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Article

GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE PAMPAS FOX, *Lycalopex gymnocercus*, IN A HUMAN-DOMINATED LANDSCAPE OF SOUTHERN ESPINAL, ARGENTINA

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ABSTRACT. Habitat fragmentation and the associated landscape connectivity loss can reduce gene flow among populations, which could lead to a decrease in genetic variability, and an increase in the extinction risk of a species. The main goal of this study was to provide genetic diversity and population structure data for Pampas foxes from the southern Argentine Espinal ecoregion. Through the analyses of 30 tissue samples collected during 2013-2018, we were able to genotype 23 individuals at 18 microsatellite loci. Results indicate high diversity values, and exhibiting an average of 10 alleles per locus (min= 6, max= 13), 0.71 (range= 0.35-0.91) of mean observed heterozygosity and 0.83 (min= 0.61, max= 0.91) of mean expected heterozygosity. Bayesian analyses suggest no population structure. Finally, we did not detect a pattern of isolation by distance. The high habitat adaptability and the generalist feeding behavior of Pampas fox may explain our findings, which are in agreement with the general patterns described for other fox species. The panel of microsatellites employed in this study proved to be suitable for Pampas fox genotyping and it can be used in further genetic assessments needed to validate and expand our understanding of its population genetics.

RESUMEN. DIVERSIDAD GENÉTICA Y ESTRUCTURA POBLACIONAL DEL ZORRO PAMPEANO *LYCALOPEX GYMNOCERCUS*, EN UN PAISAJE ANTROPIZADO DEL SUDOESTE DEL ESPINAL ARGENTINO. La fragmentación del hábitat y la pérdida asociada de conectividad del paisaje influencian el flujo génico entre poblaciones, lo que puede conducir a una disminución de la variabilidad genética, aumentando el riesgo de extinción de una especie. El objetivo principal de este estudio fue proveer datos sobre la diversidad genética y la estructura poblacional del zorro pampeano en la zona sur de la ecorregión del Espinal argentino. A través del análisis de 30 muestras de tejidos colectadas durante 2013-2018, genotipamos 23 individuos con 18 loci de microsatélites. Nuestros resultados indican valores de diversidad altos, exhibiendo en promedio 10 alelos por locus (min= 6, max= 13), 0.71 (min= 0.35; max= 0.91) de heterocigosis media observada y 0.83 (mín= 0.61; máx= 0.91) de heterocigosidad esperada. Los análisis bayesianos sugieren la ausencia de estructura poblacional. Finalmente, no se detectó un patrón de aislamiento por distancia. La elevada capacidad de adaptarse a diferentes hábitats y la dieta generalista del zorro pampeano podrían explicar nuestros resultados, los cuales concuerdan con los patrones generales descriptos para otras especies de zorros. El set de microsatélites empleado en este estudio demostró ser adecuado para genotipar al zorro pampeano y podría utilizarse en evaluaciones genéticas adicionales necesarias para validar y ampliar nuestra comprensión de la genética poblacional de la especie.

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Key words: Canidae, genetic diversity, Pampas fox, population structure.

Palabras clave: Canidae, diversidad genética, estructura poblacional, zorro gris pampeano.

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INTRODUCTION

Genetic diversity, which is the variation of alleles and genotypes within a population of a given species, is the most basic component of biodiversity (Frankham et al. 2002; Hughes et al. 2008). In order to ensure the evolutionary potential of the species, to maintain genetic diversity is one of the most important aspects of conservation biology (Frankham 2010), mainly because it provides adaptive potential, which can be crucial given the ongoing human-mediated modification of natural habitats (Doyle et al. 2015).

The effects of fragmentation (loss of habitat and connectivity) include, among others, a decrease in population sizes, which results in an increased level of inbreeding and subsequent loss of genetic diversity (Keyghobadi 2007). However, landscape characteristics do not determine alone the genetic structure of population, it also depends on species-specific biological traits (Wereszczuk et al. 2017). Actually, dispersal capacity can be directly associated with species ability to maintain genetic diversity in fragmented landscapes (Mcmanus et al. 2015; Evans et al. 2018). For habitat-specialist species with low movement capacity, landscape features are expected to have a predominant effect on gene flow and genetic structure, where gaps between suitable habitat patches would serve as barriers to dispersal (Henle et al. 2017). By contrast, habitat-generalist species with high movement capacity are likely to exhibit much less genetic differentiation between populations (Deyoung et al. 2009). However, studies conducted on broadly distributed species have revealed cryptic patterns of genetic structuring in apparently continuous populations (Geffen et al. 2004; Hapeman et al. 2011; Wereszczuk et al. 2017; Silva et al. 2018).

Globally and locally categorized as "least concern" (Lucherini 2016; Luengos Vidal et al. 2019), the Pampas fox (*Lycalopex gymnocercus*) is a medium-sized canid with a wide distribution in South America, being present in Bolivia, Paraguay, Brazil, Uruguay, and Argentina. The species has received attention in some ecological and social aspects of its biology, including diet (Farias & Kittlein 2008; Castillo et al. 2011), activity patterns (Faria-Corrêa et al. 2009), spatial and social organization (Luengos Vidal et al. 2012), and habitat use (García & Kittlein 2005; Faria-Corrêa et al. 2009; Caruso et al. 2016). Regarding genetic issues, several studies within genus *Lycalopex* with a phylogeographic or taxonomic focus have relied on mitochondrial and nuclear DNA sequencing (Bardeleben et al. 2005; Tchaicka et al. 2016; Chemisquy et al. 2019). In contrast, microsatellites-based assessments of *L. gymnocercus* population genetics are virtually absent from literature.

Microsatellites are one of the most popular and versatile genetic markers with applications in population genetics, conservation biology, and evolutionary biology studies (Haasl & Payseur 2011). The major advantages of these markers are their codominant inheritance, high polymorphism and mutation rate, and their generally small molecular size, which allows to work with low quality and quantity of DNA (Wandeler et al. 2007). They are particularly useful to evaluate genetic diversity in widely distributed and highly mobile species (Hapeman et al. 2011; Basto et al. 2016). Usually, high numbers of microsatellite markers can provide robust inferences of population genetics (Ryman et al. 2006), but panels within 8 to 20 microsatellites are considered informative enough to uncover genetic structure (Koskinen et al. 2004; Vartia et al. 2014; Tibihika et al. 2019). Finally, although the use of species-specific markers is preferable because of ascertainment bias, existing primers are often applicable across species, genera, and even families (Garner et al. 2005). Their application regardless of species boundaries allows to reduce the time and cost of the work required for the development of novel microsatellite markers (Garner et al. 2005), as well as facilitating multispecies assessments.

This study aims to investigate the patterns of genetic diversity and the degree of genetic structuring among Pampas fox living in a heavily anthropized landscape of southern Argentine Espinal ecoregion. We tested and used a set of non-specific microsatel-

lite markers, which allowed us to conduct the first population genetics survey for the species.

MATERIALS AND METHODS

Study Area

This study was carried out in southern Buenos Aires province, central-east Argentina (**Fig**. 1). The natural vegetation corresponds largely to the Espinal ecoregion, which is characterized by xerophytic deciduous woodlands (dominated by *Prosopis* spp.), prairies intermixed with natural patches of spontaneous scrub vegetation, and grassland prairies (Arturi 2005). During the last decades, this region has faced a marked intensification of agriculture and ranching activities, converting the original landscape into isolated natural vegetation patches surrounded by a cropland matrix (Caruso et al. 2016). Moreover, the intense and widespread livestock overgrazing has caused strong soil and native vegetation degradation (Fernández & Busso 1999). Although Pampas fox is protected across most of the study area, the negative perception of farmers toward the species, considered as a "plague", led to heavy human persecution of this carnivore (Caruso et al. 2016).

Sample collection, DNA extraction and microsatellites genotypes

Between 2013 and 2018, we opportunistically collected 30 tissue samples (muscle: n = 18; skin: n = 12; **Fig**. 1) from roadkill animals by inspecting paved and dirt roads. All samples were georeferenced and preserved in 96% ethanol at -20 °C until DNA extraction. We extracted DNA using the salt protocol (modified by Aljanabi & Martinez 1997) or standard phenol–chloroform procedure (Blin & Stafford 1976). We genotyped the samples using a panel of 46 dog microsatellites (Table S1). Our amplification conditions followed the ones previously used for the gray wolf (*Canis lupus*; Silva et al. 2018); (Table S2). PCR products were separated by size on an ABI 3130xl DNA analyzer (Applied Biosystems, Foster City, California) using the GeneScan500 LIZ size standard. Alleles were scored and checked manually using GENEMAPPER v5.0 (Applied Biosystems, Foster City, California).

Diversity estimation and population structure analysis

We first evaluated loci for amplification success and missing data, allowing a maximum of 10% of missing data for each locus retained in the final dataset. We estimated null allele frequencies with MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004) and the probability of identical genotypes being shared by chance among unrelated individuals (Probability of Identity, $PID_{unbiased}$) and full-sibs (PID_{Sib}) using GIMLET v1.3.3 (Valière 2002). Based on the exact test of Guo & Thompson (1992) and the sequential Bonferroni correction of the p-values for significance level (Rice 1989), we tested for deviations from Hardy-Weinberg equilibrium (HWE) and pairwise linkage disequilibrium (LD) between all pairs of loci (16×10^4 permutations) using ARLEQUIN v3.5 (Excoffier & Lischer 2010). Genetic diversity, as well as the number of alleles per locus (Na), and observed (Ho) and expected heterozygosity (He), were measured using the same software, while FSTAT v2.9.3.2 (Goudet 1995) was used to estimate the allelic richness (Ar).

We investigated population structure using a Bayesian approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000), assuming no prior population information, the admixture model, and correlated allele frequencies. We performed twenty independent runs for each putative population number (K, ranging from 1 to 5), where each run consisted of 10^6 Markov Chain Monte Carlo (MCMC) iterations with a burn-in of 100 000 steps. To identify the most likely number of genetic clusters, we observed the likelihood of the posterior probability of K [LnP(K)] using Structure Harvester (Earl & Vonholdt 2012). We tested for the presence of isolation by distance (IBD) using the Mantel test implemented in GENALEX 6.5 (Peakall & Smouse 2006) to test for correlation between genetic distance and the logarithm of geographic distance, considering 9999 permutations for significance level. Finally, we estimated the extent of spatial autocorrelation of pairwise genetic relatedness (r) in the same software. This approach compares the pairwise geographic and squared individual genetic distance matrices to calculate an autocorrelation coefficient for each of a series of predetermined distance classes. We performed this analysis using seven predetermined distance classes (50 km), 9999 random permutations of genotypes among individuals, and 10 000 bootstrap estimates of *r*.

RESULTS

Among the 46 microsatellites tested, twenty-one were successfully amplified for Pampas fox (**Table** 1. We were able to genotype 23 of the original 30 samples (76.2% were complete profiles; **Fig**. 1, **Table** 1), corresponding to 76.6% of genotyping success (88.9% muscles, 58.3% skins). Due to percentages higher than the threshold imposed for missing values, we discarded three loci for further analyses (AHT103, C14.866, and CPH14; **Table** 1).

The remaining 18 microsatellite loci provided sufficient power to distinguish unique individuals ($Pid_{unbiased} = 1.34x10^{-24}$, $Pid_{sib} = 7.56x10^{-09}$). MICRO-CHECKER suggested that null alleles may be present at four loci (C04.140, AHT121, ATH111 and INU030; **Table** 2) which were discarded for population structure analysis. Three of them (C04.140, AHT121, and INU030) showed a highly significant deviation from HWE ($p< 0.0001$ after Bonferroni correction; **Table** 2) which may be related to a high frequency of null alleles. No linkage disequilibrium was found between any pair of loci.

All 18 microsatellites retained were polymorphic, exhibiting on average 10 alleles per locus (range: 6–13) and a mean allelic richness of 9.91 (**Table** 2). We did not find rare alleles (frequency < 1%, Fig. S1). Both observed (Ho) and expected (He) heterozygosity per locus were high, with mean values of 0.71 (range= 0.35-0.91) and 0.83 (range= 0.61-0.92), respectively (**Table** 2).

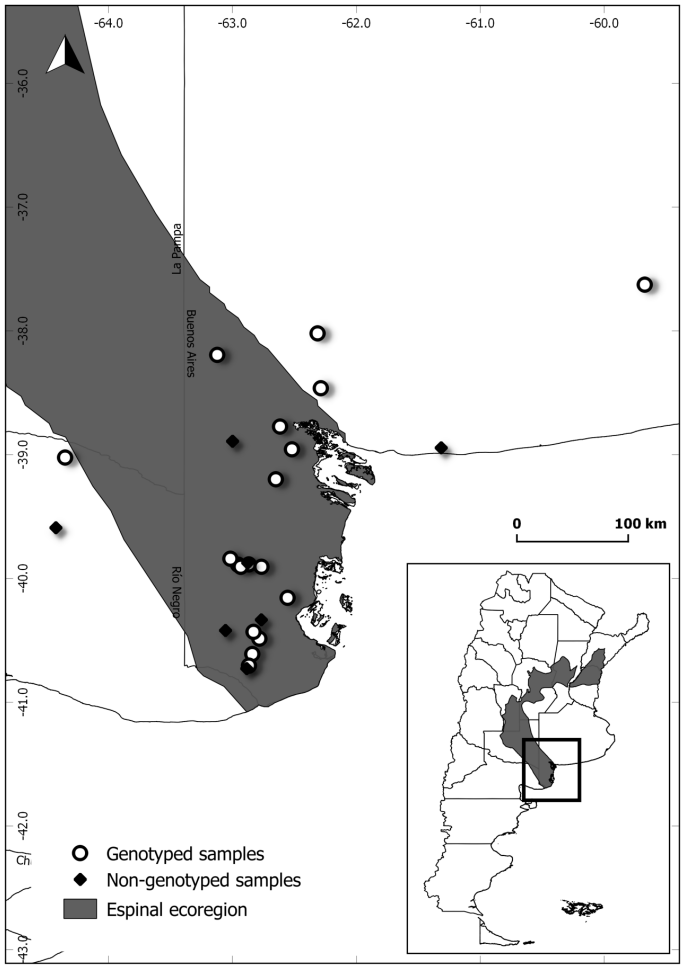

Fig. 1. Spatial distribution of the 30 samples (genotyped: n = 23; non-genotyped: n = 7) of Pampas fox collected in the southern Argentine Espinal ecoregion.

Bayesian clustering analyses could not reveal the presence of genetic structure among Pampas foxes from of southern Argentine Espinal, assigning all individuals to the same group (K= 1, exhibiting the highest log probability of the data, and thus being the most likely scenario; **Fig**. 2). Moreover, a pattern of IBD was not detected across the study area (Mantel statistic r= 0.012, p> 0.457; **Fig**. 3). Finally, spatial autocorrelation analyses showed that r is not significantly different from zero for all distances considered (**Fig**. 4).

DISCUSSION

This study provides the first insights on genetic diversity and population structure for the Pampas fox using non-specific microsatellite markers. To our knowledge, the only available set of eleven microsatellite markers for the genus *Lycalopex* was developed for *L. fulvipes*, which was also successfully amplified in two other species of the genus (*L. griseus* and *L. culpaeus*; Cabello & Dávila 2014). However, we followed the strategy of testing a larger number of markers widely used among canid species of different genera, including Neotropical canids (e.g. Da Fontoura-Rodrigues et al. 2008; Leite et al. 2015; Tensen et al. 2019; Eddine et al. 2020), allowing comparisons with other members of the Canidae family. Among the 46 markers tested for the Pampas fox, 18 were consistently amplified and exhibited

Table 1
Allele size range and amplification success of 21 heterologous (dog) microsatellite loci for Pampas fox.

Locus	Allele size range (bp)	Amplification success rate (%)
AHT103	100-108	73.91
AHT111	100-124	100
C04.140	162-186	100
C13.758	246-262	95.65
C14.866	257-275	56.52
C20.253	121-145	100
C09.173	120-138	100
CPH14	209-223	69.56
VWF	139-205	100
C08.618	204-226	100
CPH05	113-147	100
FH2010	219-255	95.65
PEZ3	130-184	100
AHT121	100-150	100
AHT137	144-184	100
INRA21	105-125	100
INU030	152-178	100
INU055	225-243	100
REN169D1	218-228	100
REN169O18	156-186	100
REN247M23	279-305	100

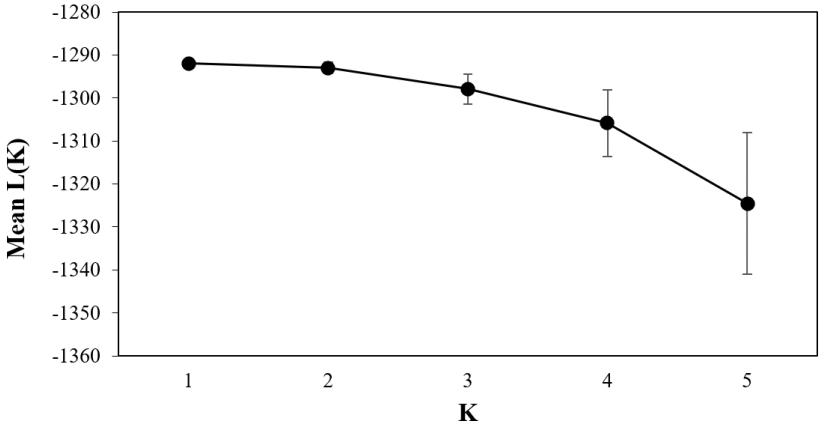

Fig. 2. The average likelihood of the posterior probability of K [LnP(K)] ± *SD* obtained considering an increasing number of clusters (*K*= 1-5) for 23 genotyped Pampas foxes from the southern Argentine Espinal ecoregion. Results indicate K= 1 as the best scenario.

high polymorphism, making them suitable for the study of our target species.

The opportunistic sampling of roadkill animals represents a good source of tissue samples for molecular studies of elusive carnivore species (Dixon et al. 2007; Deyoung et al. 2009; Wereszczuk et al. 2017). However, the quality of DNA obtained from this source is often low because of the post-mortem de-

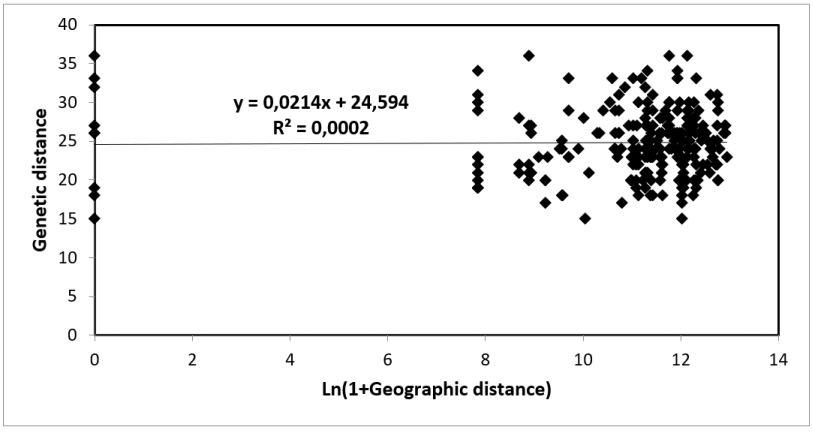

Fig. 3. Scatterplot showing the results of the Mantel test between the matrix of genetic distances and the matrix of geographic distances to test for the presence of isolation by distance for Pampas foxes from southern Argentine Espinal ecoregion.

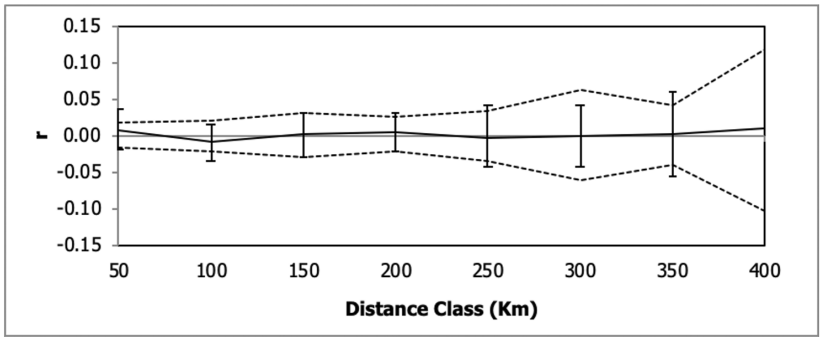

Fig. 4. Correlogram of spatial autocorrelation coefficient (r) for Pampas foxes from southern Argentine Espinal ecoregion. Dashed lines indicate a 95% significance envelope (obtained through 9999 permutations) surrounding the null hypothesis of randomly distributed genotypes, and vertical bars correspond to the 95% bootstrap distribution (10000 iterations) of r.

composition process (Rodríguez-Castro et al. 2017). Despite that, we could achieve a high percentage (76.6%) of genotyped samples, though muscles were usually fresher and performed better than skins.

All the diversity estimators showed high genetic variability for Pampas foxes of southern Espinal, which is consistent with those reported for other medium-sized wild canids (Lassevich 2014; Basto et al. 2016; Zecchin et al. 2019). Similarly, the absence of population structure observed for the Pampas fox across the southern Espinal is congruent with the patterns described for other widely and continuously distributed canids, such as the red fox (*Vulpes vulpes*) and the African wolf (*Canis lupaster*). Their long-distance dispersal capacity, habitat adaptability, and generalist feeding behavior have been pointed to explain the weak structuring and high gene flow described for these carnivores (Teacher et al. 2011; Galov et al. 2014; Mullins et al. 2014; Karssene et al. 2019; Zecchin et al. 2019; Eddine et al. 2020). All of these characteristics are also exhibited by Pampas fox (Lucherini & Luengos Vidal 2008), which can show dispersal movements up to 15 km in Pampas grassland areas (Luengos Vidal 2009). In addition, its generalist and opportunistic feeding behavior (García & Kittlein 2005; Castillo et al. 2011) allows the species to inhabit rural areas with high human-disturbance levels (Canel et al. 2016; Caruso et al. 2016).

Despite the habitat loss and degradation of the Espinal ecoregion, our results indicated that the genetic status of Pampas fox does not yet reflect the

Table 2

Genetic diversity at 18 microsatellite loci of Pampas foxes from southern Argentine Espinal ecoregion. N= number of individuals, Na= number of alleles, Ar= allelic richness, Ho= observed heterozygosity, He= expected heterozygosity, NAF= null-allele frequency, (p) HWE= Hardy-Weinberg equilibrium p-values. *Significant deviation from HWE after sequential Bonferroni correction ($p < 0.01$).

Locus	N	Na	Ar	Ho	He	NAF	(p)HWE
AHT111	23	13	12.87	0.70	0.91	0.108	0.01
C04.140	23	11	10.83	0.57	0.88	0.168	0.00*
C13.758	22	9	9	0.82	0.86	0.009	0.40
C20.253	23	12	11.83	0.91	0.90	-0.017	0.82
C9.173	23	9	8.91	0.61	0.69	0.061	0.25
VWF	23	8	7.91	0.61	0.73	0.085	0.19
C08.618	23	10	9.96	0.83	0.88	-0.016	0.52
CPH05	23	9	8.91	0.91	0.81	-0.075	0.48
FH2010	22	7	7	0.82	0.82	-0.011	0.46
PEZ03	23	13	12.82	0.74	0.88	0.066	0.01
AHT121	23	11	10.87	0.35	0.89	0.295	0.00*
AHT137	23	13	12.87	0.87	0.92	0.014	0.59
INRA21	23	11	10.95	0.74	0.89	0.076	0.08
INU030	23	12	11.95	0.57	0.91	0.178	0.00*
INU055	23	8	7.99	0.74	0.82	0.034	0.04
REN169D01	23	6	5.96	0.74	0.72	-0.031	0.18
REN169O18	23	9	8.82	0.52	0.61	0.057	0.46
REN247M23	23	9	8.96	0.74	0.88	0.064	0.02
Mean (±SD)		10 (± 2.11)	9.91 (± 2.07)	0.71 (± 0.15)	0.83 (±0.09)		

human disturbances in the area. This agrees with ecological evidence showing that Pampas fox is a species with resilience to natural habitat modifications, as well as to a certain level of hunting pressure (Farias & Kittlein 2008; Caruso et al. 2016; Lucherini 2016). However, acknowledging our limited sample size and the potential future synergic effect of human disturbances over this population of Pampas fox (Luengos Vidal et al. 2019), our results are meant to be a preliminary evaluation of its population genetics; caution is required for its consideration concerning the conservation of the species.

Further assessments on population genetics, based on larger and more comprehensive sampling efforts across the Pampas fox distribution range, will help to better understand the population structure of this carnivore, the spatial distribution of its genetic diversity, and both functional and landscape connectivity. Filling these knowledge gaps is paramount for the development of adequate conservation strategies.

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