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Corresponding Author:	Maximiliano Nardelli Universidad Nacional de Luján Luján, ARGENTINA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidad Nacional de Luján
Corresponding Author's Secondary Institution:	
First Author:	Maximiliano Nardelli
First Author Secondary Information:	
Order of Authors:	Maximiliano Nardelli Ezequiel Alejandro Ibáñez Dara Dobler Fabienne Justy Frédéric Delsuc Agustín Manuel Abba Marcelo Hernán Cassini Juan Ignacio Túnez
Order of Authors Secondary Information:	
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Suggested Reviewers:	Andrea Premoli andrea.premoli@crub.uncoma.edu.ar Researcher with vast experience in molecular ecology
	Bettina Mahler bemahler@ege.fcen.uba.ar Researcher with vast experience in molecular ecology
Response to Reviewers:	

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

Maximiliano Nardelli[1]; Ezequiel Alejandro Ibáñez[1]; Dara Dobler[1]; Fabienne Justy[2]; Frédéric Delsuc[2]; Agustín Manuel Abba[3]; Marcelo Hernán Cassini[1,4]; Juan Ignacio Túnez[1].

[1] Departamento de Ciencias Básicas, Universidad Nacional de Luján, Luján, Argentina.

[2] Institut des Sciences de l'Evolution, UMR 5554, CNRS, IRD, EPHE, Université de Montpellier, Montpellier, France.

[3] Centro de Estudios Parasitológicos y de Vectores (CEPAVE), CCT-CONICET, Universidad Nacional de La Plata, La Plata, Argentina.

[4] Laboratorio de Biología del Comportamiento, IBYME-CONICET, Buenos Aires, Argentina.

Corresponding autor: Maximiliano Nardelli, mnardelli83@yahoo.com.ar

Abstract

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 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 (*Chaetophractus nationi*).

Key words

Molecular markers, armadillos, habitat fragmentation, molecular ecology

Introduction

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

The screaming hairy armadillo (*Chaetophractus vellerosus*; Xenarthra, Chlamyphoridae) has been recently shown to include populations inhabiting high altitude grasslands of Bolivia, Chile, Peru, and northern Argentina, all of them previously recognized as a separate species, the Andean hairy armadillo (*Chaetophractus nationi*; Abba et al. 2015). Its geographical distribution once restricted to arid and semiarid regions with loose, sandy soil of southeastern Bolivia, northeastern Paraguay and central Argentina (Abba and Cassini 2010; Abba et al. 2011), has thus been largely expanded (Figure 1). In Bolivia, the high-altitude isolated populations are threatened by their overexploitation for traditional purposes and habitat degradation due to agricultural activities (Pérez-Zubieta 2011). In Argentina, a disjunct population of screaming hairy armadillo exists in the northeast of the Pampa region, which is separated from the main distribution area by about 500 km (Crespo 1974; Carlini and Vizcaíno 1987; Abba et al. 2011) (Figure 1). This relictual population is associated with the shelly beach ridges on the coast of the Río de la Plata Estuary, covering an area of less than 900 km^2 (Abba and Superina 2010). It is currently at high risk of extinction because the environment is being heavily modified by human activities such as farming, cattle raising, and mining activities (Abba et al. 2011). Such disturbances are thought to affect both individual behavior and population dynamics. For example, Pagnutti et al. (2014) analyzed the home range of the screaming hairy armadillo in the same study area that we analyzed here, which is divided in two pastures with different use intensity (see Materials and Methods for details). Their results showed that the home range of the species was reduced by human disturbance and that individuals from the most disturbed pasture presented a more aggregated distribution. In addition, the authors did not observe or recaptured the same marked individual in both pastures (AM Abba, personal communication), suggesting limited dispersal between the two areas. From these previous results, some degree of genetic differentiation might be expected between the two areas with different use intensity.

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

Materials and Methods

Microsatellites development

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

Study area, sampling and DNA extraction

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

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

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

Microsatellite amplification

Optimal PCR conditions for 11 candidate loci were initially assayed using DNA obtained from 10 individuals. PCR amplifications were successful for seven of the 11 loci tested in all 69 samples. The PCR amplification protocol consisted of one step of denaturation at 95°C for 3 min; followed by 35 cycles, each involving denaturation at 95°C for 30 sec, 45 sec at annealing temperature (Table 1) and extension at 72°C for 30 sec; with a final extension step at 72°C for 5 min. PCR amplifications were carried out in 25 µl volumes containing 10 ng of DNA, 1× PCR buffer (PB-L, Argentina), 3 mM $MgCl_2$, 0.2 mM of dNTPs mix (Genbiotech, Argentina), 0.4 µM of each primer (Genbiotech, Argentina), 0.5 U of *Taq* DNA polymerase (PB-L, Argentina) and sterile distilled water to reach final volume. One of the primers of each pair was dyed with FAM or HEX fluorochromes (Table 1). Amplification products were visualized by migration on 2% agarose gel electrophoresis at 4 V/cm.

Data analyses

Genotypes were determined using GeneMarker v. 2.2.0 (Softgenetics). Allelic richness, probability of identity, probability of identity among siblings, and observed and expected heterozygosities, were estimated with GenAlEx v. 6.5 (Peakall and Smouse 2012). Adjustment to Hardy-Weinberg Equilibrium (HWE) and F_{IS} values for all loci were calculated using GENEPOP v.4.2 (Raymond and Rousset 1995). Polymorphic Information Content (PIC) was evaluated using Microsatellite Toolkit v. 3.3.1 (Park 2001). Null allele frequency was estimated using FreeNA (Chapuis and Estoup 2007). An AMOVA analysis was performed with Arlequin v. 3.5 (Excoffier et al. 2010) in order to evaluate potential genetic differences between the southern and northern pastures. A corrected F_{ST} value was obtained with FreeNA in order to determine the effect of null alleles on genetic structure estimation. Finally, population structuring in our data set was tested using STRUCTURE 2.3.4 (Pritchard et al., 2000). This approach uses a Bayesian clustering analysis to assign individuals to clusters (K) without prior knowledge of their population affinities. STRUCTURE simulations were performed with the number of presumed clusters ranging from $K = 1$ to $K = 7$ and 20 runs per tested K value following the recommendations of Evanno et al. (2005). For each run, the initial burn-in period was set to 100,000 followed by 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. The most probable number of clusters was determined by plotting Delta K as a function of K using Structure Harvester (Earl and vonHoldt 2012), an on-line application of the Evanno´s method (Evanno et al. 2005). We chose a proportion of membership threshold value of $q \geq 0.8$ to assign individuals to clusters. This value provides a statistical cut-off within the range of suggested values in the literature (Manel et al. 2002) and indicates that $\geq 80\%$ of ancestry can be attributed to the respective subpopulation. Finally, using the Alleles in Space (AIS) software (Miller 2005), we performed a Genetic Landscape Shape interpolation analysis in order to relate genetic data with the geographic coordinates of individuals.

Results and Discussion

Microsatellites characterization

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

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

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

Population structure

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

Comparison with other xenarthrans

The screaming hairy armadillo belongs to Xenarthra, a superorder of Neotropical mammals grouping armadillos, anteaters, and sloths, which are notably understudied (Superina et al. 2014). Few studies have been previously conducted to estimate genetic diversity in xenarthrans using microsatellites as molecular markers (Table 2). In this handful of studies, observed heterozygosity values range from 0.06 to 0.71. The lowest value was registered in an endangered population of the giant anteater (*Myrmecophaga tridactyla*), which suffered from high inbreeding (Collevatti et al. 2007). The estimated heterozygosity for our population (0.58) is comparable with that obtained for populations of the nine-banded armadillo (*Dasypus novemcinctus*) that are abundant and inter-connected with other populations (Prodöhl et al. 1996; Loughry et al. 2009; Chinchilla et al. 2010; Arteaga et al. 2012). This result is somewhat unexpected considering that our population occupies a relatively restricted area with high level of geographic isolation. Future studies will be necessary to understand the underlying mechanisms involved in such a high level of genetic variability in the screaming hairy armadillo.

Conclusions

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

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Figure legends

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

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

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

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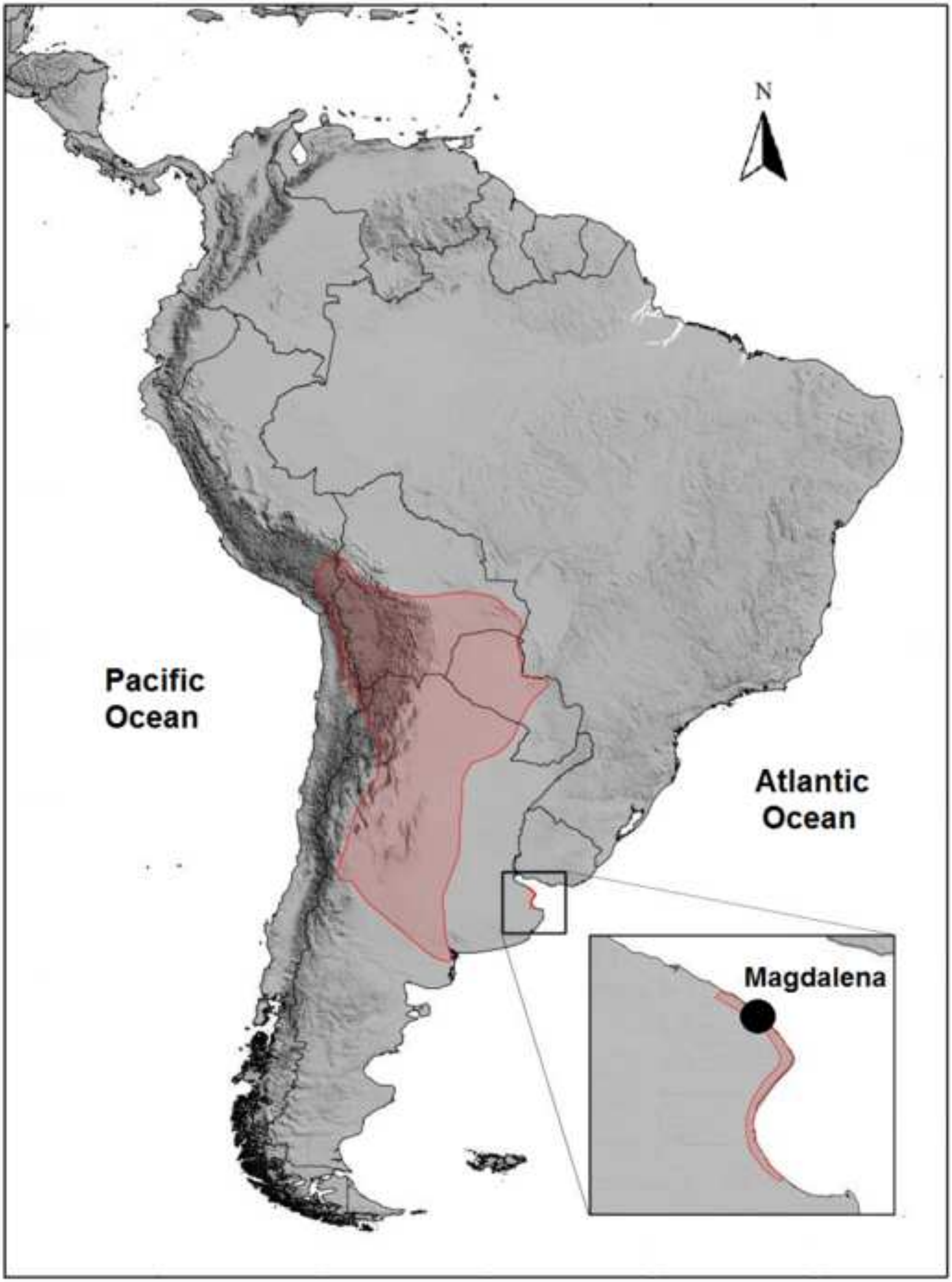
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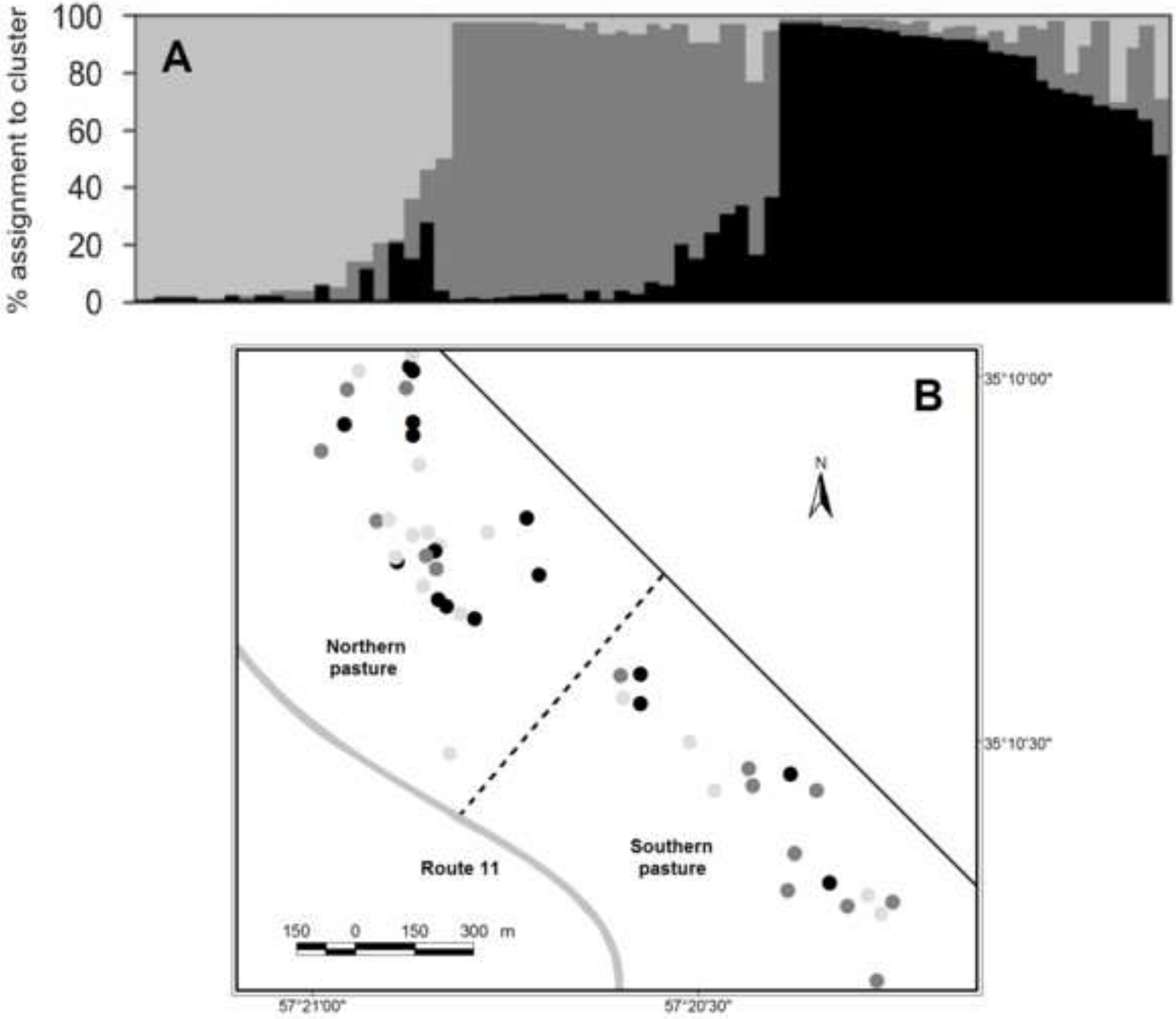
Atlantic Ocean
Magdalena

Figure 2


% assignment to cluster
100
80
60
40
20
0
A
B
35°10'00"
35°10'30"
N
Northern pasture
Southern pasture
Route 11
150
0
150
300
m
57°21'00"
57°20'30"

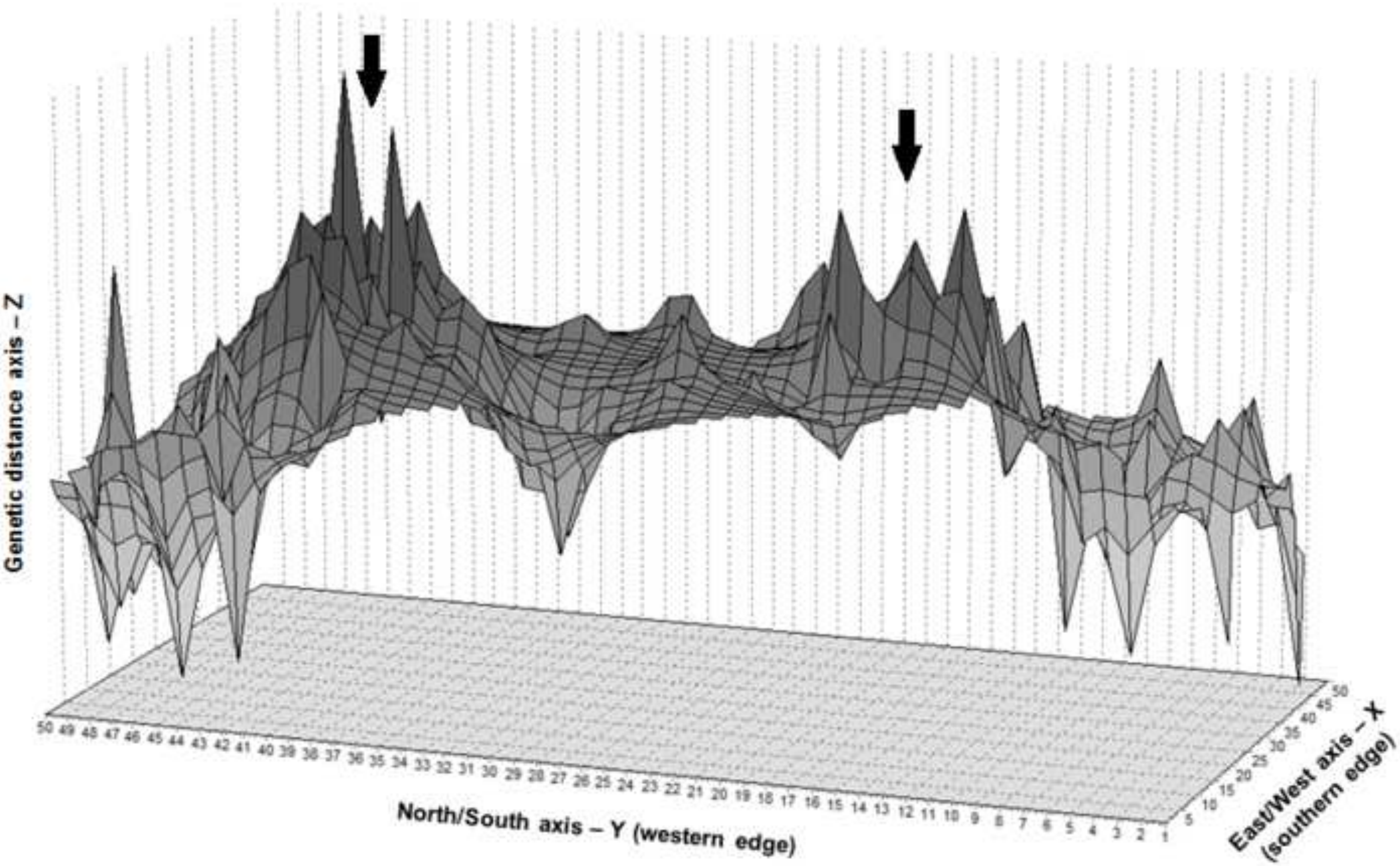

Genetic distance axis – Z
North/South axis – Y (western edge)
50 49 48 47 46 45 44 43 42 41 40 39 38 37 36 35 34 33 32 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
East/West axis – X (southern edge)
5 10 15 20 25 30 35 40 45 50

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	TCAAAGACAATGTCCCCTA ATTTTCCAGCCTTGATCTG	$(AC)_{15}$	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	Ho	Reference
Chaetophractus vellerosus	**69**	**6**	**0.58**	**This study**
Dasypus novemcinctus	310	7	0.49	Prodöhl et al. (1996)
Dasypus novemcinctus	139	4	0.64	Loughry et al. (2009)
Dasypus novemcinctus	40	9	0.46	Chinchilla et al. (2010)
Dasypus novemcinctus	116	5	0.62	Arteaga et al. (2012)
Bradypus variegatus	32	18	0.71	Moss et al. (2012)
Choloepus hoffmannii	23	16	0.55	Moss et al. (2011)
Myrmecophaga tridactyla	15	6	0.61	García et al. (2005)
Myrmecophaga tridactyla	27	5	0.059	Collevatti et al. (2007)

n, individuals. H_o, observed heterozygosity.