Mastozoología Neotropical, 28(1), Mendoza, 2021
Versión on-line ISSN 1666-0536
https://doi.org/10.31687/saremMN.21.28.1.0.20

Sección Especial
EL ÚLTIMO NATURALISTA TIPÓLOGO:
CONTRIBUCIONES EN HONOR A ELIO MASSOIA (1936-2001)
Editores: Ulyses F. J. Pardiñas y Carlos Galliari
Artículo

GENETIC DIVERSITY OF THE WHITE-EARED OPOSSUM *Didelphis albiventris* (DIDELPHIMORPHIA: DIDELPHIDAE) IN ARGENTINA

M. Amelia Chemisquy[1], Facundo M. Morinigo[2], Alberto Fameli[3], and Raúl E. González-Ittig[4,5]

[1]Museo de Ciencias Antropológicas y Naturales, Universidad Nacional de La Rioja, Av. Luis M. de la Fuente S/N, Ciudad Universitaria de la Ciencia y de la Técnica, F5300, La Rioja, Argentina.
[Correspondence: M. Amelia Chemisquy <amelych80@gmail.com>]
[2]Departamento Científico Antropología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina.
[3]Grupo de Genética y Ecología en Conservación y Biodiversidad, División Mastozoología, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Ciudad Autónoma de Buenos Aires, Argentina.
[4]Instituto de Diversidad y Ecología Animal (IDEA), CONICET-Universidad Nacional de Córdoba, Córdoba, Argentina.
[5]Cátedra de Genética de Poblaciones y Evolución, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Córdoba, Argentina.

ABSTRACT. The white-eared opossum *Didelphis albiventris* Lund, 1840, is one the largest and most common species of marsupial in Argentina, distributed from the north of the country up to Neuquén and Río Negro provinces in the south. The species is present in contrasting environments, such as the Monte (desert) and the Parana Forest (rainforest), and is also adapted to live in large cities and agricultural fields. Although there are some phylogeographic analyses of Brazilian populations of *D. albiventris*, showing little variation and some geographic structure, up to now none of them included samples from Argentina. The aim of this contribution is to analyze the genetic structure of the species, using two mitochondrial markers (cytochrome b and D-loop) on a wide geographic coverage in Argentina (> 10° S). Results showed little genetic variation and low haplotype diversity, with lesser values than those reported for Brazilian populations. This small variation could be due to a sudden expansion, which is supported by field observations of the species expanding to the south. Unfortunately, the low genetic variability resulted in low statistical power to give conclusive results in the mismatch analysis or the Bayesian skyline plot. Another possibility is high levels of gene flow, which is consistent flow, which is consistent with the low correlation beween genetic and geographic distances detected in the Mantel test (althogh statistically signicant) and the wide home range that the species has. A different approach, using a different set of markers, is needed in order to analyze the phylogeographic history of *D. albiventris*.

RESUMEN. Diversidad genética de la comadreja overa *Didelphis albiventris* (Didelphimorphia: Didelphidae) en Argentina. La comadreja overa *Didelphis albiventris* Lund, 1840, es una de las especies de marsupiales más grandes y comunes en Argentina y se distribuye desde el norte del país hasta las provincias del Neuquén y Río Negro en el sur. La especie está presente en ambientes dispares, como el Monte (desierto) y la Selva Paranaense (bosque lluvioso) y también está adaptada a vivir en grandes ciudades y regiones agrícolas. Aunque existen algunos análisis filogeográficos de poblaciones brasileñas de *D. albiventris* que

Recibido: 24 septiembre 2020. Aceptado: 19 enero 2021. Editor asociado: U. F. J. Pardiñas & C. A. Galliari.

muestran poca variación y cierta estructura geográfica, hasta el momento ninguno de ellos incluyó muestras provenientes de Argentina. El objetivo de esta contribución es analizar la estructura genética de la especie utilizando dos marcadores mitocondriales (citocromo b y D-loop) y una amplia muestra geográfica proveniente de Argentina (> 10°S). Los resultados mostraron poca variación genética y baja diversidad de haplotipos, con valores inferiores a los descriptos para las poblaciones brasileñas. Esta escasa variabilidad podría deberse a una expansión reciente, hipótesis respaldada por observaciones de campo de la especie expandiéndose hacia el sur. Sin embargo, la baja variabilidad encontrada resultó en una falta de poder estadístico para dar resultados concluyentes en el análisis de Mismatch o en el Bayesian skyline plot. Otra posibilidad serían altos niveles de flujo génico, algo consistente con la baja correlación (aunque estadísticamente significativa) entre distancias genéticas y geográficas en la prueba de Mantel y el amplio rango de acción que tiene la especie. Se necesita un enfoque diferente, utilizando otros marcadores más variables, para analizar la historia filogeográfica de *D. albiventris*.

Palabras clave: estructura genética poblacional, filogeografía, marcadores mitocondriales, marsupiales, variabilidad genética.
Key words: genetic variability, marsupials, mitochondrial markers, phylogeography, population genetic structure

INTRODUCTION

The white-eared opossum, *Didelphis albiventris* Lund, 1840, is one of the largest species of South American opossums and inhabits southern Bolivia, Paraguay, Uruguay, northeastern, central and southern Brazil, and Argentina southward to Río Negro and Neuquén provinces (Cerqueira & Tribe 2008; Carrera & Udrizar Sauthier 2014; Astúa 2015; Bianchini 2018). Due to this large distribution, the species is adapted to a wide range of environments, from gallery forests and wet areas such as the Paranaense Forest to the dry Monte Desert (Astúa 2015). Also, the species is highly adapted to urban environments, including large cities, as well as other human-modified environments such as croplands (Smith 2007; Costa et al. 2015).

Regarding its diet, *D. albiventris* is a generalist species, feeding on arthropods, vertebrates, fruits, and other plant items (Vieira & Astúa 2003). It plays a role in seed dispersal in several environments, and consequently, it may be an important part in the recovery of degraded areas through the dispersal of seeds of pioneer plants (e.g., Cantor et al. 2010; Rodrigues Da Silva et al. 2014). The white-eared opossum is also a generalist regarding the use of the vertical strata, being found both on the canopy and on the ground (Vieira & Camargo 2012), and it occupies a large home range of 2.33±2.32 ha on average (Sanches et al. 2012).

Despite being such a common species, little is known about its genetic variability and the phylogeographic structure of its populations. For Brazil, there are some analyses using cytochrome oxidase I (COI), including samples from Rio Grande do Sul, Minas Gerais, and the northeast of the country (Paraíba, Sergipe, Pernambuco, Ceará, Piauí, and Maranhão), which found two separated groups, one including samples from Rio Grande do Sul, and the other for the remaining localities (Sousa et al. 2012; Nascimento et al. 2019). Both groups, however, showed little variation, and only the one from the northeast revealed some phylogeographic structure (Nascimento et al. 2019).

In another study, Rocha et al. (2015) analyzed, using cytochrome b, samples from Brazil and Bolivia, although their sampling was much smaller than the ones from Sousa et al. (2012) and Nascimento et al. (2019). For Brazil, they included samples from Minas Gerais, Mato Grosso, Tocantins, Pará, and Paraná, and for Bolivia, samples from Chuquisaca and Tarija. Again, two groups were found, one including specimens from Paraná and Bolivia, and other comprising samples from central Brazil. Although the analyses showed some genetic structuring, which separated a group from the Cerrado from another group encompassing samples from the ecotone Amazonia-Cerrado, there were some haplotypes from one region that were more closely related to those of the other region (Rocha et al. 2015).

To date, there are no published phylogeographic analyses of *D. albiventris* based on populations from Argentina. So the aim of this contribution is to analyze the genetic structure of the species in this country, using two mitochondrial markers (cytochrome b and D-loop) and a wide geographic sample.

MATERIALS AND METHODS

Samples and molecular methods

We analyzed sequences of 32 specimens of *D. albiventris* from most of its distribution range in Argentina (Misiones, Buenos Aires, Córdoba, La Rioja, Santa Fe, Entre Ríos, Salta, Chaco, and Tucumán; **Table** 1; **Fig**. 1). Tissues were collected from road-killed animals or obtained from specimens housed at scientific collections (**Table** 1).

Total DNA was extracted using an SDS-proteinase K-NaCl protocol (modified from Miller et al. 1988). We amplified two mitochondrial markers: a fragment of cytochrome b (cyt b from here on), using primers CYTB-F1-Didelphidae, CYTB-R1-Didelphidae, PF, and RF (Giarla et al. 2010; Chemisquy & Flores 2012), and D-loop using primers L0 and E3 (Huchon et al. 1999). Polymerase chain reactions (PCR) were performed in a final volume of 15 μl. Each reaction contained between 50 and 100ng of DNA, 1.5 units of Taq polymerase, 1x PCR Buffer, 5 mM MgCl2, 0.2 μM of each primer, and 0.025 mM dNTP each. PCR amplifications were carried out as follows: a first denaturation period at 94° C for 5 min, followed by 35 cycles of denaturation at 94° C for 45 s, annealing at 50-56° C for 1 min, and extension at 72° C for 1 min. A final extension at 72° C for 6 min terminated the reactions. A negative control with no template was included for each series of amplifications to test for contamination. PCR products were electrophoresed on a 1% TBE agarose gel stained with ethidium bromide. Sequencing was performed by MACROGEN (Korea). All sequences were deposited in GenBank (see accession numbers on **Table** 1).

Data analyses

Sequences were edited and hand-aligned (since the alignments were trivial and showed no gaps or insertions) using BioEdit (Hall 1999). Matrices were combined using SequenceMatrix 1.8 (Vaidya et al. 2011). Some analyses were performed on each marker separately, while others were conducted only for the combined matrix. For the cyt b matrix, we included sequences from GenBank of samples from Brazil, Bolivia, and Paraguay (**Table** 1). We could not do the same for the D-loop since there are no sequences of that marker available for *D. albiventris*.

We analyzed four different matrices: 1) D-loop only (30 individuals); 2) cyt b only (27 individuals); 3) the combined matrix (25 individuals that have both markers). In this latter matrix individuals that only have one genetic marker were discarded and consequently, the dataset does not have missing data; this matrix was used in most of the analyses; 4) the full matrix (38 individuals), consisting of all individuals having both markers and those having only cyt b or only D-loop; therefore, it has a high percentage of missing data; this matrix was only used for the phylogenetic analyses.

The number of haplotypes (h), the number of polymorphic sites (S), haplotype (Hd), and nucleotide (π) diversity values were estimated using DnaSP 6.12.03 (Rozas et al. 2017). Pairwise distances among haplotypes were calculated using the Kimura 2-parameter implemented in Mega X (Kumar et al. 2018). In order to check if there is a correlation between genetic distances and geographic distances, we performed a Mantel test in R, using the packages vegan, Ape, and geosphere (Paradis et al. 2004; Oksanen et al. 2019; Hijmans 2019).

Deviation from neutrality was checked using the indexes D (Tajima 1989) and Fs (Fu 1997) calculated in Arlequin 3.5 (Excoffier & Lischer 2010). We also performed the R_2 test, which has a better performance in small data sets (Ramos-Onsins & Rozas 2002) using the package pegas for R (Paradis 2010). Historical demography was analyzed by performing a mismatch distribution analysis, which tests for recent range expansion, using Arlequin 3.5 (Excoffier & Lischer 2010). We also conducted a Bayesian skyline plot (BSP) reconstruction in BEAST 1.10.4 (Suchard et al. 2018). Coalescent reconstructions used a lognormal relaxed clock with a substitution rate of 0.017 per site per million years (Rocha et al. 2015), the HKY substitution model as determined by jModeltest 2 (Darriba et al. 2012), and a TMRCA (time to the most recent common ancestor of all the taxa) of 0.76 Myr BP (taken from Rocha et al. 2015). Analyses were run for 10 million MCMC steps, discarding the first 10% as "burn-in." Results were checked for convergence to a stationary distribution using Tracer 1.7 (Rambaut et al. 2018). Mismatch distribution and BSP were performed on the combined matrix only.

To analyze patterns of geographical distribution and haplotype relationships, we performed a Median-Joining network implemented in PopART 1.7 (Leigh & Bryant 2015) using the combined matrix. We also built a network using the cyt b matrix in order to analyze the placement of sequences from Bolivia, Brazil and Paraguay. We tried to run a network analysis using the full matrix (i.e., including sequences from GenBank), but due to the large amount of missing data many sequences could not be placed correctly and results were inconclusive (data not shown). For the biogeographic grouping of the samples we followed the biogeographic provinces delimited by Cabrera & Willink (1980).

To estimate the number of genetic clusters and their geographic distribution on the combined matrix, we used the software Geneland 3.0 (Guillot et al. 2005), testing up to k=6, running 20 million MCMC steps and 2,000 thinnings. Final results were checked by performing three independent runs with a fixed k to avoid ghost populations.

We also performed a maximum likelihood phylogenetic tree using the estimated haplotypes for each matrix. Sequences of *D. marsupialis*, downloaded from GenBank, were used as outgroups (accession numbers KT447521 and JQ478421). Phylogenetic analyses were performed on the cyt b matrix, the D-loop matrix and the full matrix. Analyses were run on raxmlGUI 2.0 (Edler et al. 2021) using the HKY substitution model, and 10,000 bootstrap replicates.

RESULTS

For the D-loop, we successfully amplified and sequenced 460 bp from 30 individuals of *D. albiventris*, obtaining 11 variable sites that defined 11 haplotypes (Hd=0.75 [sd=0.089]; π=0.0041 [sd=0.0007]). The most common haplotype, H1, was present in 15 individuals; H5 was present in three individuals, and the remaining haplotypes were found in one or two individuals (**Table** 1). The overall mean distance among sequences of D-loop was 0.39%, with pairwise

Table 1

Analyzed specimens of *Didelphis albiventris* and their haplotypes and their respective GenBank accession numbers of the cyt b and D-loop sequences. Asterisks indicate haplotypes only present in the full matrix (38 individuals), but not in the combined matrix (25 individuals), corresponding to individuals with a single molecular marker. CDS: combined dataset.

Seq. #	Sequence code / voucher #	Locality	Coordinates	Haplotypes			GenBank code	
				cyt b	D-loop	CDS	cyt b	D-loop
1	DA 1/ CRILAR-Ma-CT 166	San Nicolás de los Arroyos, Buenos Aires, Argentina	33.40° S; 60.19° W	H1	-	H15*	MW392620	
2	DA 2/ MACN-CT-487	Chascomús, Buenos Aires, Argentina	35.58° S; 58.01° W	H2	H1	H1	MW392621	MW392590
3	DA 3/ CRILAR-Ma-CT 163	Lezama, Buenos Aires, Argentina	35.96° S; 57.85° W	H2	H1	H1	MW392622	MW392591
4	DA 4/ MACN-CT-319	Mar de Cobos, Buenos Aires, Argentina	37.82° S; 57.72° W	H2	H1	H1	MW392623	MW392592
5	DA 5/ MACN-CT-428	Mar Chiquita Buenos Aires, Argentina	37.74° S; 57.43° W	H2	H1	H1	MW392624	MW392593
6	DA 6/ MACN-CT-461	Maipú Buenos Aires, Argentina	36.86° S; 57.88° W	H2	H1	H1	MW392625	MW392594
7	DA 7/ MACN-CT-064	Puerto Canoas, Misiones, Argentina	25.72° S; 54.40° W	H3	H2	H2	MW392626	MW392595
8	DA 8/ MACN-CT-277	Llavallol, Buenos Aires, Argentina	34.80° S; 58.43° W	H2	H1	H1	MW392627	MW392596
9	DA 11/ CRILAR-Ma-CT 164	Comodoro Py, Buenos Aires, Argentina	35.33° S; 60.52° W	H2	H1	H1	MW392628	MW392597
10	DA 12/ CRILAR-Ma-CT 165	San Carlos Sur, Santa Fe, Argentina	31.80° S; 61.10° W	H2	H1	H1	MW392629	MW392598
11	DA 14/ CRILAR-Ma-CT 5	Las Breñas, Chaco, Argentina	27.37° S; 59.29° W	H4	H3	H3	MW392630	MW392599
12	DA 15/ CRILAR-Ma-CT 15	La Para, Córdoba, Argentina	30.89° S; 63.05° W	-	H1	H12*		MW392600
13	DA 16/ CRILAR-Ma-CT 22	Anillaco, La Rioja, Argentina	28.81° S; 66.94° W	-	H4	H13*		MW392601
14	DA 17/ CRILAR-Ma-CT 28	Anillaco, La Rioja, Argentina	28.81° S; 66.94° W	H2	H4	H4	MW392631	MW392602
15	DA 18/ CRILAR-Ma-CT 12	Juan Bautista Alberdi, Tucumán, Argentina	27.56° S; 65.62° W	H5	H5	H5	MW392632	MW392603
16	DA 19/ CRILAR-Ma-CT 81	Cuatro Bocas, Entre Ríos, Argentina	33.11° S; 60.32° W	H2	H6	H6	MW392633	MW392604
17	DA 20/ CRILAR-Ma-CT 10	Agua Blanca, La Rioja, Argentina	28.96 °S; 66.99° W	-	H1	H12*		MW392605
18	DA 21/ CRILAR-Ma-CT16	Anillaco, La Rioja, Argentina	28.81° S; 66.94° W	H2	H7	H7	MW392634	MW392606
19	DA 22/ CRILAR-Ma-CT13	Rafael García, Córdoba, Argentina	31.67° S; 64.28° W	H5	H5	H5	MW392635	MW392607
20	DA 23/ MG-ZV-M-187	Rosario, Santa Fe, Argentina	32.94° S; 60.65° W	H2	H1	H1	MW392636	MW392608
21	DA 25/ ZV-M-H 16	Arroyo Leyes, Santa Fe, Argentina	31.50° S; 60.49° W	-	H1	H12*		MW392609
22	DA 26/ ZV-M-H 25	Sauce Viejo, Santa Fe, Argentina	31.77° S; 60.84° W	H2	H1	H1	MW392637	MW392610
23	DA 27/ ZV-M.H 26	Sauce Viejo, Santa Fe, Argentina	31.77° S; 60.83° W	H2	H8	H8	MW392638	MW392611
24	DA 29/ CRILAR-Ma-CT20	Córdoba, Córdoba, Argentina	31.50° S; 64.13° W	H2	H9	H1	MW392639	MW392612
25	DA 30/ CRILAR-Ma-CT26	San Pedro, Buenos Aires, Argentina	33.66° S; 59.70° W	H2	H1	H1	MW392640	MW392613
26	DA 34/ CRILAR-Ma-CT74	Anzulón, La Rioja, Argentina	28.80° S; 66.95° W	H2	-	H17*	MW392641	
27	DA 35/ CRILAR-Ma-CT 82	San Antonio, La Rioja, Argentina	31.08° S; 66.74° W	-	H10	H11*		MW392614
28	DA a5/ MACN-CT 194	San Isidro, Buenos Aires, Argentina	34.47° S; 58.51° W	H2	H1	H1	MW392642	MW392615
29	DA a6/ MACN-CT 65	Puerto Canoas, Misiones, Argentina	25.71° S; 54.40° W	H3	H2	H2	MW392643	MW392616
30	DA 464/ LIEB 464	Salta, Salta, Argentina	24.79° S; 65.38° W	H5	H11	H9	MW392644	MW392617
31	DA 493/ LIEB 493	Salta, Salta, Argentina	24.83° S; 65.46° W	H5	H11	H9	MW392645	MW392618
32	DA YB/ CRILAR-Ma-CT 45	Yerba Buena, Tucumán, Argentina	26.82° S; 65.32° W	H5	H5	H5	MW392646	MW392619
33	GB 1	Foz do Iguaçu, Paraná, Brazil	25.42° S; 54.53° W	H6	-	H16*	JF280991	
34	GB 2	Presidente Hayes, Paraguay	23.35° S; 58.74° W	H4	-	H10*	MG491973	
35	GB 3	Chuquisaca, Bolivia	20.75° S; 63.22° W	H7	-	H14*	KT153569	
36	GB 4	Chuquisaca, Bolivia	20.75° S; 63.22° W	H7	-	H14*	JF280993	
37	GB 5	Tarija, Bolivia	21.50° S; 64.00° W	H4	-	H10*	KT153568	
38	GB 6	Tarija, Bolivia	21.50° S; 64.00° W	H4	-	H10*	JF280992	

Collection acronyms: CRILAR-Ma-CT: tissue collection of the mammalogy division of the CRILAR, La Rioja, Argentina; MACN-CT: tissue collection of the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina; LIEB: Laboratorio de Investigaciones en Evolución y Biodiversidad (LIEB), CIEMEP, Esquel, Argentina; MG-ZV-M: Museo Provincial de Ciencias Naturales Dr. Angel Gallardo, Rosario, Argentina; ZV-M-H: Museo Provincial de Ciencias Naturales "Florentino Ameghino", Santa Fe, Argentina.

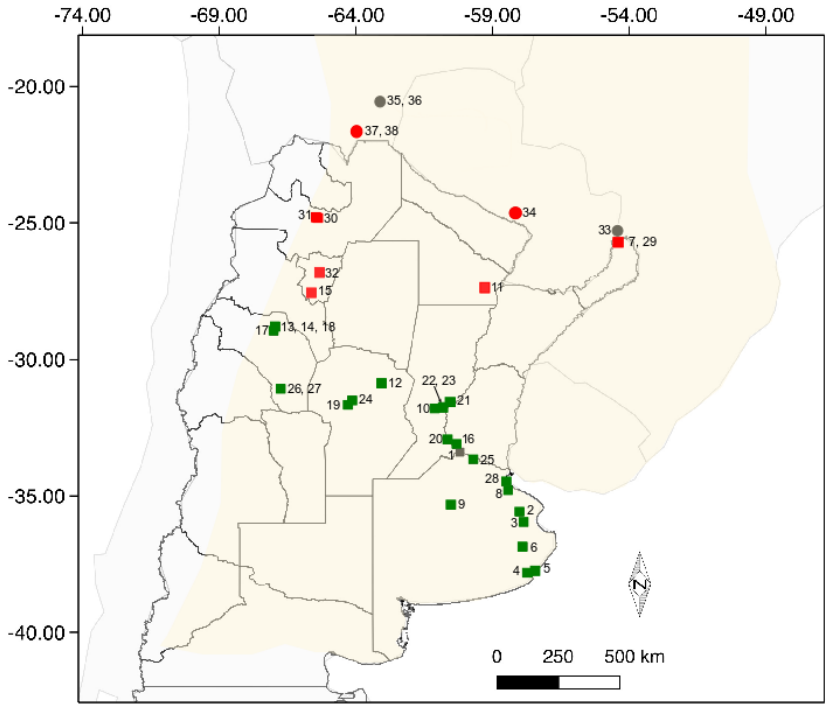

Fig. 1. Geographic location of the samples analyzed of *Didelphis albiventris*. The yellow mask indicates the schematic geographic distribution of the species. Squares indicate sequences acquired during this study and circles indicate GenBank sequences; green icons indicate sequences from haplogroup 1 and red icons indicate sequences from haplogroup 2; grey icons could not be placed in any of the mentioned haplogroups. Numbers identify each specimen as detailed in **Table**1.

distances between sequences ranging from 0 to 0.66% (**Table** S1).

We successfully amplified and sequenced 511 bp from 27 individuals, and with the addition of six sequences from GenBank, resulted in 8 variable sites defining 7 haplotypes (Hd=0.676 [sd=0.079]; π=0.0027 [sd=0.0004]). The most common haplotype, H2, was present in 18 individuals, followed by H5 in five individuals, H4 in four individuals, H3 and H7 in two individuals, and the remaining haplotypes present in one individual each (**Table** 1). The overall mean distance among sequences of cyt b was 0.27%, with pairwise distance values ranging from 0 to 0.8% (**Table** S2). Due to technical difficulties, we were not able to amplify the same number of samples for both markers, and consequently, we have fewer samples for the cyt b and the combined matrix.

The combined matrix consisted of 25 individuals (the ones that only have one marker were discarded for most of the analyses) and 971 bp, with 14 variable sites defining 9 haplotypes (Hd=0.76 [sd=0.092]; π=0.003 [sd=0.0005]). The most common haplotype, H1, was present in 13 individuals, H5 in three, and the remaining in one or two individuals (**Table** 1). The overall mean distance among individuals in the combined matrix was 0.3%, with pairwise distance values between sequences ranging from 0 to 0.73% (**Table** S3).

Neutrality tests were consistent with a constant population model (i.e., were not statistically significant) for the combined data set: D=-0.192, Fs=-1.003, R_2=0.083; the cyt b: D=-0.927, Fs=-1.537, R_2=0.097; and the D-loop: D=-1.299, Fs=-1.332. The only exception was the R_2 that was statistically significant (although marginally) for the D-loop (R_2=0.065, p=0.034).

The Mantel test showed a significant correlation between geographic and genetic distances in the three data sets (cyt b r=0.3125, p=0.0001; D-loop r=0.3036, p=0.0011; combined matrix r=0.51, p=0.00009). All the analyses performed in Geneland using the combined matrix found strong support for the subdivision of the sample in two populations, but since we have little genetic variation, the analyses did not have enough power to discriminate the individuals correctly in two populations, and the dif-

ferent runs resulted in an inconsistent placement of the boundaries among populations (data not shown).

The mismatch distribution analysis was also consistent with a model of demographic equilibrium, instead of an expanding model (θ_0=0; θ_1=4.5; SSD=0.056, p>0.5; raggedness index=0.11, p>0.5; τ=5.92; **Fig**. 2a). The same pattern was observed in the Bayesian skyline plot where the effective population size has apparently been kept stable during the last 600,000 years (**Fig**. 2b).

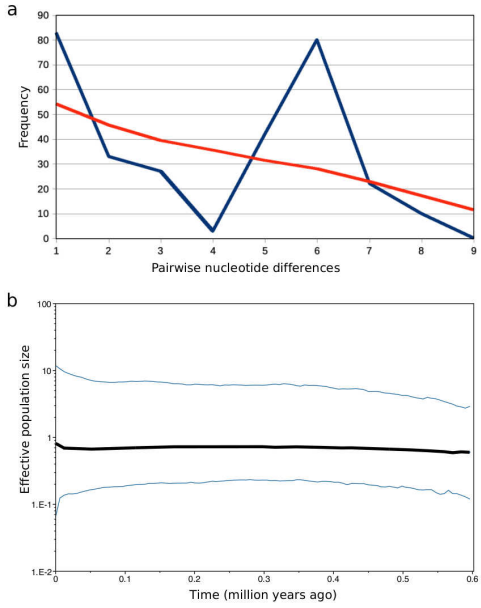

Fig. 2. Historical demography analyses. (a) Mismatch distribution; the blue line shows the observed distribution, and the red line shows the expected distribution; (b) Bayesian skyline plot; the black line is the media estimated, and the blue lines show the 95% highest posterior density intervals.

The haplotype network of the combined matrix showed that haplotypes were mostly separated by one or two mutational steps. Notwithstanding, two haplogroups, separated by two mutational steps, can be identified. The largest group (**Fig**. 3) included haplotypes from the Pampas, Chaco, Monte, and Espinal phytogeographic provinces. H1 (which is present in Pampas, Espinal, and Chaco), occupying a central position in the network, would be the most ancestral one, while derived haplotypes would have a more recent origin. The second group has a more heterogeneous composition, given that haplotypes are related through a median vector (a possible unsampled sequence or extinct ancestral sequence; **Fig**. 3). This second group enclosed haplotypes from more humid environments like the Yungas and the Parana forest. The Chaco biogeographic province presented haplotypes distributed in the two haplogroups (H3 and H5 from haplogroup 2 and H1 from haplogroup 1; see **Table** 1) and thus, admixture of the two haplogroups could be occurring in this ecoregion (**Fig**. 3).

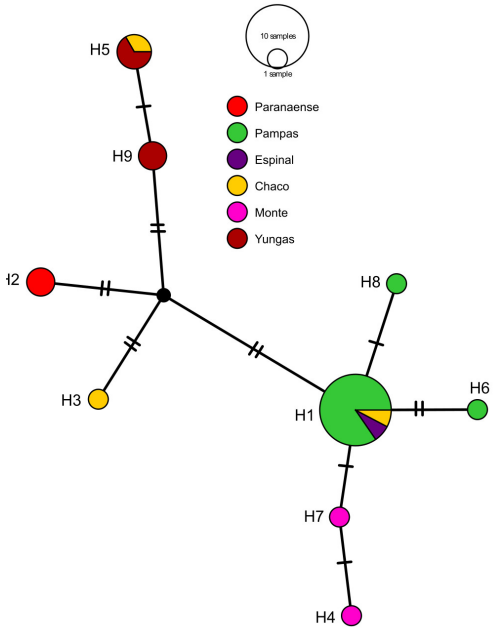

Fig. 3. Median-joining network displaying the variation of *Didelphis albiventris* in Argentina based on the combined matrix. Haplotypes are represented with discs and colors that indicate the biogeographic provinces from which the samples came from; mutational steps are indicated with stripes.

The haplotype network of the cyt b matrix was star-shaped (**Fig**. 4). The central haplotype (H7) corresponds to sequences from Chuquisaca (Bolivia). The most common haplotype (H2; see **Table** 1) is present in several biogeographic provinces: Pampas, Espinal, Monte and Chaco. Sequences from Presidente Hayes (Paraguay) and Tarija (Bolivia) shared the haplotype (H4) with sequence DA14 from Chaco, and contrary to what we expected, the sequence from Foz do Iguaçu (Brazil) did not share the haplotype with sequences from Puerto Iguazú (Argentina) (H6 and H3, respectively; **Table** 1).

The haplotype phylogenetic trees were quite uninformative, and the three trees have different structure (**Figs**. S1-S3). Regarding the haplogroups of the network analyses, we only recovered haplogroup

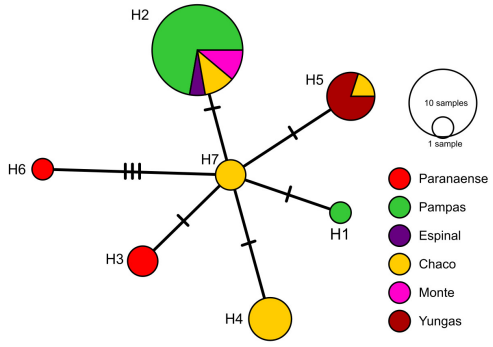

Fig. 4. Median-joining network displaying the variation of *Didelphis albiventris* in Argentina based on the cyt b matrix. Haplotypes are represented with discs and colors that indicate the biogeographic provinces from which the samples came from; mutational steps are indicated with stripes.

two (formed by haplotypes H2, H3, H5, and H9) in the analysis of the D-loop matrix, where H11 of the D-loop matrix have the same individuals as H9 of the combined matrix (**Fig**. S2; **Table** 1), while none of the other analyses recover any of the haplogroups from the network. In the analysis of the full matrix (**Fig**. S1), the haplotype that includes specimens from Paraguay and Tarija (Bolivia) (H10*; **Table** 1) was grouped with haplotype H3 (which includes a sample from Chaco Province, Argentina) while the haplotypes that included sequences from Chuquisaca (Bolivia) and Foz do Iguaçu (Brazil) (H14* and H16*, respectively) could not be correctly assigned to any group and were placed in a politomy (**Fig**. S1). The same pattern was observed in the phylogenetic analysis of the cyt b matrix (**Fig**. S3). Branch lengths were short in all the analyses, confirming the lack of information of these markers found in other analyses, as well as their lack of phylogenetic signal (**Figs**. S1-S3).

DISCUSSION

The main finding of this contribution is the little genetic variation present in the Argentinean populations of *D. albiventris*. Although we sampled a relatively small number of individuals, we covered a large portion of the species distribution in the country (**Fig**. 1), including samples from different environments, and consequently, we expected to find more variation.

The D-loop is known to be a variable marker in placental mammals, while in marsupials Nilsson (2009) found that it is more conserved. Despite those findings, the D-loop was used in phylogeographic analyses of Australian marsupials (e.g., Neaves et al. 2016; Umbrello et al. 2020) showing high levels of variation. In American marsupials, the D-loop has been mostly disregarded in phylogeographic analyses, and the few published articles, both in the genus *Marmosa*, reported low levels of genetic variation (Steiner & Catzefliz 2003; Rocha et al. 2012). On the contrary, cyt b has been widely used in phylogeographic analyses of American marsupials (e.g., Carvalho et al. 2011; Gutiérrez et al. 2014; Rocha et al. 2015; Sartorato Zanchetta et al. 2019). In addition, Steiner & Catzefliz (2003) found in their analyses in *Marmosa murina* that cyt b was more variable than D-loop.

Our findings in *D. albiventris* contrast with what was previously reported for South American marsupials since the cyt b data showed extremely low variation (only eight variable sites), and contrary to what Steiner & Catzefliz (2003) reported, the D-loop was more variable than the cyt b. Voss & Jansa (2019) analyzed cyt b sequences of five specimens of *Chironectes minimus* from widely separated samples across the distribution range of the species and found low levels of genetic variation (uncorrected pairwise distances from 0.1 to 0.6 % in the studied samples from South America). The overall mean genetic distance among our samples (Kimura 2-parameter distance of 0.3 % for the combined data set) was on the lower bound of the values reported by Voss & Jansa (2019), and individuals from widely separated localities and completely different environments (such as Buenos Aires and La Rioja) shared the same haplotype (**Table** 1).

The genetic variation found in this contribution also contrasts with the findings of Rocha et al. (2015) for specimens of *Didelphis albiventris* from the Amazonia and Cerrado of Brazil, which showed higher levels of variation in the cyt b sequences (between 1 and 6 % of genetic variation), a between clades divergence of 6%, a higher haplotype diversity (0.87 versus 0.53 reported here), and more polymorphic sites (18 versus 5 reported here). It is important to mention that Rocha et al. (2015) only analyzed 15 specimens, so differences could not be explained by sample size. On the contrary, the findings of Rocha et al. (2015) for the closely related species, *Didelphis marsupialis*, are similar in levels of haplotype diversity and polymorphic sites to the ones reported here (5 haplotypes with 6 polymorphic sites for *D. marsupialis*, while we found 8 polymorphic sites defining 7 haplotypes).

The reason for these low levels of genetic differentiation among populations found in our analyses, and the lack of phylogeographic structure could be caused by high levels of gene flow, as discussed by Nascimento et al. (2019). *D. albiventris* has a broad home range (mean 2.33±2.32 ha but with measurements of up to 7 ha) with an overlap between individuals of up to 34%, without differences between sexes (Sanches et al. 2012), so high levels of gene flow are expected, at least among close populations (Centeno-Cuadros et al. 2011; Richardson et al. 2021). Moreover, the species is ecologically flexible, being a generalist feeder and adapted to different environments, including large cities (Astúa 2015; Costa et al. 2015); so it is difficult to consider ecological factors as barriers to gene flow (e.g., Tammeleht et al. 2010; Latch et al. 2014; Levy et al. 2019). In the sister species, *Didelphis virginiana*, Beatty et al. (2012), using microsatellite loci, found no genetic structure in an agriculturally fragmented system of Indiana (USA), which was attributed to the ecological characteristics of the species, and common to *D. albiventris*.

The analysis of the haplotypes show that distant localities shared the same haplotype, for example haplotype H1 of the combined matrix is present in localities 1 000 km apart, and haplotype H2 of the cyt b matrix is present in contrasting environments such as Pampa and Monte (**Table 1**). Similar results were described for Brazilian populations of *D. albiventris*, where Sousa et al. (2012) found haplotypes shared between distant localities in their larger haplogroup from Minas Gerais, which were interpreted as lack of impediments to gene flow. On a taxonomic distant species (the rodent *Lagostomus maximus*), but with a shared geographic distribution with *D. albiventris*, Gariboldi et al. (2019) found a similar pattern of low genetic diversity and shared haplotypes among distant populations, coupled with high levels of gene flow. However, due to the life history of the species and their home range, the authors discarded ongoing gene flow among distant populations and propose a scenario of recent range expansion which homogenized populations (Gariboldi et al. 2019).

At a macrogeographic scale, we found a subtle correlation between geographic and genetic distance. To achieve this pattern, sufficient time must have passed for a balance between drift and gene flow, which is in line with a stable population history and contradicting empirical observations. Similar results were described for the rodent *Oligoryzomys flavescens*, where the observed isolation-by-distance pattern suggested that the population reached stability after the range expansion, resulting in low levels of geographic differentiation (although not as low as the levels found by us; Rivera et al. 2018).

Another possible explanation for these low levels of mitochondrial gene divergence could be found in the phylogeographic history of the Argentinean populations of *D. albiventris*. A demographic expansion is usually associated with low genetic variability (see, for example, Poljak et al. 2018; Gariboldi et al. 2019). The analyses performed here to test for population expansion (i.e., mismatch analysis and BSP) suggest that the species in Argentina is in a demographic equilibrium. However, both analyses are affected by low levels of sequence polymorphism and small sample sizes (Grant 2015), and consequently, the most likely explanation is that the result we are obtaining is that we do not have enough statistical power to discriminate among the possible evolutionary scenarios. Something similar happens with some of the neutrality tests used (Tajima's D, Fu's Fs), that lack of statistical power when analyzing small samples (Ramos-Onsins & Rozas 2002). So again, results should be carefully analyzed when discarding an expansion scenario due to the non-significant values, especially since they showed negative signs in all the analyses (and even relatively large negative values in the D-loop). The R_2 is more robust when dealing with small samples (Ramos-Onsins & Rozas 2002), and for the D-loop showed a statistically significant result indicating population growth. Perhaps, the species is going through an expansion period, but we do not have enough genetic signal in both mitochondrial genes of *D. albiventris* to detect the putative expansion.

In fact, a scenario of population growth would be consistent with recent reports that suggest that *D. albiventris* is expanding its geographic range to the south of Argentina and is currently found in the provinces of Neuquén and Río Negro, with its southern limit approximately in the 40° 40' S (Carrera & Udrizar Sauthier 2014; Bianchini 2018). Also, the species is highly adapted to urban and rural environments (Smith 2007; Costa et al. 2015), so it is possible to think that it went through a demographic expansion, both caused by climate change and also following the enlargement of the agricultural frontier, especially in the Pampas region. It is noteworthy that the sister species, *D. virginiana*, is expanding northward in North America, and although the low temperatures were once a limitation, the global climate warming plus the increase of agricultural settlements in cold areas helped the species to overcome that limitation and allowed

the northern expansion of *D. virginiana* (Walsh & Tucker 2018). Consequently, it is expected a similar behavior in *D. albiventris*, since both species share most ecological characteristics.

In Brazil, Sousa et al. (2012), Rocha et al. (2015), and Nascimento et al. (2019) found a similar pattern regarding neutrality indexes to the one recovered for Argentina (but with higher levels of genetic variation). Neutrality tests (D, R_2, and Fu & Li's F) were not significant in all their analyses (except for one of the demes in Nascimento et al.'s analyses), and the authors interpreted that result as a constant population indicator. However, Rocha et al. (2015) only analyzed 15 samples, so the failure in finding any signal (both in the analyses above mentioned and in the BSP) could be an artifact caused by the small sample size, similar to what happens in this contribution. Sousa et al. (2012) and Nascimento et al. (2019) analyzed a larger number of individuals (93 and 142, respectively), but since there is little genetic variation, D, and Fu & Li's F might have lacked statistical power to find a significant result, and the authors did not perform the Ramos-Onsins and Rozas' R_2 test. Also, it is possible that a BSP or a mismatch analysis could have shown evidence of demographic change, but those analyses were not performed by the authors. Contrary to what we detected in Argentina, Nascimento et al. (2019) found evidence of population structuring, but this structure was not correlated with the biomes analyzed. In fact, they found haplotypes shared between the Uruguayan district (part of the Pampas biogeographic province) and the Paranaense Forest while in our analyses, both provinces (Pampas and Paranaense) were in separate groups in the haplotype network. This would suggest that there could be geographic structuring in a regional scale when considering different biogeographic regions of South America.

Finally, it is clear that the data gathered from mitochondrial markers is not sufficient enough to get a better understanding of the phylogeographic history of *D. albiventris*. A comprehensive phylogeographic approach would be necessary, including collecting sites from throughout its distribution range, including not only other countries apart from Argentina, but also samples from the southern limit of its distribution, currently missing in this contribution. Using a global point of view, it could be evaluated if the mitochondrial markers are really adequate to know the evolutionary history of this species. In addition, new analyses of highly variable markers, such as microsatellites or SNPs, would be recommended to improve our knowledge of the genetic structure of the white-eared opossum in Argentina in particular, and South America in general.

ACKNOWLEDGMENTS

This article is part of a special section in honor of Elio Massoia, who made important contributions to the knowledge of Argentinean marsupials (among other mammals). We thank Javier Pereira, Andrés Pautasso, Eugenia Montani, David Flores, Gabriel Martin and Sergio Lucero for the donation of tissues of *D. albiventris*. Patricia Mirol, Jimena Gómez and Thamara Fariñas provided technical support in the lab. This is a contribution to PICT 2016-3151.

LITERATURE CITED

Astúa, D. 2015. Order Didelphimorphia. Handbook of the mammals of the world, vol. 5 Monotremes and Marsupials (D. E. Wilson & R. A. Mittermeier eds.). Lynx Editions, Barcelona.

Beatty W. S., J. C. Beasley, G. Dharmarajan, & O. E. Rhodes, Jr. 2012. Genetic structure of a Virginia opossum (*Didelphis virginiana*) population inhabiting a fragmented agricultural ecosystem. Canadian Journal of Zoology 90:101–109. https://doi.org/10.1139/z11-119

Bianchini, M. 2018. Comadreja overa (*Didelphis albiventris*): ampliación de su distribución geográfica en su extremo austral (Patagonia) e información de su antigüedad, y aportes a su límite occidental en la Argentina. Nótulas Faunísticas - Segunda Serie 238:1–13.

Cabrera, A. L., & A. Willink. 1980. Biogeografía de América Latina. Serie de Biología, Monografía 13. Organización de los Estados Americanos, Washington, DC.

Cantor, M., L. A, Ferreira, W. R. Silva, & E. Z. F. Setz. 2010. Potential seed dispersal by *Didelphis albiventris* (Marsupialia, Didelphidae) in highly disturbed environment. Biota Neotropica 10. https://doi.org/10.1590/s1676-06032010000200004

Carrera, M., & D. E. Udrizar Sauthier. 2014. Enlarging the knowledge on *Didelphis albiventris* (Didelphimorphia: Didelphidae) in northern Patagonia: New records and distribution extension. Historia Natural (Tercera Serie) 4:111–115.

Carvalho, B. A., L. F. B. Oliveira, A. Langguth, C. C. Freygang, R. S. Ferraz, & M. S. Mattevi. 2011. Phylogenetic relationships and phylogeographic patterns in *Monodelphis* (Didelphimorphia: Didelphidae). Journal of Mammalogy 92:121–133. https://doi.org/10.1644/10-mamm-a-075.1

Centeno-Cuadros A., J. Román, M. Delibes, & J. A. Godoy. 2011. Prisoners in their habitat? Generalist dispersal by habitat specialists: A case study in Southern Water Vole (*Arvicola sapidus*). PLoS ONE 6:e24613. https://doi.org/10.1371/journal.pone.0024613

Cerqueira, R., & C. J. Tribe. 2008. Genus *Didelphis*. Mammals of South America Vol. 1. Marsupials, Xenarthrans, Shrews, and Bats (A. L. Gardner, ed.). Chicago University Press, Chicago. https://doi.org/10.7208/chicago/9780226282428.001.0001

Chemisquy, M. A., & D. A. Flores. 2012. Taxonomy of the southernmost populations of *Philander* (Didelphimorphia, Didelphidae), with implications for the systematics of the genus. Zootaxa 3841:60–72. https://doi.org/10.11646/zootaxa.3481.1.5

Costa, L. P., D. Astúa, D. Brito, P. J. Soriano, & D. Lew. 2015. *Didelphis albiventris*. IUCN Red List. https://doi.org/10.2305/iucn.uk.2015-4.rlts.t40489a22176404.en

Darriba, D., G. L. Taboada, R. Doallo, & D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9:772. https://doi.org/10.1038/nmeth.2109

Edler, D., J. Klein, A. Antonelli, & D. Silvestro. 2021. raxmlGUI 2.0: A graphical interface and toolkit for phylogenetic analyses

using RAxML. Methods in Ecology and Evolution 12:373–377.https://doi.org/10.1111/2041-210x.13512

Excoffier, L., & H. E. L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Fu, Y. X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925. https://doi.org/10.1093/genetics/147.2.915

Gariboldi, M. C., P. I. F. Inserra, S. Lucero, M. Failla, S. I. Perez, & A. D. Vitullo. 2019. Unexpected low genetic variation in the South American hystricognath rodent *Lagostomus maximus* (Rodentia: Chinchillidae). PLoS ONE 14:e0221559. https://doi.org/10.1371/journal.pone.0221559

Giarla, T.C., R. S. Voss, & S. A. Jansa. 2010. Species limits and phylogenetic relationships in the didelphid marsupial genus *Thylamys* based on mitochondrial DNA sequences and morphology. Bulletin of the American Museum of Natural History 346: 1–67.

Grant, W. S. 2015. Problems and cautions with sequence mismatch analysis and Bayesian skyline plots to infer historical demography. Journal of Heredity 106:333–346. https://doi.org/10.1093/jhered/esv020

Guillot, G., F. Mortier, & A. Estoup. 2005. GENELAND: a computer package for landscape genetics. Molecular Ecology Notes 5:712–715. https://doi.org/10.1111/j.1471-8286.2005.01031.x

Gutiérrez E. E., R. P. Anderson, R. S. Voss, J. Ochoa-G., M. Aguilera, & S. A. Jansa. 2014. Phylogeography of *Marmosa robinsoni*: insights into the biogeography of dry forests in northern South America. Journal of Mammalogy 95:1175–1188. https://doi.org/10.1644/14-mamm-a-069

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95–98.

Hijmans, R. J. 2019. geosphere: Spherical Trigonometry. R package version 1.5-10. .

Huchon, D., F. Delsuc, F. M. Catzeflis, & E. J. P. Douzery. 1999. Armadillos exhibit less genetic polymorphism in North America than in South America: Nuclear and mitochondrial data confirm a founder effect in *Dasypus novemcinctus* (Xenarthra). Molecular Ecology 8:1743–1748. https://doi.org/10.1046/j.1365-294x.1999.00768.x

Kumar, S., G. Stecher, M. Li, C. Knyaz, & K. Tamura. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547–1549. https://doi.org/10.1093/molbev/msy096

Latch, E. K., D. M. Reding, J. R. Heffelfinger, C. H. Alcala-Galván, & O. E. Rhodes Jr. 2014. Range-wide analysis of genetic structure in a widespread, highly mobile species (*Odocoileus hemionus*) reveals the importance of historical biogeography. Molecular Ecology 23:3171–3190. https://doi.org/10.1111/mec.12803

Leigh, J. W., & D. Bryant. 2015. PopART: Full-feature software for haplotype network construction. Methods in Ecology and Evolution 6:1110–1116. https://doi.org/10.1111/2041-210x.12410

Levy, E., M. Byrne, J. A. Huey, M. J. Hillyer, R. C. Firman, & K. M. Ottewell. 2019. Limited influence of landscape on the genetic structure of three small mammals in a heterogeneous arid environment. Journal of Biogeography 46:539–551. https://doi.org/10.1111/jbi.13523

Miller, S., D. Diker, & H. Polesky. 1988. A Simple Salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Research 6:1215. https://doi.org/10.1093/nar/16.3.1215

Nascimento, D. C., B. A. T. P. Campos, E. C. Fraga, & M. C. Barros. 2019. Genetic variability of populations of the white-eared opossum, *Didelphis albiventris* Lund 1840 (Didelphimorphia; Didelphidae) in Brazil. Brazilian Journal of Biology 79:594–602. https://doi.org/10.1590/1519-6984.184842

Neaves, L. E. et al. 2016. Phylogeography of the Koala, (*Phascolarctos cinereus*), and harmonizing data to inform conservation. PLoS ONE 11:e0162207. https://doi.org/10.1371/journal.pone.0162207

Nilsson, M. A. 2009. The structure of the Australian and South American marsupial mitochondrial control region. Mitochondrial DNA 20:126–138. https://doi.org/10.3109/19401730903180112

Oksanen, J. et al. 2019. vegan: Community Ecology Package. R package version 2.5-6. .

Paradis, E. 2010. pegas: an R package for population genetics with an integrated–modular approach, Bioinformatics 26:419–420. https://doi.org/10.1093/bioinformatics/btp696

Paradis, E., J. Claude, & K. Strimmer. 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20:289–290. https://doi.org/10.1093/bioinformatics/btg412

Poljak, S., A. M. Ferreiro, M. B. Chiappero, J. Sánchez, M. Gabrielli, & M. S. Lizarralde. 2018. Phylogeography of screaming hairy armadillo *Chaetophractus vellerosus*: Successive disjunctions and extinctions due to cyclical climatic changes in southern South America. PLoS ONE 13:e0190944. https://doi.org/10.1371/journal.pone.0190944

Ramos-Onsins, S., & J. Rozas. 2002. Statistical properties of new neutrality tests against population growth. Molecular Biology and Evolution 19:2092–2100. https://doi.org/10.1093/oxfordjournals.molbev.a004034

Richardson, J. L. et al. 2021. Dispersal ability predicts spatial genetic structure in native mammals persisting across an urbanization gradient. Evolutionary Applications 14:163–17. https://doi.org/10.1111/eva.13133

Rivera P. C. et al. 2018. Molecular phylogenetics and environmental niche modeling reveal a cryptic species in the *Oligoryzomys flavescens* complex (Rodentia, Cricetidae). Journal of Mammalogy 99:363–376. https://doi.org/10.1093/jmammal/gyx186

Rocha, R. G., Y. L. R. Leite, E. Ferreira, J. Justino, & L. Pires Costa. 2012. Highly conserved d-loop sequences in woolly mouse opossums *Marmosa* (*Micoureus*). Mitochondrial DNA 23:77–83. https://doi.org/10.3109/19401736.2012.660922

Rocha, R. G., E. Ferreira, A. C. Loss, R. Heller, C. Fonseca, & L. Pires Costa. 2015. The Araguaia river as an important biogeographical divide for didelphid marsupials in Central Brazil. Journal of Heredity 106:593–607. https://doi.org/10.1093/jhered/esv058

Rodrigues Da Silva, A., E. Dias Forneck, S. A. De Loretto Bordignon, & C. Vargas Cademartori. 2014. Diet of *Didelphis albiventris* Lund, 1840 (Didelphimorphia, Didelphidae) in two periurban areas in southern Brazil. Acta Scientiarum, Biological Sciences 36:241–247. https://doi.org/10.4025/actascibiolsci.v36i2.20444

Rozas, J., et al. 2017. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Datasets. Molecular Biology and Evolution 34:3299–3302.

Rambaut, A., A. J. Drummond, D. Xie, G. Baele, & M. A. Suchard. 2018. Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. Systematic Biology 67:901–904. https://doi.org/10.1093/sysbio/syy032

Sanches, V. Q. A., M. M. A. Gomes, F. C. Passos, G. Graciolli, & A. C. A. Ribas. 2012. Home-range and space use by *Didelphis albiventris* (Lund 1840) (Marsupialia, Didelphidae) in Mutum Island, Paraná river, Brazil. Biota Neotropica 12(4) . https://doi.org/10.1590/s1676-06032012000400004

Sartorato Zanchetta, L., R. Gomes Rocha, & Y. L. Reis Leite. 2019. Comparative phylogeography and demographic history of two marsupials of the Atlantic Forest in eastern Brazil. Journal of Zoological Systematics and Evolutionary Research 57:632–641. https://doi.org/10.1111/jzs.12280

Smith, P. 2007. White-eared opossum *Didelphis albiventris* (Lund, 1840). FAUNA Paraguay, Handbook of Mammals of Paraguay 1–19.

Sousa, L. C. C., C. M. F. Gontijo, H. A. Botelho, & C. C. Fonseca. 2012. Mitochondrial genetic variability of *Didelphis albiventris* (Didelphimorphia, Didelphidae) in Brazilian localities. Genetics and Molecular Biology 35:522–529. https://doi.org/10.1590/s1415-47572012005000035

Steiner, C., & F. M. Catzefliz. 2003. Mitochondrial diversity and morphological variation of *Marmosa murina* (Didelphidae) in French Guiana. Journal of Mammalogy 84:822–831. https://doi.org/10.1644/bme-019

Suchard, M. A., P. Lemey, G. Baele, D. L. Ayres, A. J. Drummond, & A. Rambaut. 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10 Virus Evolution 4:vey016. https://doi.org/10.1093/ve/vey016

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595. https://doi.org/10.1093/genetics/123.3.585

Tammeleht, E. et al. 2010. Genetic structure in large, continuous mammal populations: the example of brown bears in northwestern Eurasia. Molecular Ecology 19:5359–5370. https://doi.org/10.1111/j.1365-294x.2010.04885.x

Umbrello, L. S., R. K. Didham, R. A. How, & Joel A. Huey. 2020. Multi-Species phylogeography of arid-zone Sminthopsinae (Marsupialia: Dasyuridae) reveals evidence of refugia and population expansion in response to Quaternary change. Genes 11:963. https://doi.org/10.3390/genes11090963

Vaidya, G., D. J. Lohman, & R. Meier. 2011. SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27:171–180. https://doi.org/10.1111/j.1096-0031.2010.00329.x

Vieira, E. M., & D. Astúa. 2003. Carnivory and insectivory in Neotropical marsupials. Predators with Pouches (M. E. Jones, C. R. Dickman & M. Archer eds.). CSIRO Publishing, Collingwood. https://doi.org/10.1071/9780643069862

Vieira, E. M., & N. F. Camargo. 2012. Uso do espaço vertical por marsupiais brasileiros. Os marsupiais do Brasil: Biologia, ecologia e conservação, 2o Edition (N. C. Cáceres ed.). Editora UFMS, Campo Grande.

Voss, R. S., & S. A. Jansa. 2019. Minimal genetic divergence among South American samples of the water opossum *Chironectes minimus*: evidence for transcontinental gene flow? Mammalia 83:190–192 https://doi.org/10.1515/mammalia-2018-0073

Walsh, L. L., & P. K. Tucker. 2018. Contemporary range expansion of the Virginia opossum (*Didelphis virginiana*) impacted by humans and snow cover. Canadian Journal of Zoology 96:63–169. https://doi.org/10.1139/cjz-2017-0071

ONLINE SUPPLEMENTARY MATERIAL

Supplement 1

Table S1. Estimates of evolutionary divergence between sequences of D-loop. Values are expressed as percentage.

Table S2. Estimates of evolutionary divergence between sequences of cyt b. Values are expressed as percentage.

Table S3. Estimates of evolutionary divergence between sequences of the combined data set. Values are expressed as percentage.

Supplement 2

Fig. S1. Phylogenetic reconstruction of the haplotypes of the full matrix. Haplotype numbers follow **Table** 1.

Fig. S2. Phylogenetic reconstruction of the D-loop haplotypes of *Didelphis albiventris*.

Fig. S3. Phylogenetic reconstruction of the cyt b haplotypes of *Didelphis albiventris*.