other small mammal genera [6].

Mitochondrial DNA reflects an important component of the species history: the matriarchal phylogeny, although it does not represent a complete characterization of intraspecific phylogeny [1]. Phylogeography faces criticism for its excessive dependence on the mtDNA haplotypic system, but maternal inheritance, high substitution rates and smaller coalescent time contributed to consolidate mtDNA as the most useful tool to evolutionary studies [1, 2, 24]. Populations of the slender mouse opossum from southern areas outside refugia had higher collective diversity than those located within the range of the hypothesized Bahia refugia, what is against the expectation under the forest refugia hypotheses, conflicting with the stability model predictions [4]. Mitochondrial markers have been used to analyze genetic variability and differentiation in the Atlantic Forest sigmodontine rodents *Oligoryzomys nigripes* and *Euryoryzomys russatus* [15], in the short-tailled bats *Carollia brevicauda*, *C. perspicillata*, *C. sowelli* and *C. castanea* [20], and in the Brazilian opossums *Didelphis albiventris* and *D. aurita* [38, 39].

The cytochrome c oxidase subunit I gene has been used for species discrimination, as the marker of choice by the Barcode of Life Database (BOLD), for phylogeographic differentiation of intraspecific lineages and to study differentiation in large scale structure studies [19, 41]. Genetic differentiation between subpopulations, subspecies and species of marsupials seems to be smaller than those observed for eutherians [36]. This study aims to

contribute to the understanding of *M. incanus* genetics emphasizing the analysis of spatial distribution of COI molecular diversity and its phylogeographic patterns.

MATERIALS AND METHODS

Sampling

This study was developed under license ICMBIO / SISBIO, number 20170-2, renewed on February, 2011. The institutions that collaborated with samples donations also have their own scientific licenses.

In this study, a total of 58 specimens from 17 localities in the Brazilian Southeastern states of Espírito Santo (ES), Minas Gerais (MG) and São Paulo (SP) were sampled (Figure 1), representing different biomes and transitional areas. The Atlantic Forest of the states of Minas Gerais (Jacinto, João Monlevade, RPPNC, PESB), Espírito Santo (Ibiraçu, Santa Teresa, Domingos Martins, Castelo) and São Paulo (Cotia, Ibiúna); the Cerrado of the state of Minas Gerais (UHI). Transitional áreas (ecotones) between Atlantic Forest and Cerrado of Minas Gerais State (Itabira, São Gonçalo do Rio Abaixo, PESRM, FNR); between Cerrado and Caatinga (RIX) and between Cerrado, Caatinga and Atlantic Forest (Mato Verde) were also sampled [22].

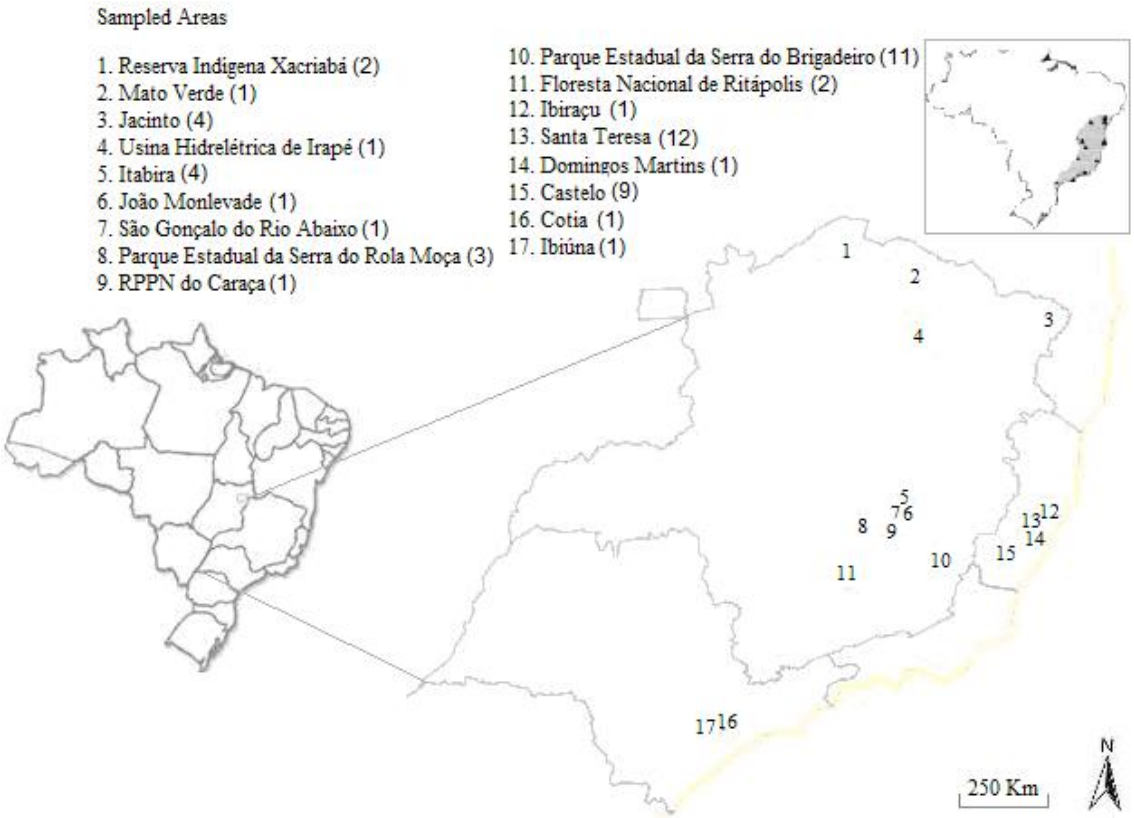

Figure 1. Map with sampled localities, and sample numbers (in parentheses). Numbers indicate sampled areas. The map in the inset represents *M. incanus* geographic distribution based on Gardner [14].

Sampled localities approximate coordinates were plotted (Figure 2) using the AIS software [30].

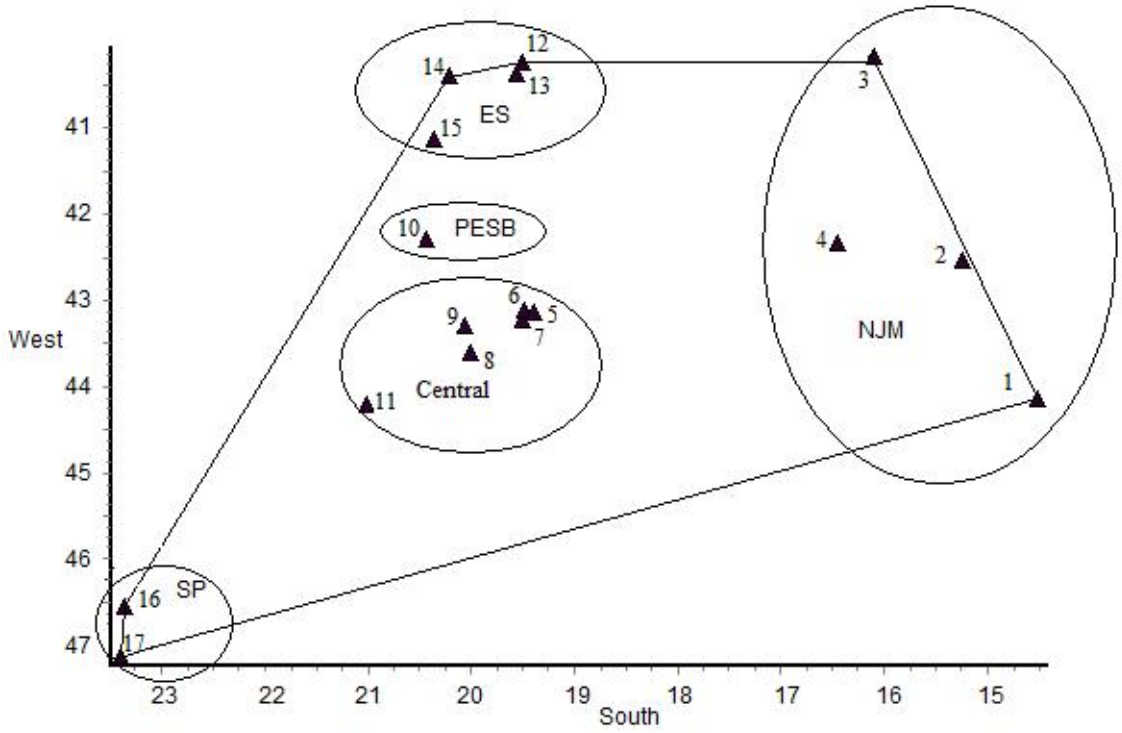

Figure 2. Plot of sample location in degrees of latitude (horizontal axis) and longitude (vertical axis). Numbers represent localities as in Figure 1. We grouped localities in five geographic clusters.

Five sample groups were recognized. The population treatment was not applicable to the SP group (Ibiúna and Cotia demes) because of the small sample size (only two samples), and for NJM (Norte and Jequitinhonha Mucuri) group (wich includes RIX, Mato Verde, Jacinto and UHI demes) because the geographic distances between demes were considerably higher in this group than in the other groups. Three sample groups will be treated in this work as populations: the Central population with six demes (Itabira, João Monlevade, São Gonçalo do Rio Abaixo, RPPNC, FNR and PESRM); the PESB population (with samples from the PESB deme); and the ES population with four demes (Santa Teresa, Castelo, Domingos Martins and Ibiraçu). The PESB population is geographically intermediate between the two other populations.

DNA extraction, DNA amplification and sequencing

We utilize tissue samples, most of them liver, and in few cases spleen, muscle or ear fragment. Most samples were kindly supplied by researchers from Museums and other institutions. Almost all samples were collected between 1990 and 2011. DNA from macerated tissue fragments was extracted following standard phenol-chloroform protocols, as described in Sambrook *et al.* [35].

DNA sequences of the mitochondrial gene of cytochrome c oxidase subunit I (COI) 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' [12]. Each PCR were performed in a 20 µl final volume, containing 50 ng of genomic DNA, 10x Buffer III B (Phoneutria®: 100mM$(NH_4)_2SO_4$, 100mM KCl, 100mM Tris-HCl pH 8,4, 1% Triton-X, 15mM $MgCl_2$ 10x), 0.8 µM dNTPs, 0.5 µM of each primer, 1% bovine serum albumin (BSA) and 1 unit of *Taq* DNA polymerase (Phoneutria®). After an initial denaturing step of 3 min at 94°C, the PCR conditions followed a standard three-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 extension step for 5 min at 72°C. Satisfactory amplifications were visualized in 6% polyacrylamide gel. Amplified DNA products were cleaned using 20% polyethylene-glycol (PEG 8000) and 2.5M NaCl according to Sambrook *et al*. [35].

PCR products were sequenced in both directions using the same primers: LCO 1490 or HCO 2198 [12] on ABI3100® automated sequencer using BigDye Terminator Kit v3 (Applied Biosystems). Alternatively, some sequences were obtained on a MegaBACE automated capillary sequencer, using ET® dye terminator kit (GE Healthcare).

Produced COI mtDNA sequences were base called with the software Phred [9, 10], checked for quality using the Phrap [17] software, and the assembled chromatograms were verified and edited in Consed [16]. Chromatograms peaks for each sequence were visually verified to ensure consensus fidelity. Sequence group was aligned using the Clustal W algorithm

implemented in MEGA 4.1 [23], a 509 pb fragment shows high levels of sequence quality for all individuals. All studied sequences were produced in this work and deposited in GenBank (JQ030920 to JQ030977).

Statistical Data Analyses

We used MEGA 4.1 [23], DNAsp v.5 [27] and Arlequin v.3.1 [11] to analyze intrapopulation genetic diversity and to find standard indices of genetic variation such as haplotype diversity (*h*) and nucleotide diversity (π). DNAsp v.5 [27] and Arlequin v.3.1 [11] are also used to calculate differentiation indices, like conventional F_{ST} and its analogues, using a matrix of Tamura & Nei distance, and 10100 permutations. Arlequin v.3.1 [11] was used to perform global test of differentiation among populations and Analysis of Molecular Variance (AMOVA), wich estimate genetic structure indices and variation partitioning using haplotype content and frequencies information. We use Arlequin v.3.1 [11] either to test demographic expansion inferences performing Mismatch Distribution, Tajima's D and Fu's Fs tests of neutrality wich tests whether mutations are neutral or under influence of selection.

Haplotype network was constructed based on statistical parsimony. The maximum number of steps connecting parsimoniously two haplotypes is indicated by TCS v.1.21 [5], wich estimate genealogical relationships among sequences [5].

Alleles in Space (AIS) [30] was used to analyze the relationship between inter-individual spatial and genetic information, performing Mantel test and spatial autocorrelation analysis to verify the correlation between genetic and geographical distances.

The best evolutionary model was found using Modeltest v.3.7 [33]. Filogeographic inference using Neighbor Joining, Maximum Parsimony, Maximum Likelihood and Bayesian analysis were performed using Mega 4, PAUP* 4.0 [40], Phy ML 3.0 [18] and Mr Bayes [21] respectively.

RESULTS

The analysis of 509 bp of 58 mtDNA COI sequences revealed 445 monomorphic and 64 polymorphic sites, corresponding to 70 nucleotide substitutions. We observed four singleton and 60 parsimony informative sites (55 with two variants, four sites with three variants and one with four variants). The substitutions were in their great majority synonymous changes (69), with only one of them corresponding to a nonsynonymous transition. This substitution was observed in samples from Ibiúna and Cotia, grouped in this work as a single geographic cluster (SP group). Eighteen haplotypes, a great haplotype diversity of 93% and a high nucleotide diversity of 2.6% were observed. Nucleotide composition exhibited 33.6% of thymine, 23.2% cytosine, 28.4% adenine and 14.9% guanine.

The haplotype network for 18 haplotypes showed five haplotypes (28%) shared between two or three demes, while the majority (13 haplotypes, representing 72%) was found in a single deme (private forms) (figure 3). The large number of steps between haplotypes indicates that the *M. incanus* populations are genetically structured (figure 3 and table 1).

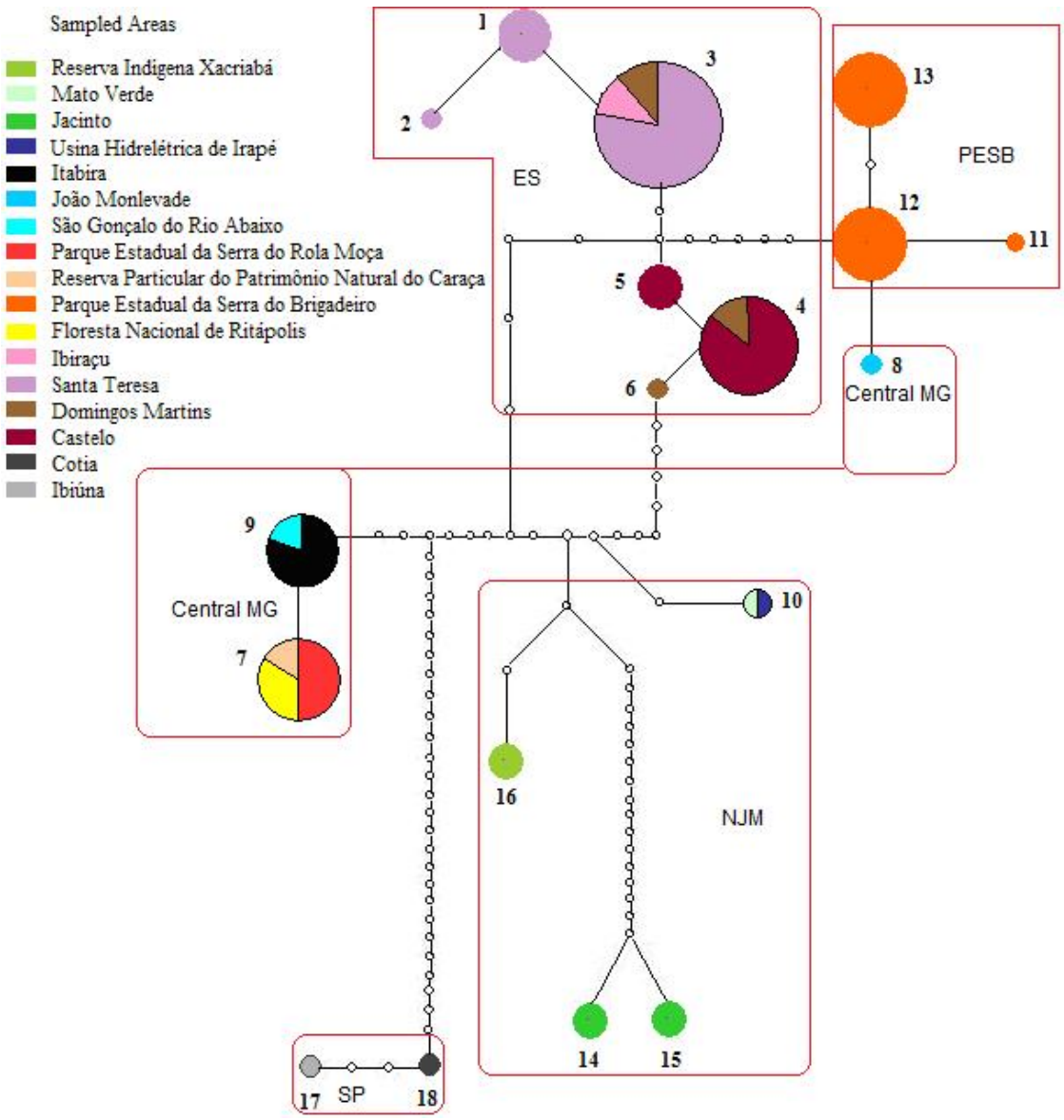

Figure 3. Haplotype network. Numbers identify 18 haplotypes, 13 of them being private to a single deme.

Table 1. Average percentage distance between haplotypes.

Comparing	NJM MG	Central	PESB	ES
NJM MG				
Central MG	4.1			
PESB	4.8	3.7		
ES	4.0	2.8	1.8	
SP	7.0	6.9	8.7	7.9

Mantel Test (Figure 4) and spatial autocorrelation analysis results exhibited a great correlation between genetic and geographical distances of 0.77. Monmonier Maximum difference

Algorithm detected a barrier separating SP samples from the others. Mismatch distribution, Fu's Fs test and Tajima's D test results were not significant.

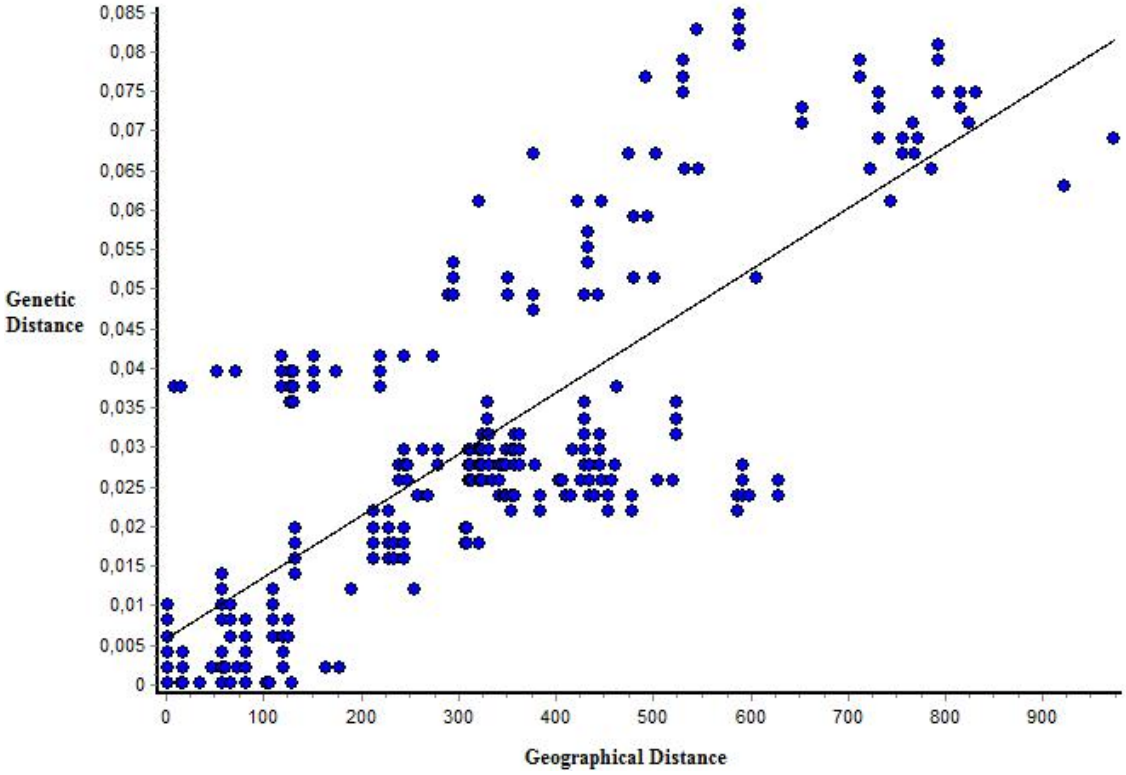

Figure 4. Mantel Test showing the great correlation between genetic and geographical distances.

For all population analysis we worked with Central, PESB and ES. As cited in "Materials and Methods", group exclusions (NJM and SP) were necessary to avoid statistical analysis bias. Values obtained with Tamura & Nei were near identical to those obtained with other distance method, such as Kimura 2 Parameters (K2P) and Jukes & Cantor. Large and highly significant F_{ST} values were obtained for pairwise populations comparisons (table 2) and, in AMOVA, the great part of variation (80.4%) was among proposed populations (table 3).

Table 2. Pairwise F_{ST} significant values.

Population	Pairwise F_{ST}	P-value
ES x PESB	0.77	0.00±0.00
PESB x MG Central	0.86	0.00±0.00
ES x MG Central	0.79	0.00±0.00

Table 3. AMOVA variation partitioning, with the Tamura & Nei distance method.

Source of variation	Percentage of variation
Among populations	80.4
Within populations	19.6
F_{ST}= 0.804 (p-value = 0.00±0.00)	

The GTR (General Time Reversible) was the most appropriate model of nucleotide substitution for the *M. incanus* analyzed data set, using the Akaike informative criterion. Phylogeographic analysis with Maximum Parsimony (figure 5), Neighbor Joining, Maximum Likelihood and Bayesian inference showed similar results, all of them revealing a strong phylogeographic pattern for *M. incanus*, with clear differentiation into distinct haplogroups.

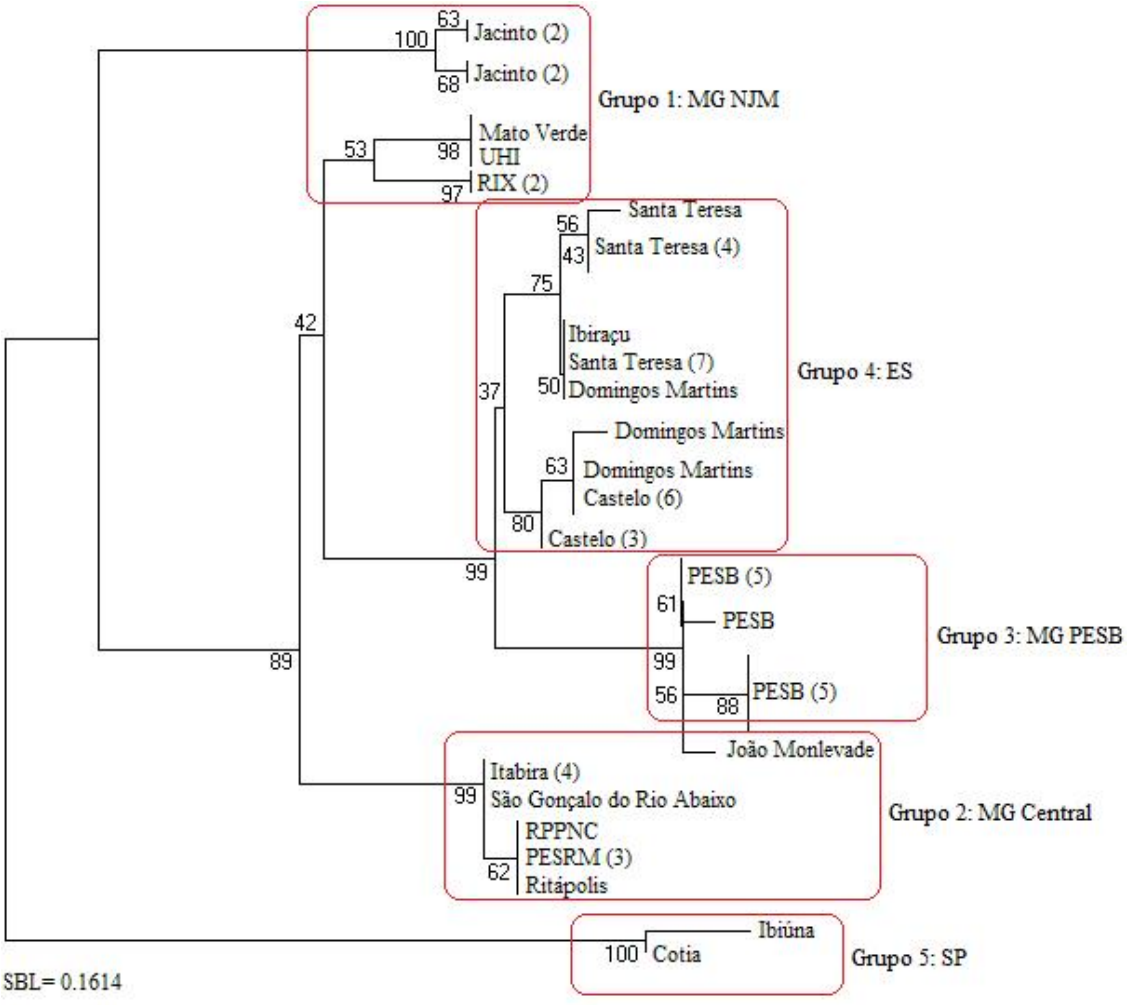

Figure 5. Maximum Parsimony tree. When more than one sample was represented, haplotype occurrence number for each deme is exhibited in parentheses.

DISCUSSION

A great haplotype diversity (Hd= 93%) and a nucleotide diversity of 2.62% characterized our data analysis and resulted from the high number of haplotypes, nucleotide substitutions (70) and polymorphic sites (64). Most substitutions were synonymous, which is expected as we are working with a conserved coding gene region.

We found 18 haplotypes from 17 sampled areas, 13 (72 %) were exclusive of a single deme, and the other five were shared by two or three different demes. The expressive presence of a majority of privative haplotypes, and the high genetic distance between some of these forms produced an interesting haplotype network, with a large number of steps between some studied haplotypes (figure 3). The João Monlevade haplotype didn't group with other Central MG region haplotypes, but with PESB haplotypes (see figure 3 and 5); in this isolated case, the haplogroup is not concordant with geographic clustering. Domingos Martins appears in the haplotype net as an intermediate area, geographically and molecularly connecting demes from the ES population.

The PESB population is geographically situated between the Central MG and ES populations; molecularly, the PESB population haplotypes were closer to ES haplotypes, indicating genetic proximity. Genetic mean distance analysis showed greater genetic distances in comparisons involving the SP group, which is partially explained by geographic distance effect, but requires geological and ecological studies to be better understood. The smallest interpopulation distance was exhibited between the PESB and ES populations (a result concordant with that observed by Yazbeck and colleagues [42], for Akodon cursor), and, surprisingly, we observed higher genetic distances between Central and PESB than between the Central MG and ES populations, what is unexpected because PESB is geographically located between the Central and ES populations.

Our results, specially the high levels of intraspecific genetic variation and high mean distance between haplotypes in intergroups analysis observed, strongly support the ancient fragmentation event inference. The COI region is molecularly conserved and, despite this, we observed high rates of genetic divergence between the studied demes. Nevertheless, the most recent mtDNA small mammals surveys used the cytochrome b gene, for which wide phylogenetic studies including substitution rates inferences are easily found, which do not occur with COI gene. As mtDNA substitution rates are extremely variable between mammalian species, varying up to two orders of magnitude, the famous "2% per site per million year" should not be generally trusted; as it has implications to molecular phylogenies, molecular dating and population genetics knowledge [31].

The species life history is important to explain our results. These are mostly influenced by events that happened before anthropogenic fragmentation, but now are governed by genetic drift and by insufficient gene flow, that allows the maintenance of high levels of genetic differentiation. As a result, we observed the large and highly significant F_{ST} values; the great amount of variation being among populations (> 80% in AMOVA); the elevated correlation between genetic and geographical distances; the haplotype network and the phylogeographic trees clearly showing molecular haplogroups mostly compatible to geographic clustering, which is confirmed by preliminary results obtained with the d-loop region of some of this populations.

We obtained a high correlation rate between geographic and genetic distances, translated into an observed distance effect between populations. In observed phylogenies (figure 5), geographical clustering was exactly compatible with genetic phylogroups except in two situations: firstly, the João Monlevade grouping with the PESB population instead of the Central population (as discussed above), and secondly, the Jacinto deme located apart from other NJM group localities. In the second case, the observed pattern seems understandable

because the distances between demes are very large and the group unity is thus unlikely (we did not consider the NJM group for population analysis). A strong phylogeographic pattern characterizes *M. incanus* space distribution.

Biological and ecological characteristics of *M. incanus*, like semelparity [29], semi-arboreal habits [7, 8] and its sensitivity to the landscape fragmentation [34] impact gene flow. Ecological features are important to species biology promoting or avoiding interpopulational unity. Gene flow has not been enough to erase the phylogeographic information. The strong genetic structure (resulting in high interpopulations F_{ST}) and the great variation among phylogroups characterized the *M. incanus* analyses, as observed for some other small mammals by Costa [6], and seem to be related to ancient processes that influenced the lineages geographic distribution, reflecting fragmentation events that predate human driven habitat destruction. It is notable that the very large genetic distance observed between haplotypes among the *M. incanus* SP group and the others makes imperative an important question: we are working with a single species or it can be a group of species? More than turn wider the studied area and the sample number, an integration between morphological and molecular data analysis is necessary to support a taxonomic discussion about this interesting neotropical marsupial.

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CONCLUSÕES GERAIS

A caracterização molecular da população de *D. albiventris* da Região Metropolitana de Belo Horizonte (BHMR), um fragmento urbano da Mata Atlântica Brasileira, produziu uma rede de haplótipos composta por três haplótipos COI intimamente ligados; a distribuição espacial dos haplótipos na área amostrada e sua ocorrência demonstram que se trata de uma unidade populacional. A simpatria entre *D. albiventris* e *D. aurita* foi confirmada para BHMR, o mesmo ocorrendo para Piracema, outro fragmento deste ameaçado bioma. A utilização de sequências COI para a realização de análises filogenéticas facultou a nítida separação entre as espécies, demonstrando a aplicabilidade da metodologia de código de barras de DNA (*DNA barcode*) na discriminação molecular entre espécies de gambás da Mata Atlantica brasileira.

Os elevados índices de estruturação genética (F_{ST}) observados entre populações de *D. albiventris* de localidades distintas são inesperados e incompatíveis com características ecológicas como ampla distribuição, hábitos generalistas, alta adaptabilidade, capacidade de percorrer longas distâncias (Gentile & Cerqueira 1995; Chiarello 2000; Pires *et al*. 2002) e elevada taxa de mobilidade entre fragmentos florestais (Pires *et al*. 2002), que potencialmente conduzem a uma expectativa de que as populações, ainda que geograficamente afastadas, possam estar geneticamente ligadas. Concluímos que uma outra característica de *D. albiventris* é fundamental para explicar a elevada diferenciação genética observada entre os demes estudados: a área de vida média muitas vezes maior para machos do que para fêmeas de gambás (Sunquist *et al*. 1987). A utilização de um marcador genético de herança materna, não nos permite argumentar sobre a história completa da diversidade genética em *D. albiventris*, nossos resultados informam sobre a história mutacional que corresponde às linhagens genealógicas maternas. A elevada diferenciação genética observada sugere que, no contexto mitocondrial, o fluxo gênico é insuficiente para a manutenção da unidade

populacional e para promover a aproximação genética entre os demes estudados. Assim, a rede de haplótipos e os altos valores de diferenciação sugerem fortemente que o fluxo gênico realizado pelas fêmeas não é suficiente para conectar e efetivamente aproximar as populações de *D. albiventris* estudadas. Apesar dos elevados valores de diferenciação interpopulacional, o padrão filogeográfico em *D. albiventris* é muito fraco, não permitindo a separação entre haplótipos de demes separados por distâncias de até 700 km. A separação em haplogrupos diferentes só foi possível quando escalas geográficas muito mais amplas foram consideradas, neste caso os *clusters* geográficos de MG e de RS, foram totalmente compatíveis com *clusters* genéticos, evidenciando um nítido padrão de distribuição geográfica das linhagens genealógicas para a espécie; mas apenas para análises em ampla escala, quando a comparação incluiu demes muito afastados (distâncias lineares maiores do que 1200 Km) e lacunas amostrais entre estes. Pesquisas adicionais com marcadores microssatélites e ou sequências nucleares podem completar nossas conclusões, propiciando um melhor entendimento da genética de populações da espécie.

Nossas conclusões sobre a variabilidade genética e a diferenciação interdemes para *M. incanus* foram completamente diferentes das expostas para *D. albiventris*. Características biológicas e ecológicas de *M. incanus*, como semelparidade (Lorini *et al*. 1994), hábitos semi-arbóreos (Emmons & Feer 1990; D'Elia 1999) e sensibilidade à fragmentação de habitats (Rocha *et al*. 2011) restringem a ocorrência de fluxo gênico e dificultam a aproximação genética entre as populações. Analisando populações de *M. incanus*, obtivemos uma elevada correlação entre as distâncias geográficas e genéticas. Os filogrupos genéticos apresentaram alta compatibilidade com o agrupamento geográfico. O forte padrão filogeográfico que caracteriza a distribuição espacial de *M. incanus* indica que o fluxo gênico não foi suficiente para apagar os vestígios da história das linhagens desta espécie. A forte estruturação genética e a grande variação entre filogrupos parecem estar relacionadas a eventos antigos de fragmentação, que influenciaram a distribuição geográfica das linhagens, e que antecedem a

destruição de habitat causada pela exploração humana. O contato com *D. albiventris* e com *M. incanus* torna evidente a demanda por pesquisas adicionais para ampliar a compreensão de sua interessante Biologia.

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Abordagens Iniciadas e Resultados Preliminares de Linhas de Pesquisa Relacionadas

DNA barcoding confirma o registro morfológico de Simpatria entre *M. incanus* e *M. paulensis* no Parque Estadual da Serra do Brigadeiro

Testamos molecularmente o registro morfológico de simpatria entre *M. incanus* e *M. paulensis* observado no Parque Estadual da Serra do Brigadeiro. Utilizando um fragmento de 541 pb do gene da COI foi possível constatar a nítida separação filogenética entre os 11 espécimes de *M. incanus* e o espécime único de *M. paulensis*, e a elevada divergência nucleotídica entre os dois grupos taxonômicos simpátricos (Figura 1). Para embasar a aplicabilidade da tecnologia de DNA barcoding na identificação destas espécies do gênero *Marmosops*, incluimos espécimes de *M. incanus* e de *M. paulensis* coletados em outras localidades. Utilizamos sequências COI de *M. incanus* coletados em outras 16 localidades do sudeste brasileiro (capítulo IV desta tese). Nos falta ainda produzir sequências de *M. paulensis* de outras localidades, para esta espécie contamos com dois espécimes coletados em São Paulo, e não há nenhuma sequência depositada no Genbank. A nossa perspectiva é reunir amostras de *M. paulensis* de outras cinco localidades, sequenciá-las e após análise filogenética referendar ou refutar a aplicabilidade da metodologia de DNA barcode para a identificação destas espécies. O trabalho final será submetido ao periódico *Molecular Ecology Resources*.

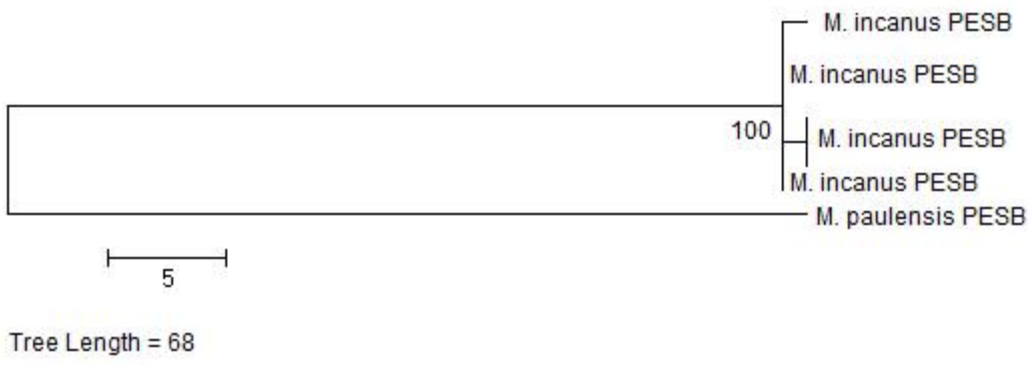

Figura 1. Nítida separação filogenética entre *M. incanus* e *M. paulensis*

Resultados preliminares obtidos em análises da Região Controle do mtDNA

Os primers E3 e Lo (Douzery & Randi 1997; Huchon *et al*. 1999) amplificam adequadamente a região controle (D-loop ou HVSI - região hipervariável) das amostras de *M. incanus*, facultando o acesso a sequências de alta qualidade com aproximadamente 400 pb. Em concordância com o observado para COI, observamos para a região controle do mtDNA nítido padrão filogeográfico caracterizando a distribuição geográfica das linhagens genealógicas de *M. incanus* (Figura 1). Esta abordagem será ampliada de modo a facultar a inferência dos tempos de divergência entre as linhagens, assim poderemos contrastar resultados obtidos com as duas regiões do mtDNA estudadas, o que confere maior credibilidade a análises que incluem a inferência embasada em relógios moleculares.

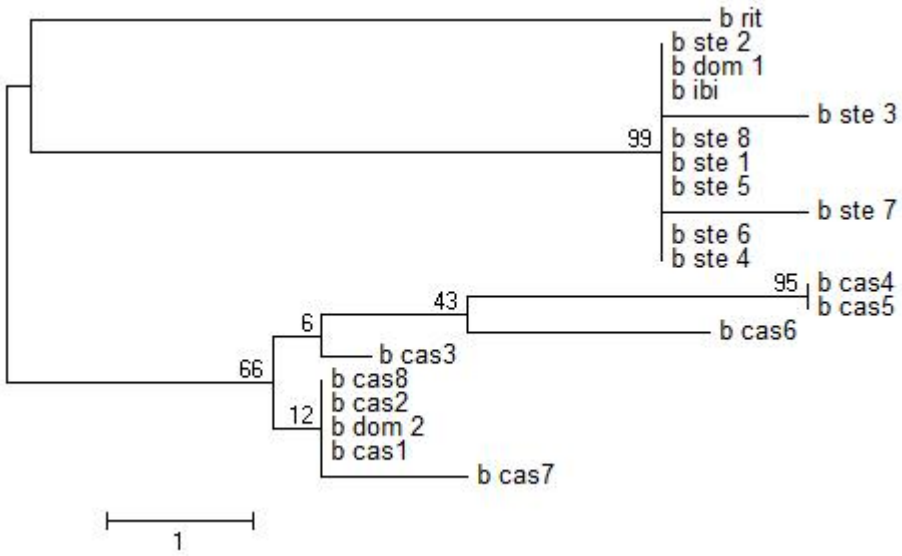

Figura 2. Padrão filogeográfico observado para *M. incanus* utilizando a região controle do mtDNA.

Seleção de primers para a amplificação de loci microssatélites, padronização das PCRs e constatação do caráter polimórfico dos marcadores selecionados

Sequências de mtDNA e *loci* nucleares microssatélites exibem características distintas e analisados em conjunto conferem maior qualidade e informação à pesquisa. A utilização de dois tipos de marcadores moleculares para acessar a variabilidade genética associada a *D. albiventris* torna-se ainda mais importante quando consideramos que os machos de *Didelphis* migram mais, resultando em uso diferenciado do espaço entre os sexos (Loretto & Vieira, 2005); os efeitos do fluxo gênico ocasionado pelo deslocamento dos machos na aproximação entre as populações é imperceptível pela análise de mtDNA, devido ao mecanismo de herança materna deste.

Os locos DM1, DM2, DM3, DM4, DM5 (Lavergne *et al*., 1999), DM7, DM8, DM9 (Guillemin *et al*., 2000), descritos para *Didelphis marsupialis*, PO2, PO3 e PO4 (Guillemin *et al*., 2000), descritos para *Philander opossum* foram testados quanto à aplicabilidade na amplificação de *loci* nucleares microsatélites. Observamos que nove dos onze pares de iniciadores testados apresentaram amplificação em *D. albiventris* e em *D. aurita*, e nenhum deles apresentou amplificação em *M. incanus*. Apenas PO3 e PO4 (ambos desenvolvidos para *P. opossum*) não apresentaram amplificação para o gênero *Didelphis*. A padronização das PCRs de nove *loci* para *D. albiventris* e para *D. aurita* foi concluída com sucesso.

Os produtos amplificados foram submetidos à eletroforese por 2 horas a 1000 V em gel de poliacrilamida desnaturante 6%, corado com prata, procedimento que permitiu a nítida constatação da presença de polimorfismos entre os indivíduos. No entanto, a genotipagem feita deste modo apresenta alguns vieses decorrentes da interpretação errônea, quando os alelos presentes no heterozigoto têm um número muito próximo de repetições do motivo (especialmente para motivos menores). Assim, optamos por realizar a genotipagem dos loci microssatélites em seqüenciador automático (procedimento ainda não iniciado), utilizando os

primers selecionados com cauda M13 marcada com fluorescência. Para agregar significância estatística às análises, estas serão realizadas preferencialmente com 30 indivíduos de cada localidade.

As PCRs geraram fragmentos amplificados de tamanho e intensidade desejados nas condições descritas: uma unidade de Taq DNA polimerase, 8 mM dNTPs, 5 pM primer, tampão IB 10x ou IVB 5x. Condições de ciclagem: Desnaturação inicial: 5 min a 94°C; 30 ciclos de (94°C por 1 min; temperatura de anelamento por 1 min; 72°C por 1 min); Extensão final: 72°C por 25 minutos. As condições de tamponamento (Tampão IB 10x e ou tampão IVB 5x, Phoneutria Biotecnologia) e de temperatura de anelamento ideais para cada *loci* encontram-se na tabela 1.

Tabela 1. Condições ideais: tamponamento e temperatura de anelamento. Tampões IB 10x e tampão IVB 5x, Phoneutria Biotecnologia.

Primer	IB10x	IVB5x	Amplificação
DM1	62,3 °C	-	Presente
DM2	58,6 °C	-	Presente
DM3	59,8 °C	-	Presente
DM4	51 °C	-	Presente
DM5	56,3 °C	56,3 °C	Presente
DM7	-	52,3 °C	Presente
DM8	57,4 °C	58,6 °C	Presente
DM9	56,3 °C	56,3 °C	Presente
PO2	55 °C	55 °C	Presente
PO3	-	-	Ausente
PO4	-	-	Ausente

ANEXOS

ANEXO I: Autorização para atividades com finalidade científica - IBAMA/ICMBIO/SISBIO, e renovação desta (nº 20170-1).

Ministério do Meio Ambiente - MMA
Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 20170-1	Data da Emissão: 03/07/2009 17:28	Data de Validade: 03/07/2010

Dados do titular

Registro no Ibama: 1991274	Nome: Cleusa Graça da Fonseca	CPF: 129.237.296-68
Título do Projeto: Variabilidade Genética, Estrutura Populacional, Filogeografia e Análise de Caracteres Quantitativos dos gêneros Didelphis e Marmosops – Confrontando Subpopulações de Regiões Silvestres e Urbanas do Sudeste do Brasil		
Nome da Instituição : UFMG - UNIVERSIDADE FEDERAL DE MINAS GERAIS		CNPJ: 17.217.985/0001-04

Observações, ressalvas e condicionantes

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passa da, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização não exime o titular e a sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade.
3	Esta autorização não poderá ser utilizada para fins comerciais, industriais, esportivos ou para realização de atividades inerentes ao processo de licenciamento ambiental de empreendimentos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES). Em caso de material consignado, consulte www.ibama.gov.br/sisbio - menu Exportação.
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico.
7	Em caso de pesquisa em Unidade de Conservação Federal, o pesquisador titular deverá contactar a administração dessa unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.
8	As atividades contempladas nesta autorização NÃO abrangem espécies brasileiras constante de listas oficiais (de abrangência nacional, estadual ou municipal) de espécies ameaçadas de extinção, sobreexplotadas ou ameaçadas de sobreexplotação.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Luciene Cássia Corrêa de Sousa	Aluna de doutorado envolvida em todas as etapas do trabalho	039.014.266-20	MG 8 863 879 SSP MG-MG	Brasileira

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 20170-2	Data da Emissão: 14/02/2011 15:35

Dados do titular

Nome: Cleusa Graça da Fonseca	CPF: 129.237.296-68
Título do Projeto: PEDIDO DE RENOVAÇÃO DE LICENÇA PARA FINS CIENTÍFICOS (Para continuidade de Pós-Graduação em Genética Nível Doutorado) Projeto Variabilidade Genética, Estrutura Populacional, Filogeografia e Análise de Caracteres Quantitativos dos gêneros Didelphis e Marmosops	
Nome da Instituição : UFMG - UNIVERSIDADE FEDERAL DE MINAS GERAIS	CNPJ: 17.217.985/0001-04

ANEXO II: Números de acesso no Genbank

Tabela 1. Correspondência entre os números de acesso no Genbank e dados de 161 amostras seqüenciadas (93 *D. albiventris*, 10 *D. aurita*, 58 *M. incanus*)

Número de Acesso	ID	Espécie	UF	Local de Coleta	Origem
JN638891	BH01	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638892	BH02	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638893	BH03	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638894	BH04	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638895	BH05	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638896	BH06	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638897	BH07	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638898	BH08	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638899	BH09	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638900	BH10	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638901	BH11	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638902	BH12	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638903	BH13	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638904	BH14	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638905	BH15	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638906	BH16	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638907	BH17	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638908	BH18	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638909	BH19	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638910	BH20	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638911	BH21	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638912	BH22	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638913	BH23	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638914	BH24	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638915	BH25	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638916	BH26	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638917	BH27	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638918	BH28	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638919	BH29	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638920	BH30	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638921	BH31	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638922	BH32	*D. albiventris*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638923	Div01	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638924	Div02	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638925	Div03	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638926	Div04	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638927	Div05	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638928	Div06	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638929	Div07	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638930	Div08	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638931	Div09	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638932	Div10	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638933	Div11	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638934	Div12	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638935	Div13	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638936	Div14	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638937	Div15	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638938	Div16	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638939	Div17	*D. albiventris*	MG	Divinópolis	CPqRR LALEI

JN638940	Div18	*D. albiventris*	MG	Divinópolis	CPqRR LALEI
JN638941	RIX01	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638942	RIX02	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638943	RIX03	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638944	RIX04	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638945	RIX05	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638946	RIX06	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638947	RIX07	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638948	RIX08	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638949	RIX09	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638950	RIX10	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638951	RIX11	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638952	RIX12	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638953	RIX13	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638954	RIX14	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638955	RIX15	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638956	RIX16	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638957	RIX17	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638958	RIX18	*D. albiventris*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JN638959	Bam01	*D. albiventris*	MG	Bambuí	CPqRR LTEDC
JN638960	Bam02	*D. albiventris*	MG	Bambuí	CPqRR LTEDC
JN638961	Bam03	*D. albiventris*	MG	Bambuí	CPqRR LTEDC
JN638962	Pir01	*D. albiventris*	MG	Piracema	CPqRR LTEDC
JN638963	Pir02	*D. albiventris*	MG	Piracema	CPqRR LTEDC
JN638964	Pir03	*D. albiventris*	MG	Piracema	CPqRR LTEDC
JN638965	RS01	*D. albiventris*	RS	Rio Grande do Sul	PUC RS
JN638966	RS02	*D. albiventris*	RS	Rio Grande do Sul	PUC RS
JN638967	RS03	*D. albiventris*	RS	Rio Grande do Sul	PUC RS
JN638968	RS04	*D. albiventris*	RS	Porto Alegre	FZB RS
JN638969	RS05	*D. albiventris*	RS	Porto Alegre	FZB RS
JN638970	RS06	*D. albiventris*	RS	Machadinho	FZB RS
JN638971	RS07	*D. albiventris*	RS	Caxias do Sul	FZB RS
JN638972	RS08	*D. albiventris*	RS	Triunfo	FZB RS
JN638973	RS09	*D. albiventris*	RS	Triunfo	FZB RS
JN638974	Alm01	*D. albiventris*	MG	Almenara	MCN PUC Minas
JN638975	Alm02	*D. albiventris*	MG	Almenara	MCN PUC Minas
JN638976	BH33	*D. albiventris*	MG	BHMR Coração Eucarístico	MCN PUC Minas
JN638977	BH34	*D. albiventris*	MG	BHMR Coração Eucarístico	MCN PUC Minas
JN638978	BH35	*D. albiventris*	MG	BHMR Coração Eucarístico	MCN PUC Minas
JN638979	BH36	*D. albiventris*	MG	BHMR Jardim Canadá	Road killed animal
JN638980	BH37	*D. albiventris*	MG	BHMR Instituto Agronômico	Road killed animal
JN638981	BH38	*D. albiventris*	MG	BHMR Mangabeiras	Road killed animal
JN638982	BH39	*D. albiventris*	MG	BHMR Instituto Agronômico	Road killed animal
JN638983	BH40	*D. albiventris*	MG	BHMR Jardim Canadá	Road killed animal
JN638984	BH41	*D. aurita*	MG	BHMR Capitão Eduardo	CPqRR LALEI
JN638985	Gov01	*D. aurita*	MG	Serra do Ibituruna	CPqRR
JN638986	PirD	*D. aurita*	MG	Piracema	CPqRR LTEDC
JN638987	Vic01	*D. aurita*	MG	Viçosa	UFV MZJM
JN638988	CBa01	*D. aurita*	ES	Conceição da Barra	MBML
JN638989	Mac01	*D. aurita*	RJ	Macaé	UFRJ Campus Macaé
JN638990	Cot01	*D. aurita*	SP	Cotia	USP
JN638991	Rib01	*D. aurita*	SP	Ribeirão Grande	USP
JQ738373	PirE	*D. aurita*	MG	Piracema	CPqRR LTEDC
JQ738374	PirF	*D. aurita*	MG	Piracema	CPqRR LTEDC
JQ030920	Ste1	*M. incanus*	ES	Santa Teresa	MBML
JQ030921	Ste2	*M. incanus*	ES	Santa Teresa	MBML

JQ030922	Ste3	*M. incanus*	ES	Santa Teresa	MBML
JQ030923	Ste4	*M. incanus*	ES	Santa Teresa	MBML
JQ030924	Ste5	*M. incanus*	ES	Santa Teresa	MBML
JQ030925	Ste6	*M. incanus*	ES	Santa Teresa	MBML
JQ030926	Ste7	*M. incanus*	ES	Santa Teresa	MBML
JQ030927	Ste8	*M. incanus*	ES	Santa Teresa	MBML
JQ030928	Ste9	*M. incanus*	ES	Santa Teresa	MBML
JQ030929	Ste10	*M. incanus*	ES	Santa Teresa	MBML
JQ030930	Ste11	*M. incanus*	ES	Santa Teresa	MBML
JQ030931	Ste12	*M. incanus*	ES	Santa Teresa	MBML
JQ030932	Cas1	*M. incanus*	ES	Castelo	MBML
JQ030933	Cas2	*M. incanus*	ES	Castelo	MBML
JQ030934	Cas3	*M. incanus*	ES	Castelo	MBML
JQ030935	Cas4	*M. incanus*	ES	Castelo	MBML
JQ030936	Cas5	*M. incanus*	ES	Castelo	MBML
JQ030937	Cas6	*M. incanus*	ES	Castelo	MBML
JQ030938	Cas7	*M. incanus*	ES	Castelo	MBML
JQ030939	Cas8	*M. incanus*	ES	Castelo	MBML
JQ030940	Cas9	*M. incanus*	ES	Castelo	MBML
JQ030941	Dom1	*M. incanus*	ES	Domingos Martins	MBML
JQ030942	Dom2	*M. incanus*	ES	Domingos Martins	MBML
JQ030943	Dom3	*M. incanus*	ES	Domingos Martins	UFRJ Campus Macaé
JQ030944	Ibi	*M. incanus*	ES	Ibiraçu	MBML
JQ030945	FNR1	*M. incanus*	MG	Floresta Nacional de Ritápolis	MBML
JQ030946	FNR2	*M. incanus*	MG	Floresta Nacional de Ritápolis	MBML
JQ030947	JM	*M. incanus*	MG	João Monlevade	MCN PUC Minas
JQ030948	Ita1	*M. incanus*	MG	Itabira	MCN PUC Minas
JQ030949	Ita2	*M. incanus*	MG	Itabira	MCN PUC Minas
JQ030950	Ita3	*M. incanus*	MG	Itabira	MCN PUC Minas
JQ030951	Ita4	*M. incanus*	MG	Itabira	MCN PUC Minas
JQ030952	PESRM1	*M. incanus*	MG	P. E. da Serra do Rola Moça	MCN PUC Minas
JQ030953	Mat	*M. incanus*	MG	Mato Verde	MCN PUC Minas
JQ030954	PESB1	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFRJ Campus Macaé
JQ030955	PESB2	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFRJ Campus Macaé
JQ030956	PESB3	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFRJ Campus Macaé
JQ030957	PESB4	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFRJ Campus Macaé
JQ030958	PESB5	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFRJ Campus Macaé
JQ030959	PESB6	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFRJ Campus Macaé
JQ030960	PESB7	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFRJ Campus Macaé
JQ030961	PESB8	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFRJ Campus Macaé
JQ030962	PESB9	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFV MZJM
JQ030963	PESB10	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFV MZJM
JQ030964	PESB11	*M. incanus*	MG	P. E. da Serra do Brigadeiro	UFV MZJM
JQ030965	RPPNC	*M. incanus*	MG	R.P.P.N. do Caraça	MCN PUC Minas
JQ030966	UHI	*M. incanus*	MG	Usina Hidrelétrica de Irapé	MCN PUC Minas
JQ030967	Jac1	*M. incanus*	MG	Jacinto	MCN PUC Minas
JQ030968	Jac2	*M. incanus*	MG	Jacinto	MCN PUC Minas
JQ030969	Jac3	*M. incanus*	MG	Jacinto	MCN PUC Minas
JQ030970	Jac4	*M. incanus*	MG	Jacinto	MCN PUC Minas
JQ030971	PESRM2	*M. incanus*	MG	P. E. da Serra do Rola Moça	MCN PUC Minas
JQ030972	PESRM3	*M. incanus*	MG	P. E. da Serra do Rola Moça	MCN PUC Minas
JQ030973	RIX1	*M. incanus*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JQ030974	RIX2	*M. incanus*	MG	Reserva Indígena Xacriabá	CPqRR LALEI
JQ030975	Sgon	*M. incanus*	MG	São Gonçalo do Rio Abaixo	MCN PUC Minas
JQ030976	SPIb	*M. incanus*	SP	Ibiúna	USP
JQ030977	Cot	*M. incanus*	SP	Cotia	USP

VERSÕES NOS FORMATOS PUBLICADOS DOS ARTIGOS DESCRITOS NOS CAPÍTULOS I E III