

Genetic structuring in a relictual population of screaming hairy armadillo (*Chaetophractus vellerosus*) in Argentina revealed by a set of novel microsatellite loci

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Genetic structuring in a relictual population of screaming hairy armadillo (Chaetophractus vellerosus) in Argentina revealed by a set of novel microsatellite loci --Manuscript Draft--

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Abstract:	<p>The screaming hairy armadillo (Chaetophractus vellerosus) is a mammal species containing disjunct and isolated populations. In order to assess the effect of habitat fragmentation and geographic isolation, we developed seven new microsatellite loci isolated from low-coverage genome shotgun sequencing data for this species. Among these loci, six microsatellites were found to be polymorphic with 8 to 26 alleles per locus detected across 69 samples analyzed from a relictual population of the species located in the northeast of the Buenos Aires Province (Argentina). Mean allelic richness and polymorphic information content were 15 and 0.75, with observed and expected heterozygosities ranging from 0.40 to 0.67 and 0.58 to 0.90, respectively. All loci showed departures from Hardy-Weinberg equilibrium. The analysis of population</p>	

	<p>structure in this relictual population revealed three groups of individuals that are genetically differentiated. These newly developed microsatellites will constitute a very useful tool for the estimation of genetic diversity and structure, population dynamics, social structure, parentage and mating system in this little-studied armadillo species. Such genetic data will be particularly helpful for the development of conservation strategies for this isolated population and also for the endangered Bolivian populations previously recognized as a distinct species (<i>Chaetophractus nationi</i>).</p>
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1 **Genetic structuring in a relictual population of screaming hairy armadillo (*Chaetophractus vellerosus*)**
2 **in Argentina revealed by a set of novel microsatellite loci**

3

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13 **Abstract**

14 The screaming hairy armadillo (*Chaetophractus vellerosus*) is a mammal species containing disjunct and
15 isolated populations. In order to assess the effect of habitat fragmentation and geographic isolation, we
16 developed seven new microsatellite loci isolated from low-coverage genome shotgun sequencing data for this
17 species. Among these loci, six microsatellites were found to be polymorphic with 8 to 26 alleles per locus
18 detected across 69 samples analyzed from a relictual population of the species located in the northeast of the
19 Buenos Aires Province (Argentina). Mean allelic richness and polymorphic information content were 15 and
20 0.75, with observed and expected heterozygosities ranging from 0.40 to 0.67 and 0.58 to 0.90, respectively.
21 All loci showed departures from Hardy-Weinberg equilibrium. The analysis of population structure in this
22 relictual population revealed three groups of individuals that are genetically differentiated. These newly
23 developed microsatellites will constitute a very useful tool for the estimation of genetic diversity and
24 structure, population dynamics, social structure, parentage and mating system in this little-studied armadillo
25 species. Such genetic data will be particularly helpful for the development of conservation strategies for this
26 isolated population and also for the endangered Bolivian populations previously recognized as a distinct
27 species (*Chaetophractus nationi*).

28

29 **Key words**

30 Molecular markers, armadillos, habitat fragmentation, molecular ecology

31

32 **Introduction**

33 Reduced population size can cause loss of genetic diversity within populations and the emergence of harmful
34 genetic effects associated with this genetic load. Small isolated populations can suffer from the effects of
35 inbreeding and loss of heterozygosity, leading to a decrease in reproductive success and an increase in
36 extinction probability (Frankham et al. 2002). The deleterious effects of isolation and low effective population
37 size are often exacerbated by habitat loss or fragmentation, a situation experienced by many wild mammal
38 populations in the Argentinean Pampas due to human activities related to cattle raising and farming (Viglizzo
39 et al. 2011; Bilenca et al. 2012). Early detection of potentially deleterious genetic load and loss of genetic
40 variability maximizes our ability to implement a management approach aims at limiting or reversing these
41 effects before they become substantial or irreversible (Hedrick 2001).

42 The screaming hairy armadillo (*ChaetophRACTUS vellerosus*; Xenarthra, Chlamyphoridae) has been
43 recently shown to include populations inhabiting high altitude grasslands of Bolivia, Chile, Peru, and northern
44 Argentina, all of them previously recognized as a separate species, the Andean hairy armadillo
45 (*ChaetophRACTUS nationi*; Abba et al. 2015). Its geographical distribution once restricted to arid and semiarid
46 regions with loose, sandy soil of southeastern Bolivia, northeastern Paraguay and central Argentina (Abba and
47 Cassini 2010; Abba et al. 2011), has thus been largely expanded (Figure 1). In Bolivia, the high-altitude
48 isolated populations are threatened by their overexploitation for traditional purposes and habitat degradation
49 due to agricultural activities (Pérez-Zubieta 2011). In Argentina, a disjunct population of screaming hairy
50 armadillo exists in the northeast of the Pampa region, which is separated from the main distribution area by
51 about 500 km (Crespo 1974; Carlini and Vizcaíno 1987; Abba et al. 2011) (Figure 1). This relictual
52 population is associated with the shelly beach ridges on the coast of the Río de la Plata Estuary, covering an
53 area of less than 900 km² (Abba and Superina 2010). It is currently at high risk of extinction because the
54 environment is being heavily modified by human activities such as farming, cattle raising, and mining
55 activities (Abba et al. 2011). Such disturbances are thought to affect both individual behavior and population
56 dynamics. For example, Pagnutti et al. (2014) analyzed the home range of the screaming hairy armadillo in
57 the same study area that we analyzed here, which is divided in two pastures with different use intensity (see
58 Materials and Methods for details). Their results showed that the home range of the species was reduced by
59 human disturbance and that individuals from the most disturbed pasture presented a more aggregated

60 distribution. In addition, the authors did not observe or recaptured the same marked individual in both
61 pastures (AM Abba, personal communication), suggesting limited dispersal between the two areas. From
62 these previous results, some degree of genetic differentiation might be expected between the two areas with
63 different use intensity.

64 The aim of this work is to conduct a preliminary study of genetic variation and structure in a relictual
65 population of the screaming hairy armadillo by developing a set of microsatellite markers that would be
66 useful for studying the conservation genetics of this species in wild populations. Microsatellites constitute
67 useful genetic markers for estimating genetic diversity, population structuring, demography, social structure,
68 parentage, and mating system (Awise 2004; Andrew et al. 2013). Estimating these parameters will be helpful
69 for the development of future conservation strategies of the endangered populations of screaming hairy
70 armadillos in both the northeast of the Pampas region in Argentina and the high altitude habitats of Bolivia.

71

72 **Materials and Methods**

73 *Microsatellites development*

74 We used shotgun genomic data generated in a previous study focused on xenarthran mitogenomics (Gibb et
75 al. 2016). As part of this phylogenetic study, single-end Illumina reads were produced from a *C. vellerosus*
76 individual from the Mendoza province in Argentina (1,212,063 reads) and from an individual representing the
77 high altitude populations of the Oruro department in Bolivia (790,237 reads), previously referred to as *C.*
78 *nationi* (see Abba et al. 2015). De novo assembly of these reads was performed with ABySS (Simpson et al.
79 2009). Identical contigs were collapsed using CD-HIT (Fu et al. 2012). By merging the contigs obtained from
80 the two individuals, we obtained a total set of 4,232 unique contigs of more than 150 bp. These contigs were
81 searched for di-, tri-, and tetra-nucleotide repeats using MSATCOMMANDER (Faircloth 2008). Primer
82 design from the resulting 11 candidate loci was subsequently optimized using the BatchPrimer3 web server
83 (You et al. 2008).

84

85 *Study area, sampling and DNA extraction*

86 During 8 years (2006-2013) armadillos were sampled in a 100 hectares cattle farm located in Magdalena,
87 Buenos Aires, Argentina (35° 10.45' S, 57° 20.66' W; Figure 1). The field is bounded on the west by the

88 Provincial Route #11, to the east by the Rio de la Plata Estuary and to the north and south by two artificial
89 canals that flow into this Estuary. These bounds represent physical barriers to dispersal for screaming hairy
90 armadillos. This area is in turn divided in two pastures similarly sized (approximately 50 hectares each), but
91 with different use intensity. The northern one, characterized by a low intensity of use, is mainly used for cattle
92 and sheep breeding, while the southern one, with high intensity of use, is covered by modified grassland used
93 for livestock feeding.

94 Handling technique was used to capture individuals, sometimes helped by a net. Small ear punches of
95 tissues were collected from 69 armadillos, 45 from the northern pasture and 24 from the southern one.
96 Permanent, semi-permanent and temporal marks were made in each individual in order to avoid resampling.
97 Tissue samples were used for DNA extraction using a phenol:chloroform and DNA precipitation method
98 (Sambrook et al. 1989). Precipitated DNA was resuspended in buffer TE, pH = 8.0, quantified in a
99 spectrophotometer at 260/280 nm and stored at -20 °C.

100

101 *Microsatellite amplification*

102 Optimal PCR conditions for 11 candidate loci were initially assayed using DNA obtained from 10 individuals.
103 PCR amplifications were successful for seven of the 11 loci tested in all 69 samples. The PCR amplification
104 protocol consisted of one step of denaturation at 95°C for 3 min; followed by 35 cycles, each involving
105 denaturation at 95°C for 30 sec, 45 sec at annealing temperature (Table 1) and extension at 72°C for 30 sec;
106 with a final extension step at 72°C for 5 min. PCR amplifications were carried out in 25 µl volumes
107 containing 10 ng of DNA, 1× PCR buffer (PB-L, Argentina), 3 mM MgCl₂, 0.2 mM of dNTPs mix
108 (Genbiotech, Argentina), 0.4 µM of each primer (Genbiotech, Argentina), 0.5 U of *Taq* DNA polymerase
109 (PB-L, Argentina) and sterile distilled water to reach final volume. One of the primers of each pair was dyed
110 with FAM or HEX fluorochromes (Table 1). Amplification products were visualized by migration on 2%
111 agarose gel electrophoresis at 4 V/cm.

112

113 *Data analyses*

114 Genotypes were determined using GeneMarker v. 2.2.0 (Softgenetics). Allelic richness, probability of
115 identity, probability of identity among siblings, and observed and expected heterozygosities, were estimated

116 with GenAEx v. 6.5 (Peakall and Smouse 2012). Adjustment to Hardy-Weinberg Equilibrium (HWE) and
117 F_{IS} values for all loci were calculated using GENEPOP v.4.2 (Raymond and Rousset 1995). Polymorphic
118 Information Content (PIC) was evaluated using Microsatellite Toolkit v. 3.3.1 (Park 2001). Null allele
119 frequency was estimated using FreeNA (Chapuis and Estoup 2007). An AMOVA analysis was performed
120 with Arlequin v. 3.5 (Excoffier et al. 2010) in order to evaluate potential genetic differences between the
121 southern and northern pastures. A corrected F_{ST} value was obtained with FreeNA in order to determine the
122 effect of null alleles on genetic structure estimation. Finally, population structuring in our data set was tested
123 using STRUCTURE 2.3.4 (Pritchard et al., 2000). This approach uses a Bayesian clustering analysis to assign
124 individuals to clusters (K) without prior knowledge of their population affinities. STRUCTURE simulations
125 were performed with the number of presumed clusters ranging from $K = 1$ to $K = 7$ and 20 runs per tested K
126 value following the recommendations of Evanno et al. (2005). For each run, the initial burn-in period was set
127 to 100,000 followed by 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. The most probable
128 number of clusters was determined by plotting Delta K as a function of K using Structure Harvester (Earl and
129 vonHoldt 2012), an on-line application of the Evanno's method (Evanno et al. 2005). We chose a proportion
130 of membership threshold value of $q \geq 0.8$ to assign individuals to clusters. This value provides a statistical
131 cut-off within the range of suggested values in the literature (Manel et al. 2002) and indicates that $\geq 80\%$ of
132 ancestry can be attributed to the respective subpopulation. Finally, using the Alleles in Space (AIS) software
133 (Miller 2005), we performed a Genetic Landscape Shape interpolation analysis in order to relate genetic data
134 with the geographic coordinates of individuals.

135

136 **Results and Discussion**

137 *Microsatellites characterization*

138 We developed seven microsatellite loci and used them to analyze 69 individuals from an isolated population
139 of the screaming hairy armadillo (*C. vellerosus*). The seven loci assayed were successfully amplified.
140 However, one of them (locus 5656_750_3130) was found to be monomorphic in our sample set, amplifying a
141 unique fragment of 124 bp. The other six loci were polymorphic with a number of alleles ranging from 8 to 26
142 and a mean allelic richness of 15 (Table 1). All polymorphic loci were highly informative, registering PIC
143 values greater than or equal to 0.530, with a mean of 0.752 (Table 1).

144 Probability of Identity (P_{ID}) and the Probability of Identity among Siblings ($P_{ID\text{sibs}}$) for the whole set of
145 loci were 1.0×10^{-7} and 3.2×10^{-3} , respectively. This result indicate that any individual in this population
146 could be identified, and distinguished from the other individuals in the population, with a probability greater
147 than 0.99. Individual identification is crucial for carrying out behavioral studies in wild populations aiming at
148 determining the mating system or the presence of a social structure (Prodöhl et al. 1996). The newly
149 developed microsatellites will allow such surveys in the screaming hairy armadillo for which these life-
150 history traits are poorly characterized.

151 Observed heterozygosities estimated from our microsatellite loci ranged from 0.403 to 0.672, averaging
152 0.583. Expected heterozygosities varied from 0.584 to 0.898, with a mean value of 0.766. None of the six
153 polymorphic loci adjusted to HWE ($p < 0.001$; Table 1). Five of them showed positive F_{IS} values, but only the
154 value for loci 300_304_832 was significant (Table 1). Waples (2015) conducted an exhaustive study
155 analyzing the possible causes of departures from HWE in natural populations. The possible causes include:
156 overlapping generations, population structure, endogamy, small effective population size, and genotyping
157 errors (i.e. null alleles), among others (Waples 2015). Departure from HWE in our data set could be due to an
158 overlapping generations effect, taking into account that samples used in our study were taken from 2006 to
159 2013, and that offspring, juveniles and adults were captured. Another possibility is the presence of null alleles
160 in the data set, which frequencies ranged from 0.029 to 0.261 (Table 1). However, these values should be
161 taken with caution since null alleles frequencies calculated in FreeNA and related software are obtained
162 assuming panmixia and ascribing heterozygote deficiencies to the presence of null alleles. The panmixia
163 assumption is quite hardly supported by our data given the effect of overlapping generations previously
164 mentioned. Population genetic structure (Wahlund effect) would be another possible cause of the HWE
165 deviations observed. In consequence, we carried out an AMOVA and a STRUCTURE analysis (see below) in
166 order to test the existence of population structure. Finally, we cannot reject endogamy or small effective
167 population size as possible causes of the HWE deviation.

168

169

170 *Population structure*

171 As previously mentioned, the departure from HWE and the positive F_{IS} values obtained would be explained
172 by the existence of a population structuring in our study area. Because a reduced home range due to human
173 disturbance and a more aggregated distribution of individuals in the most disturbed pasture (Pagnutti et al.
174 2014) could have restricted gene flow between pastures, we test the existence of genetic structure between the
175 northern and southern pastures by means of an AMOVA. Our results showed no significant genetic
176 differentiation between pastures ($F_{ST} = 0.007$; $p = 0.095$). The corrected F_{ST} value obtained taking into
177 account the presence of null alleles, also support the lack of genetic structuring ($F_{ST} = 0.003$; $p > 0.05$). A
178 STRUCTURE analysis was also carried out without defining subpopulations *a priori*. Results showed a
179 maximum mean Ln P value at $K = 3$ (Mean Ln P = -1423.79), suggesting the existence of three genetic groups
180 within our study area (Figure 2A). The Evanno's method confirmed this result, showing a peak at $K = 3$.
181 Forty-nine of the 69 individuals (71%) were assigned to one of the three groups. Two of them were composed
182 of 17 individuals, while the remaining was composed by 15 individuals. Figure 2B shows the geographic
183 distribution of the three genetic groups. Most individuals that composed one of these groups were found in the
184 southern pasture, while most individuals that composed the other two groups were found in the northern one.
185 In addition, the Genetic Landscape Shape interpolation analysis (Figure 3) produced a surface plot that
186 qualitatively support results from STRUCTURE. Two major ridges were observed in the landscape,
187 indicating the areas of greatest genetic distance separating the population in three genetically distinct groups.
188 However, field surveys did not detect evidence of physical barriers to dispersal in the study area that might
189 explain this genetic structuring. The observed genetic structure might thus be due to the social behavior or the
190 mating system of the species. Future studies using a higher number of samples and loci together with
191 biological data of the animals obtained during the field works (i.e. sex, age, weight) and parentage analyses,
192 could contribute to a better understanding of this surprising observation.

193

194

195 *Comparison with other xenarthrans*

196 The screaming hairy armadillo belongs to Xenarthra, a superorder of Neotropical mammals grouping
197 armadillos, anteaters, and sloths, which are notably understudied (Superina et al. 2014). Few studies have
198 been previously conducted to estimate genetic diversity in xenarthrans using microsatellites as molecular

199 markers (Table 2). In this handful of studies, observed heterozygosity values range from 0.06 to 0.71. The
200 lowest value was registered in an endangered population of the giant anteater (*Myrmecophaga tridactyla*),
201 which suffered from high inbreeding (Collevatti et al. 2007). The estimated heterozygosity for our population
202 (0.58) is comparable with that obtained for populations of the nine-banded armadillo (*Dasybus novemcinctus*)
203 that are abundant and inter-connected with other populations (Prodöhl et al. 1996; Loughry et al. 2009;
204 Chinchilla et al. 2010; Arteaga et al. 2012). This result is somewhat unexpected considering that our
205 population occupies a relatively restricted area with high level of geographic isolation. Future studies will be
206 necessary to understand the underlying mechanisms involved in such a high level of genetic variability in the
207 screaming hairy armadillo.

208

209 *Conclusions*

210 Our results show that these microsatellite loci can be useful to study this particularly isolated population and
211 other populations of *C. vellerosus*, such as the endangered populations that live in the Andean region of
212 Bolivia (Abba et al. 2015). These loci might also prove useful for the study of the population genetics of other
213 closely related euphractine armadillo species such as *Chaetophractus villosus*, *Euphractus sexcinctus*, and
214 *Zaedyus pichiy* (Abba et al. 2015). Finally, the genetic structuring described here might have to be considered
215 in future conservation actions, taking into account that this relictual population is highly impacted by human
216 activities and is about 500 Km away from the core distribution area of the species.

217

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226

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231 manuscript.

232

233 **Figure legends**

234 **Figure 1** Geographical range of *Chaetophractus vellerosus* and location of the relictual population
235 (Magdalena, Buenos Aires Province) where sampling was carried out. Map was extracted from IUCN SSC
236 Anteater, Sloth and Armadillo Specialist Group, *Chaetophractus vellerosus*, The IUCN Red List of
237 Threatened Species.

238 **Figure 2** Results of the STRUCTURE analysis. A) STRUCTURE bar plot for the screaming hairy armadillo.
239 Each bar represents one individual and each color (light grey, dark grey and black) represents the posterior
240 probability of the individual to belong to that cluster. B) Geographic distribution of the 49 individuals
241 assigned to each of three genetic groups. Colors correspond to those in Figure 2A.

242 **Figure 3** Results of the Genetic Landscape Shape interpolation analysis using a 50 x 50 grid and a distance
243 weighting parameter (α) of 1. X and Y axes correspond to geographic locations within the overall physical
244 landscape examined in this study. Surface plot heights reflect genetic distances. Arrows indicate the two
245 major ridges in the landscape (areas with the highest genetic distance).

246

247

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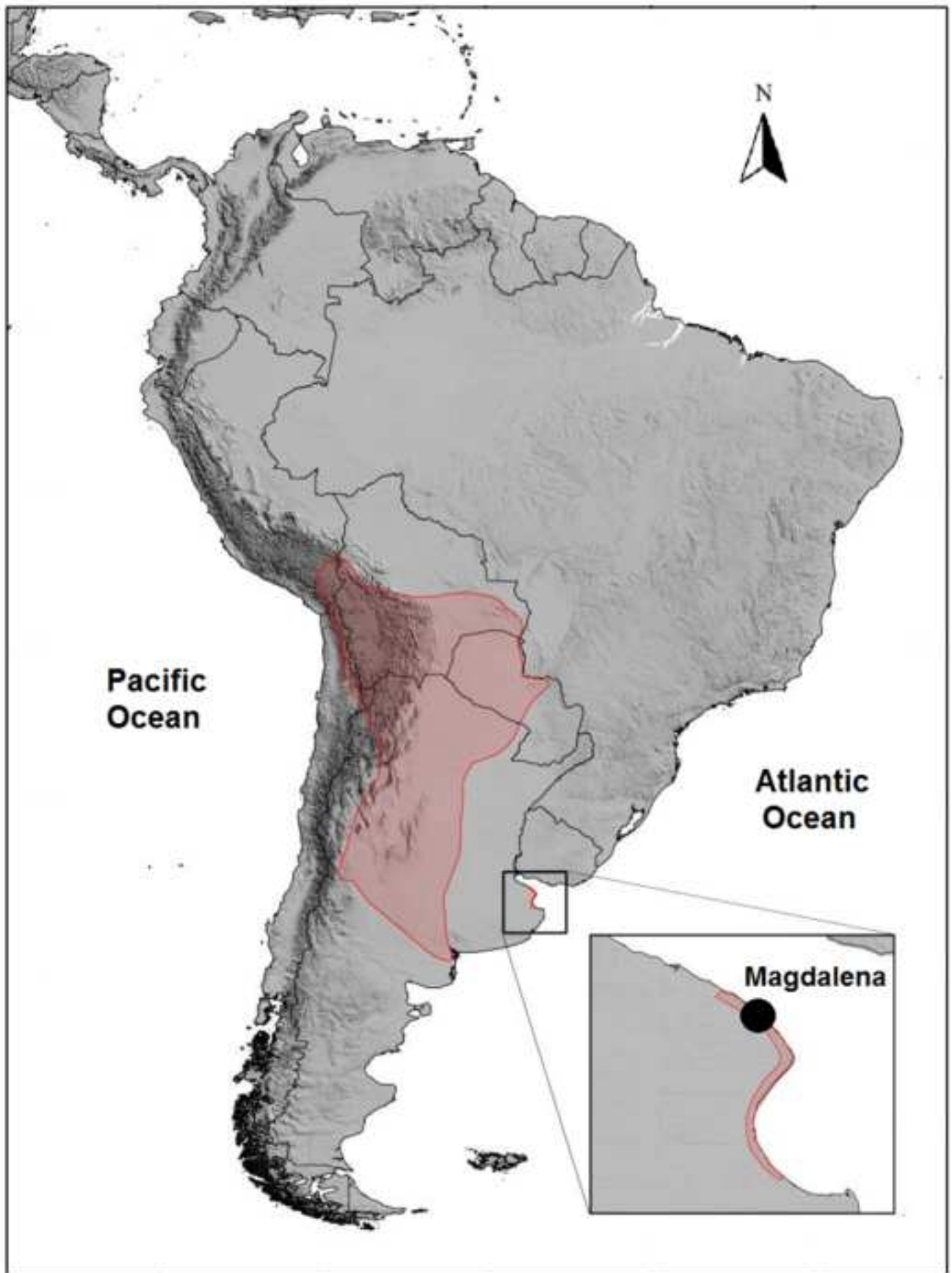
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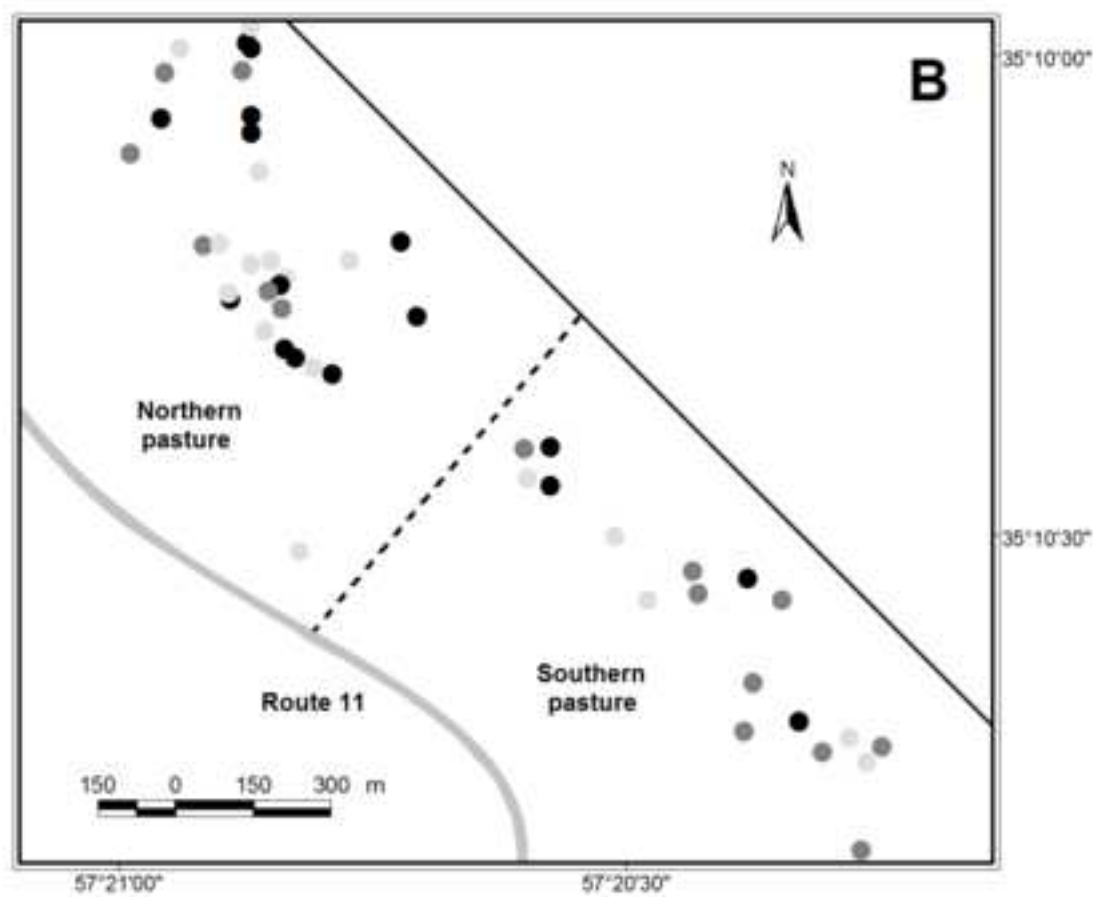
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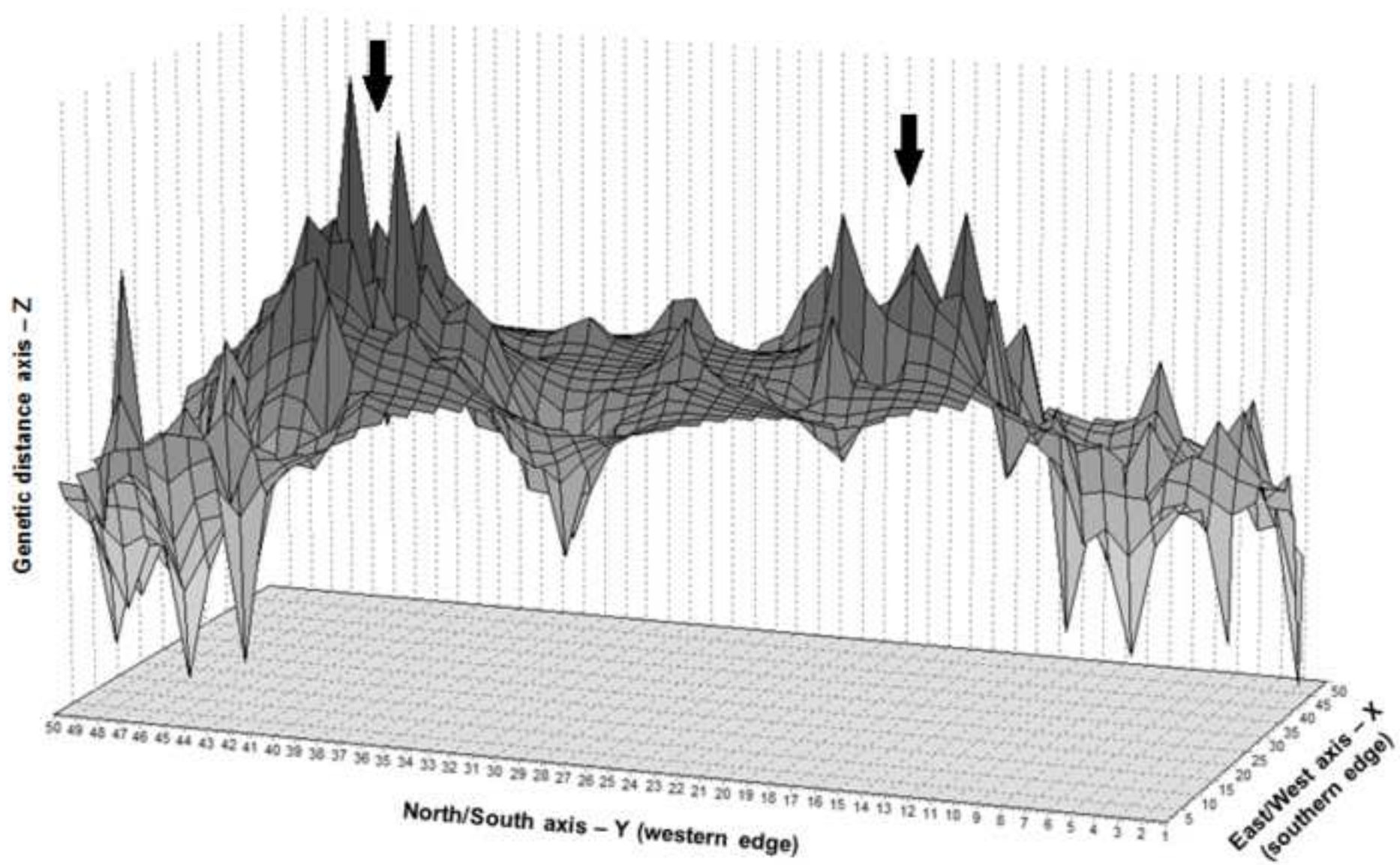


Table 1. General features of microsatellite loci for the screaming hairy armadillo (*ChaetophRACTUS vellerosus*).

Locus name	Primer sequences	Repeat motif	T _a	n	Size range (bp)	N _A	PIC	H _o	H _e	P _{HWE}	F _{IS}	Null alleles freq
376_440_1976	GACCCGGTTCGATTTAATA CACTGCTTGACATTCTCATT	(AG) ₁₃	56°C	69	95-111	10	0.708	0.551	0.738	***	0.260	0.115
2824_669_1772	CTGGGTATTCACACCAGAA GGGGTGACGAAAGTTAAAG	(AC) ₁₄	56°C	68	88-108	15	0.781	0.559	0.796	***	0.304	0.148
54997_179_933	CTAACCGTGCATTTTATGG GGCCTAAGACGGTATTACA	(TC) ₈	54°C	67	71-142	8	0.530	0.657	0.584	***	-0.117	0.029
3972_751_4333	TCAAAGACAATGTCCCTA ATTTCCAGCCTTGATCTG	(AC) ₁₅	54°C	67	77-112	13	0.789	0.672	0.812	***	0.180	0.101
17379_526_1988	CAAGCAAGCAAGCAAG GCCACGGTTTAGTTAATCA	(AAC) ₈	49°C	61	87-109	18	0.741	0.656	0.771	***	0.158	0.116
300_304_832	ACCCTTCAAAAACACTTATT TAAAAACAAGCAAGCAAGC	(TTG) ₈	48°C	67	77-168	26	0.890	0.403	0.898	***	0.556	0.261
5656_750_3130	CGATGAATCAACCCTTAGA GTGCCTGAAGATGTGTGTC	(GT) ₂₂	52°C	69	124	1	—	—	—	—	—	—
						Mean	15	0.752	0.583	0.776		

T_a, annealing temperature. n, individuals. N_A, number of alleles. PIC, polymorphic information content. H_o, observed heterozygosity. H_e, expected heterozygosity. P_{HWE}, p value for exact test of Hardy-Weinberg equilibrium. F_{IS}, inbreeding coefficient.

*** $P < 0.0001$

Table 2. Studies estimating genetic diversity in xenarthrans using microsatellites.

Species	n	# loci	H _o	Reference
<i>Chaetophractus vellerosus</i>	69	6	0.58	This study
<i>Dasypus novemcinctus</i>	310	7	0.49	Prodöhl et al. (1996)
<i>Dasypus novemcinctus</i>	139	4	0.64	Loughry et al. (2009)
<i>Dasypus novemcinctus</i>	40	9	0.46	Chinchilla et al. (2010)
<i>Dasypus novemcinctus</i>	116	5	0.62	Arteaga et al. (2012)
<i>Bradypus variegatus</i>	32	18	0.71	Moss et al. (2012)
<i>Choloepus hoffmannii</i>	23	16	0.55	Moss et al. (2011)
<i>Myrmecophaga tridactyla</i>	15	6	0.61	García et al. (2005)
<i>Myrmecophaga tridactyla</i>	27	5	0.059	Collevatti et al. (2007)

n, individuals. H_o, observed heterozygosity.