Using non–invasive genetic techniques to assist in maned wolf conservation in a remnant fragment of the Brazilian Cerrado

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Abstract

Using non–invasive genetic techniques to assist in maned wolf conservation in a remnant fragment of the Brazilian Cerrado. The maned wolf is a South American canid considered a keystone species of the Cerrado. We performed a genetic assessment of maned wolves that inhabit a small remnant fragment of the Cerrado in Brazil. We collected 84 fecal samples over a year and also included two tissue samples from road–killed animals. We successfully identified the species, sex, and individuals using molecular markers. Using microsatellite loci analysis we identified 13 different individuals, eight females and five males. The genetic variability level found and the high number of individuals detected indicates the presence of an open population.

Key words: Fecal DNA, Microsatellite loci, ZFX–ZFY, Real time PCR, Neotropical canid

Resumen

Utilización de técnicas genéticas no invasivas para contribuir a la conservación del aguará guazú en un fragmento residual del Cerrado de Brasil. El aguará guazú es una especie de cánido sudamericano que se considera clave en el Cerrado. Se realizó un estudio genético de varios individuos de aguará guazú que habitan en un pequeño fragmento del Cerrado de Brasil. Se colectaron 84 muestras fecales durante un año y se incluyeron también dos muestras de tejidos de animales atropellados. Se determinaron la especie, el sexo y los individuos mediante marcadores moleculares. Asimismo, se identificaron 13 individuos mediante la amplificación de *loci* de microsatélites, de los cuales ocho eran hembras y cinco, machos. El grado de variabilidad genética observado y el elevado número de individuos detectados indican la presencia de una población abierta.

Palabras clave: ADN fecal, *Loci* de microsatélites, ZFX–ZFY, PCR en tiempo real, Cánidos neotropicales

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Introduction

The maned wolf is the keystone canid species of the Brazilian Cerrado, the largest and richest tropical savanna in the world, and one of the most threatened biomes in South America (Klink and Machado, 2005). The IUCN listed it as Near Threatened because of the drastic reduction of suitable habitats for their survival. In Brazil it is categorized as Endangered (De Paula et al., 2008). The Cerrado is considered 'the central range of this species', and has lost approximately 50 % of its native vegetation (Klink and Machado, 2005).

In this scenario, fragmented and isolated protected areas are too small for their long–term viability (Do Passo Ramalho et al., 2014; Lion et al., 2011). It is therefore critical to understand the amount of movement in and out of these areas. However, only two studies of maned wolves have been carried out to monitor population size and genetic diversity at a local scale in these protected areas (Do Passo Ramalho et al., 2014; Lion et al., 2011). Do Passo Ramalho et al. (2014) analyzed a maned wolf population in Jataí Ecological Station (São Paulo). The geographic distance between Jataí and our study area, the Estação Ecológica de Itirapina (EEI), is about 180 km. Habitat loss and fragmentation could lead to a rapid and dramatic decline if maned wolves are unable to disperse from one Cerrado patch to another.

Our aim was to assess the population genetic diversity of maned wolves in the EEI, an area that contains a remnant fragment of the Cerrado biome in São Paulo (Brazil) near other protected areas where maned wolf population status have previously been assessed. Our results provide valuable information to evaluate the population dynamics of maned wolves at a fine, local scale.

Material and methods

The EEI (22º 11'–22º 15' S and 47º 51'–48º 00' W, São Paulo–Brazil) is inside an array of cultivated areas (fig. 1).

We conducted monthly, six–day sampling surveys using the linear transect method. Each transect was 3 m wide and 200 m long. From March 2007 to February 2008, we collected 84 feces samples. Samples were stored in 50 ml sterile plastic tubes with 100 % ethanol and deposited in a freezer at –20 °C until DNA extraction. Two tissue samples were collected from road–killed animals of opposite sexes.

DNA extractions and PCR amplifications were conducted under sterile conditions with negative controls in *Núcleo de Pesquisa e Conservação de Cervídeos* (UNESP, Brazil). We used DNA Stool Mini Kit (Qiagen Inc. Valencia–California), following the manufacturer's instructions. DNA extractions from tissues were conducted following González et al. (2015a) procedures.

For species identification, we amplified a species–specific *D–loop* fragment of mitochondrial DNA (mtDNA) using the protocols of González et al. (2015b). The PCR products were purified using Zymo Research DNA Clean and Concentrator™ and sequenced in Macrogen and Institute Pasteur–Montevideo. Sequences were compared with the GenBank database using BLAST. We used MEGA 5 software to construct the alignment by Clustal X (Tamura et al., 2011).

Sex was determined through Real Time–PCR (RT–PCR) amplification of a fragment of the ZFX–ZFY genes, and High Resolution Melting Analysis (HRMA) (González et al., 2015a). Experiments were conducted in duplicate in a Rotor Gene 6000® (Corbett Research) (software version 1.7, Qiagen, UK).

We used twelve microsatellite loci that were previously established to reliably amplify in maned wolves and had sufficient levels of polymorphism and adequate non–exclusion probabilities of identity to assign parent pairs (Mannise et al., 2017). Multiplex PCR reactions and thermal profile were conducted as suggested by Mannise et al. (2017). PCR products were run using the LIZ 500 size standard on an ABI PRISM 3100 sequencer. We analyzed fragment size and genotypes in GENMARKER V1.75 (SoftGenetics). PCRs were repeated three and two times for homozygotes and heterozygotes, respectively.

We identified capture histories and the number of maned wolves resampled from multiple scats using GENECAP software (Wilberg and Dreher, 2004). We detected matching genotypes with a probability of 0.01, assuming that individuals could be siblings (Wilberg and Dreher, 2004). The probability of identity (P(id)) was calculated for Hardy–Weinberg equilibrium (HWE) and for sibling presence.

Genotyping errors were estimated with MICRO-CHECKER (Van Oosterhout et al., 2004); genotypes were corrected following Brooksfield´s (1996) estimation if genotyping errors were detected. We used GENEPOP (Raymond and Rousset 1995) to compute HWE, linkage disequilibrium (LD) and inbreeding coefficients (Fis). In case of disequilibrium, Bonferroni corrections were applied. We calculated number of alleles, expected and observed heterozygosity and allele frequencies using CERVUS (Marshall, 1998–2007). We applied the ML–RELATE software to analyze the relatedness coefficients and pedigree relationships among individuals (Kalinowski et al., 2006).

Results

We identified 58 fecal samples as being from maned wolves and we determined the presence of three *D–loop* haplotypes previously described in other maned wolf populations from Brazil (table 1) (González et al., 2015b). Of the twelve microsatellite loci tested, one (AHTK253) showed low amplification success. Samples with at least six reliable genotypes after replicates were included in further analyses.

We determined the presence of 13 different individuals sampled with P(id) = 3.57×10^{-11} for HWE and 1.18×10^{-4} for siblings (table 1, fig. 1). P(id) values were acceptable for accurate individual identification (< 0.05) (Woods et al., 1999).

We assigned eight females and five males based on the melting curve pattern (table 1). The mean melting curve was 82.95 ºC (SD = 0.22) for ZFX, and

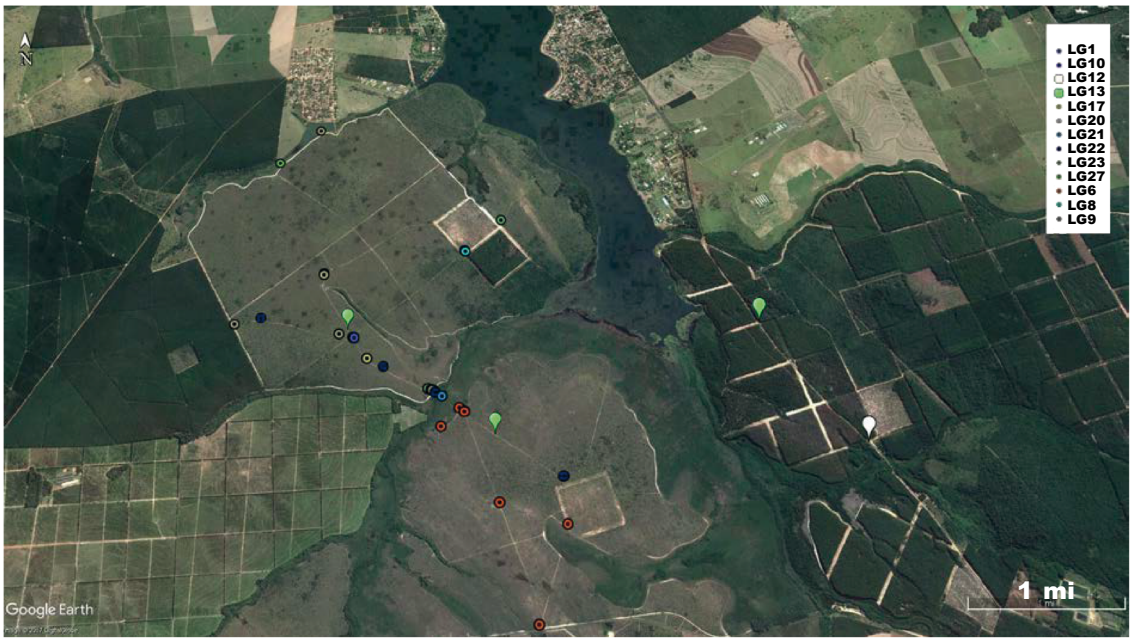

Fig. 1. Samples collected in the EEI. Each symbol represents a different individual; same shape represents re–sampled individuals. Road–killed animals and its re–sampled feces are shown by balloons.

Fig. 1. Muestras colectadas en la estación ecológica de Itidapina (EEI). Cada símbolo representa distintos individuos; la misma forma representa individuos que se han muestreado más de una vez. Los animales atropellados y sus muestras fecales muestreadas más de una vez se indican con globos.

82.08 ºC (SD = 0.17) for ZFY. The observed sex ratio was not significantly different from the expected 1:1 (X^2 = 0.69, p = 0.41).

The presence of null alleles in FH2561 has been previously detected in other maned wolf populations, for this reason we recommend that this locus should not be used in future studies.

We identified a minimum of three family groups (table 1). One comprised a mother with her son, two aunts and an uncle (table 1). Another included two full–siblings (brother and sister), and the third had two half–sisters (table 1).

Discussion

Our non–invasive genetic protocols allowed us to reliably identify species, gender, and individuals. We successfully amplified fecal DNA using microsatellite multiplex protocols to effectively genotype 67 % of the samples. Maned wolves in the EEI had high levels of polymorphism and similar levels of observed and expected heterozygosity (table 2).

We found that maned wolves in the EEI have moderate levels of mitochondrial genetic diversity and high levels of nuclear genetic diversity. Mean levels of heterozygosity (0.646) were slightly lower than other population heterozygosity estimates of maned wolf populations in Brazil (0.71 and 0.72) (Do Passo Ramalho et al., 2014; Lion et al., 2011).

The presence of three family groups in the EEI area during our surveys suggests that if dispersal in and out of this area were restricted, it would result in inbreeding and loss of genetic variability. In strong support of a recent genetic analysis conducted in maned wolves throughout their range (Mannise et al., 2017), our results here suggest that wolves maintain high levels of genetic diversity and may avoid inbreeding by dispersing through cultivated lands. Furthermore, these high levels of genetic variability coupled with the large number of individuals detected indicate the presence of an open population in the EEI. Effective conservation management strategies can benefit from approximate estimates of population size and non–invasive genetic studies such as this one (Kohn et al., 1999).

Our study provides valuable information regarding the genetic diversity of an imperiled canid species in a human–dominated landscape. It suggests that this small fragmented remnant of Cerrado can maintain several maned wolf family groups with adequate levels of genetic variability, and it highlights the essential need to protect the Cerrado habitat for their conservation. Future studies that include non–invasive

Table 1. For each individual we show: ID, identification number; ST, sample type; *D–lH, D–loop* haplotype; Genbank, Genbank accession number; sex; N, number of recaptures; and family group (PO, parent–offspring; FS, full–sibling; HS, half–sibling).

Tabla 1. Para cada individuo se muestra: ID, el número de identificación; ST, el tipo de muestra; D–lH*, el haplotipo para* D–loop*; GenBank, el número de accesión del Genbank; el sexo; N, el número de recapturas; y el parentesco (PO, padre–hijos; FS, hermanos; HS, medio hermanos).*

ID	ST	*D–lH*	Genbank	Sex	N	Family group
LG1	Feces	B	KM406503	Female	3	PO: LG10, HS: LG8 – LG21 – LG22
LG6	Feces	G	KM406508	Female	6	HS: LG12
LG8	Feces	B	KM406503	Female	1	HS: LG1 – LG21 – LG22
LG9	Feces	B	KM406503	Female	3	–
LG10	Feces	B	KM406503	Male	2	PO: LG1, HS: LG8 – LG21 – LG22
LG12	Skin	G	KM406508	Female	0	HS: LG6
LG13	Skin	G	KM406508	Male	2	–
LG17	Feces	B	KM406503	Female	1	–
Lg20	Feces	B	KM406503	Male	1	–
LG21	Feces	D	KM406505	Male	1	FS: LG22, HS: LG1 – LG8
LG22	Feces	D	KM406505	Female	3	FS: LG21, HS: LG1 – LG8
LG23	Feces	B	KM406503	Female	2	FS: LG27
LG27	Feces	B	KM406503	Male	1	FS: LG23

Table 2. For the overall dataset and for each locus: k, number of alleles; Ta, annealing temperature; Ho, observed heterozygosity; He, expected heterozygosity; Pic, polymorphic information content; Fis, inbreeding coefficient; Ge, presence/absence of genotyping errors; * loci that after Bonferroni corrections slightly deviate from HWE.

Tabla 2. Para el conjunto de datos y para cada locus se muestra: k, número de alelos; Ta, temperatura de unión al cebador; Ho, heterocigosidad observada; He, heterocigosidad esperada; Pic, contenido de información polimórfica; Fis, coeficiente de endogamia; Ge, presencia/ausencia de errores de genotipado; * loci *que después de las correcciones de Bonferroni se desvían ligeramente del equilibrio Hardy–Weinberg (HWE).*

Locus	k	Ta (ºC)	Ho	He	Pic	Fis	Ge
FH2140*	8	58	1	0.883	0.831	–0.139	No
FH2328*	10	58	0.556	0.928	0.864	0.416	Null alleles
FH2137*	6	58	0.636	0.788	0.713	0.2	No
FH2535	6	58	0.846	0.748	0.687	–0.138	No
FH2848	6	58	0.833	0.808	0.741	–0.033	No
REN105	2	58	0.25	0.489	0.359	0.5	No
PEZ19	3	58	0.444	0.386	0.327	–0.164	No
FH2561*	10	58	0.583	0.899	0.836	0.361	Null alleles
FH2226	9	58	0.846	0.886	0.836	0.047	No
FH2054*	6	60	0.25	0.757	0.683	0.679	Null alleles
REN169	3	60	0.167	0.163	0.15	–0.023	No
Overall	6.27	–	0.589	0.646	0.583	0.168	–

surveys outside these protected areas will be crucial to evaluate movement and corridor connectivity within this fragmented landscape.

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