



Species delimitation and intraspecific diversification in recently diverged South American foxes

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Abstract

The divergence between the Andean fox (*Lycalopex culpaeus*) and the South American gray fox (*L. griseus*) represents a recent speciation event in South America. These taxa are partially sympatric and share biological, morphological, and ecological traits. Previous studies failed to recover reciprocal monophyly, suggesting the occurrence of introgression or incomplete lineage sorting (ILS). Here, we obtained mitochondrial and nuclear markers for 140 *L. culpaeus* and 134 *L. griseus* from the Southern Cone of South America to assess their inter and intraspecific divergence. We recovered reciprocal monophyly of *L. culpaeus* and *L. griseus*, with mild signatures of introgression or ILS. Therefore, taxonomic misidentification and the use of a limited number of markers may be the main reason behind the past debate about the delimitation of both species. Two main divergent clades were found in *L. culpaeus* with a phylogeographical boundary in the High Plateau of northeastern Chile. The southern clade along with three northern sub-clades corresponded to four morphological subspecies. Less genetic differentiation was found in *L. griseus* with a spatial population structure that does not support the occurrence of distinct subspecies. The results found in this study suggest the extant evolutionary significant units that need to be considered for biological conservation management of these species.

Keywords Andean fox · South American gray fox · Genetic structure · Hybridization · Genetic diversity · Phylogeographic structure

Introduction

South American canids diverged from their North American counterpart after the rise of the Panamanian land bridge during the late Pliocene (Berta 1987; Perini et al. 2010; Prevosti 2010). Despite the recent expansion from North America into South America, the largest diversity of the family is found in the latter (Wozencraft 2005), favored by their opportunistic foraging including small and medium-sized preys, fruits, and seeds in their diet. This likely allowed them to persist and diversify after the extinction of large specialized canids at the end of the Pleistocene such as the *Theriodictis platensis* (Prevosti and Forasiepi 2018), when large

herbivores went extinct (Berta 1987). The diversification of *Lycalopex* occurred rapidly and recently during the Pleistocene 1.3 million years ago (Perini et al. 2010; Tchaicka et al. 2016). This genus, endemic to South America, consists of six species: the sechuran fox (*L. sechurae*, Thomas 1900), the hoary fox (*L. vetulus*, Lund 1842), the pampas fox (*L. gymnocercus*, Fischer 1814), the Darwin's fox (*L. fulvipes*, Martin 1837), the Andean fox (*L. culpaeus*, Molina 1782), and the South American gray fox (*L. griseus*, Gray 1837). The last four species occur across the Southern Cone of South America (Wozencraft 2005).

Within the evolution of *Lycalopex*, *L. culpaeus* and *L. griseus* divergence was found as the most recent (250–800 kya) (Wayne et al. 1989; Bininda-Emonds et al. 1999; Tchaicka et al. 2016; Favarini et al. 2022), but the internal taxonomy of the genus is still debated (Berta 1987; Medel et al. 1990; Zunino et al. 1995; Vilà et al. 2004; Favarini et al. 2022), leaving uncertainties about the evolutionary relationships. Therefore, phylogeographic studies represent a useful tool to

This work is dedicated to Daniel González-Acuña who passed away during the preparation of this study.

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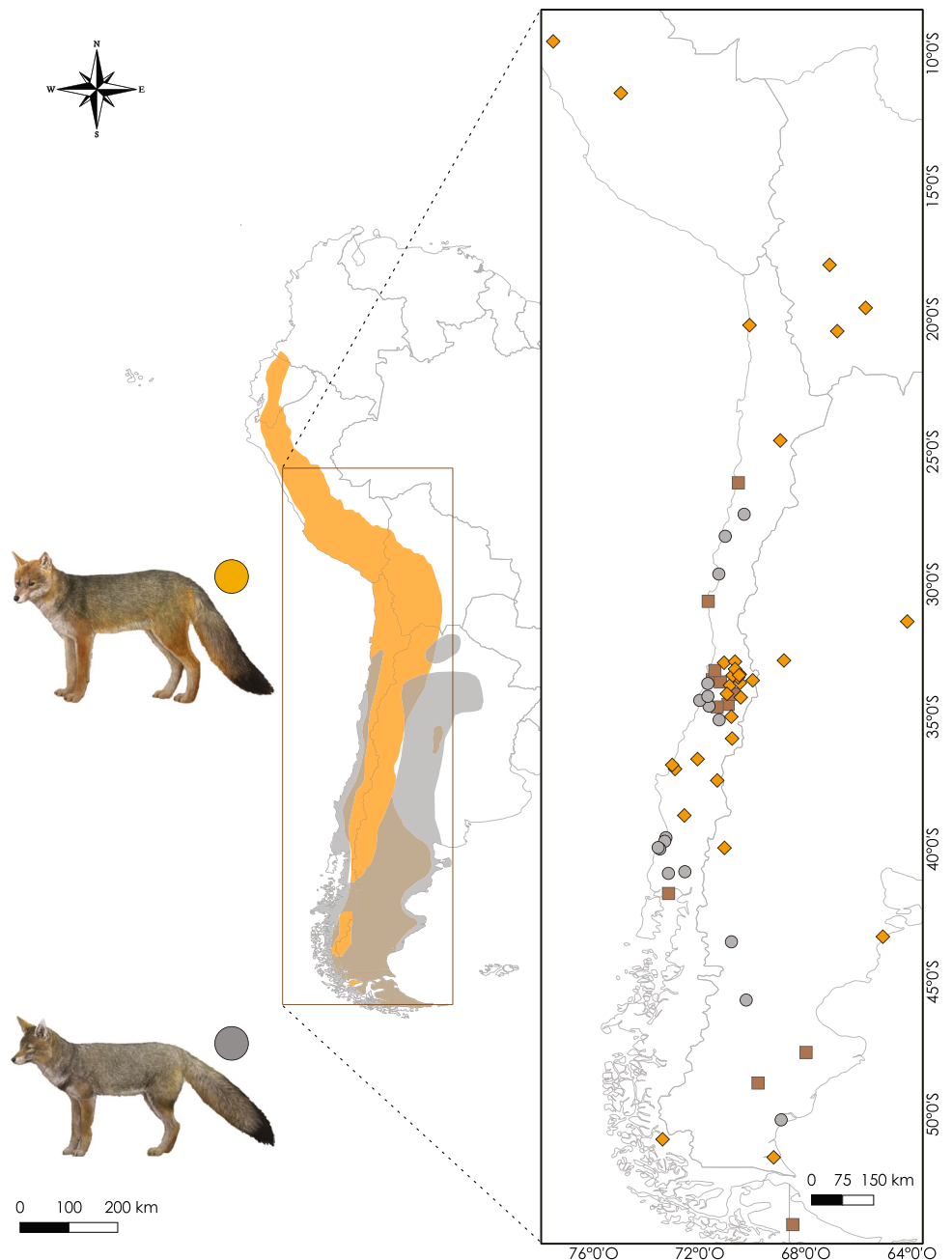
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provide an evolutionary hypothesis for the divergence of the species (Perini et al. 2010). A few studies based on nuclear and mitochondrial molecular markers failed to recover the monophyly of both species (Yahnke 1995; Yahnke et al. 1996; Prevosti et al. 2013; Ruiz-Garcia et al. 2013). More recently, Tchaicka et al. (2016) and Favarini et al. (2022) obtained reciprocal monophyly, but with low samples size by locality and species, and with some individuals found in different clades than the expected by the classification of the morphological species. Therefore, the uncertainties found may be attributed to introgression or incomplete lineage sorting (ILS), two recognized processes that have hampered

the species delimitation in canids (Gopalakrishnan et al. 2018).

Lycalopex culpaeus and *L. griseus* have a wide distribution in the Southern Cone of South America (Fig. 1). They can be distinguished from one another mostly by their body size and coat color. The former is larger (total length: 100–180 cm; weight: 4–13 kg) and displays a reddish pelage, while the latter is smaller (total length: 70–96 cm; weight: 2.5–4 kg) with a beige-grayish pelage (González del Solar and Rau 2004; Jiménez and Novaro 2004; Iriarte 2008). *L. culpaeus* is the largest fox in its genus (Jiménez and Novaro 2004) and is widely distributed across western

Fig. 1 **A** Geographical distribution of *Lycalopex culpaeus* (orange) and *L. griseus* (gray). The distributions are based on IUCN data. **B** Location of sampling sites for *L. culpaeus* (orange diamonds), *L. griseus* (gray circles), and both species (brown squares)



South America. It lives along the Andes up to 4500 masl (Iriarte 2008) in Colombia, Ecuador, Peru, Bolivia, Chile, and Argentina (Novaro 1997), ranging from Nariño Province of southern Colombia to Tierra del Fuego (Jiménez et al. 1995). In Chile, it can be found down to the Pacific shoreline in the northern desert (Jiménez and Novaro 2004), while in Argentina, it can reach the Atlantic shoreline from Río Negro to the south (Novaro 1997). Six subspecies are recognized: *L. c. andinus*, *L. c. culpaeus*, *L. c. lycoides*, *L. c. magellanicus*, *L. c. reissii*, and *L. c. smithersi* (Gray 1837; Thomas 1914a, b; Cabrera 1931), but their distributions and genetic distinctiveness have been partially defined (Martinez et al. 2018). On the other hand, *L. griseus* inhabits plains and mountains on both sides of the Andes in Chile and Argentina, down to sea level and up to 3000 masl. In Chile, this species ranges from Arica to Tierra del Fuego, while in Argentina, it can be found south of the Río Negro to the Strait of Magellan (González del Solar and Rau 2004). There is evidence that *L. griseus* is also present in Peru from central Lima to Tacna, along the coast and western slopes of the Andes (Pacheco et al. 2009; Vivar and Pacheco 2014). Four subspecies are generally recognized based primarily on geographic distribution: *L. g. griseus*, *L. g. domeykoanus*, *L. g. maullinicus*, and *L. g. gracilis* (Osgood 1943; González del Solar and Rau 2004). Noteworthy, *L. griseus* is not native to Tierra del Fuego (González del Solar and Rau 2004), since it was introduced in the 1950s to control the European rabbit (*Oryctolagus cuniculus*, Linnaeus, 1758; Atalah et al. 1980; Zurita et al. 2023).

Lycalopex culpaeus and *L. griseus* are two of the most broadly distributed mammals in Chile (Iriarte 2008). Despite their similar geographic ranges, these species do not exhibit high levels of sympatry at the local scale (Zapata et al. 2005), presumably because of interspecific territoriality and competition, with *L. culpaeus* excluding *L. griseus* given to its bigger size (Fuentes and Jaksic 1979; Medel and Jaksic 1988; Johnson and Franklin 1994; Jiménez et al. 1996; Donadio and Buskirk 2006).

The occurrence of hybrid zones had emerged in previous studies (Yahnke et al. 1996) as well as the challenges associated with species delimitation due to intermediate morphology. Also, it is expected an intermediate level of population structure, with areas of higher genetic differentiation where gene flow is limited by geographic barriers such as the Andean Mountain and the Atacama Desert in Chile. The main aim of this study was to assess the geographic distribution of genetic diversity of *L. culpaeus* and *L. griseus* in southern South America, exploring possible events of hybridization in sympatry. The specific objectives were (1) to compare the phylogeographical pattern of both species; (2) to assess the genetic diversity and degree of divergence between them; and (3) to determine intraspecific lineages of *L. culpaeus* and *L. griseus*. For this purpose, we

characterized the Control Region (CR) and the Cytochrome-b (MT-CYB) of the mitochondrial DNA (mtDNA) along with the feline sarcoma protooncogene (FES) and eight microsatellites.

Material and methods

Sampling and DNA isolation

We collected samples of 87 *L. culpaeus* and 122 *L. griseus* individuals from Chile and Argentina (Fig. 1, Tables S1 and S2) following the guidelines of the American Society of Mammalogists (Sikes et al. 2011). Blood, tissue, or hair samples were stored in sterile 95% ethanol. DNA was isolated using a salt-extraction method from Aljanabi and Martinez (1997) with modifications (Vianna et al. 2017). A total of 53 *L. culpaeus* and 12 *L. griseus* CR sequences available in GenBank were downloaded (Fig. 1, Tables S1 and S2), along with 17 sequences from *L. fulvipes*, *L. vetulus*, *L. sechurae*, and *L. gymnocercus* (Table S3). Also, for the FES intron 14, we analyzed one additional GenBank sequence from each one of the six species (Table S3).

Molecular procedures, sequencing, and genotyping

Two different mtDNA genes were amplified by PCR: (1) CR (551 bp), using primers MTLPRO2 and CCR-DR1 (Tchajcka et al. 2007), and (2) MT-CYB (952 bp), using primers MT-CYBDF1 and MT-CYBDR1 (Tchajcka et al. 2007). The FES intron 14 was also amplified using primers FES-F and FES-R (Venta et al. 1996). Finally, we amplified eight polymorphic microsatellites: 2001, 2137, 2140 (Francisco et al. 1996), Lfu 5D3a, Lfu 5F6, Lfu 5G3c, Lfu 8D5, and Lfu 8D6b (Cabello and Dávila 2014). The first three are tetranucleotide repeat loci, while the last five are dinucleotide repeat loci. All forward primers had a 5'-M13 tail (Boutin-Ganache et al. 2001). The microsatellite genotypes obtained in this study were placed in Supplementary Material (Tables S4 and S5).

For the DNA amplification of mtDNA and intron loci, PCRs were performed in 40- μ l reactions containing 1- μ l template DNA (20 ng/ μ l), 1 \times reaction buffer (Invitrogen®, Brazil), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 μ M of each primer, and 0.75 units of Taq DNA polymerase Platinum (Invitrogen®, Brazil). As for the DNA amplification of microsatellite markers, PCRs were performed in 30- μ l reactions which contained 1- μ l template DNA (20 ng/ μ l), 1 \times reaction buffer (Invitrogen®, Brazil), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.8 μ M of primer mix, and 0.8 units of Taq DNA polymerase (Invitrogen®, Brazil). Primer mix contained 3 \times forward primer with M13-tail, 40 \times reverse primer, and 40 \times M13-tail primer with fluorescence

(FAM, HEX, or NED, depending on the case). Thermocycling conditions were the same for all loci and consisted of a denaturation step at 95 °C for 10 min followed by 11 cycles (touchdown) including a denaturing step at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 45 s plus 40 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s and elongation at 72 °C for 45 s followed by a final elongation of 30 min at 72 °C. PCR products were run on agarose gel (1% for mtDNA and FES intron; 2.5% for microsatellites) stained with GelRed Nucleic Acid Stain (Biotium, San Francisco, USA) and visualized under ultraviolet light. PCR products were purified and sequenced bi-directionally (mtDNA and intron) or scored (microsatellite) at MacroGen Inc. (Seoul, South Korea).

Sequencher v. 5.4.5 (GeneCodes Corporation, Ann Arbor, MI, U.S.A. 2016) was used for sequence manual editing and alignment. MtDNA haplotypes were identified using DnaSP v. 6.12.03 (Rozas et al. 2017), while those of nuclear introns with Phase, a Bayesian approach implemented in the same program. Microsatellite allele length was determined using GeneMarker v. 2.7.0 (Piry et al. 2004), while automatic binning and microsatellite allele scoring were performed with Tandem v. 1.09 (Matschiner and Salzburger 2009). Quality control was performed using Microsatellite Toolkit for Excel to detect scoring errors (Park 2002).

Species divergence and hybridization

Maximum likelihood (ML) phylogenetic analyses for CR (Fig. S1) were performed in RAxML-HPC2 v. 8.2.12 (Stamatakis 2006) in the CIPRES Science Gateway (Miller et al. 2010) using the crab-eating fox (*Cerdocyon thous*, Linnaeus 1776) as outgroup. The reconstructions were based on 200 inferences on the original alignment and 1000 bootstrap replicates, using default parameter settings and GTR+GAMMA model for both bootstrapping and final ML optimization. Trees were visualized with FigTree v. 1.4.4 (Rambaut 2009).

Median joining networks (MJN) were constructed with NETWORK v. 10 (Bandelt et al. 1999) to explore relations among haplotypes and their possible sharing between species (Figs. 2 and 3). The first MJN was constructed with CR haplotypes of *L. culpaeus* and *L. griseus* (595 bp; Fig. 2). A second MJN was built with MT-CYB (952 bp) and a third one with the nuclear intron FES (383 bp). We also reconstructed a MJN using the concatenated marker (CR+Cybt+FES, 1978 bp; Fig. 3).

Genetic diversity and population structure

Species differentiation and possible hybridization were explored by means of the Bayesian clustering method implemented in STRUCTURE v. 2.3.4 (Pritchard et al. 2000; Fig. 4), assessing the Q-value of each sample and comparing with the species assignment of the other

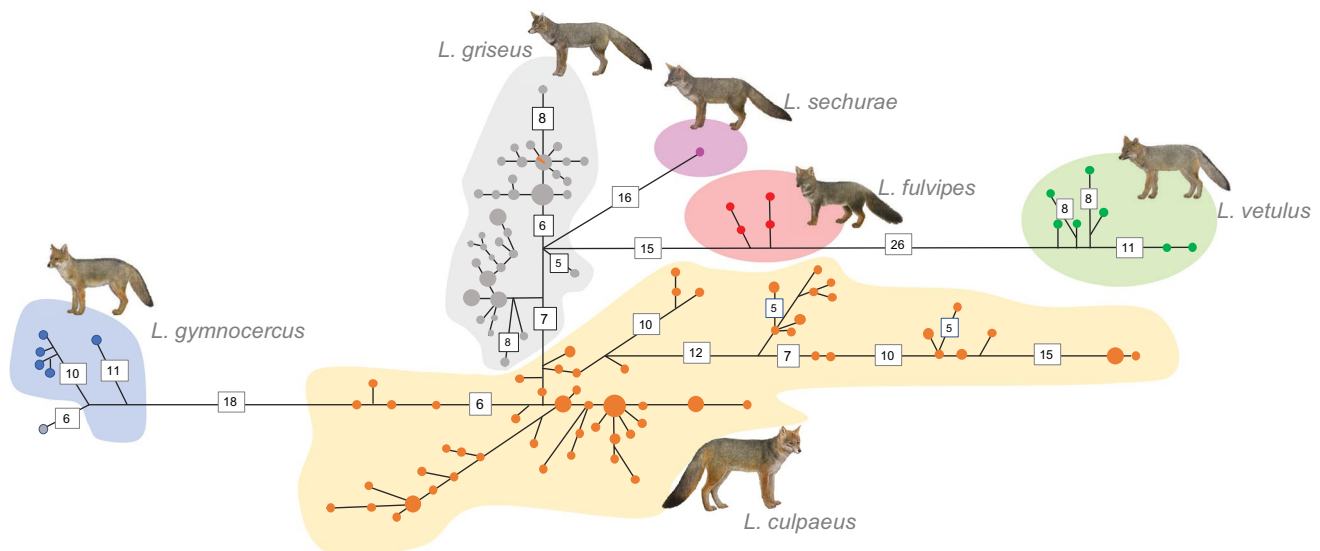


Fig. 2 CR Median joining network of *Lycalopex griseus* (gray), *L. culpaeus* (orange), *L. sechurae* (purple), *L. gymnocercus* (blue), *L. fulvipes* (red), and *L. vetulus* (green) individuals. The size of each circle is proportional to the haplotype frequency. Bars and numbers placed on connecting lines indicate the number of nucleotide differ-

ences between haplotypes. Connecting lines without bars or numbers indicate that there is only one substitution between haplotypes. The individual M2299 was assumed to be *L. culpaeus* in the network analysis

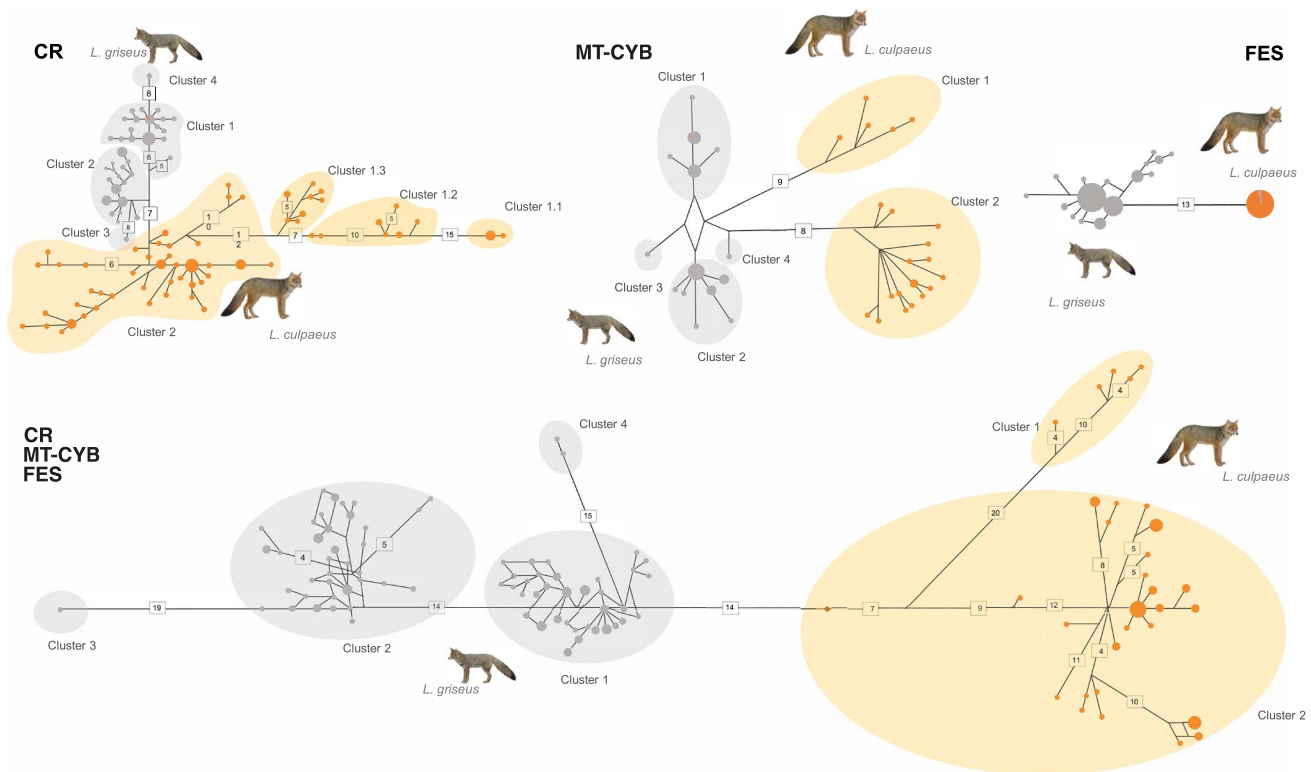


Fig. 3 Median joining network for *Lycalopex griseus* (gray), *L. culpaeus* (orange) using CR (top left), MT-CYB (top middle), the nuclear FES (top right), and the concatenated markers (CR+MT-

CYB + FES, bottom network). The individual M2299 was assumed to be *L. culpaeus* in the network analysis

markers and the visual identification. We also performed the analyses within each of the two fox species to detect population genetics structure excluding the individuals indicated as potential hybrids in the entire dataset (Figs. 5 and 6). Given their high mobility, the sampled foxes might have ancestors from multiple populations which are likely closely related (Porras–Hurtado et al. 2013); therefore, we chose the admixture ancestry model with LOCPRIOR and correlated allele frequencies (Hubisz et al. 2009). To infer lambda, the hyperparameter associated with the allele frequency model prior (Pritchard et al. 2000), we first performed one run with 150,000 iterations and 50,000 burn-in. This run calculated a lambda of 0.71, which we used in the subsequent runs. We evaluated K (i.e., the number of clusters) with values ranging from one to the total number of locations (29 locations for *L. culpaeus* and 23 locations for *L. griseus*; Tables S1 and S2) and performed five independent runs for each value of K , with 500,000 MCMC and a 50,000 burn-in period estimating the 90% probability intervals for admixture coefficients. The web version of STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used for inferring the most likely K using Evanno's method (Evanno et al. 2005) and the highest posterior mean log-likelihood (mean $\text{LnP}(K)$). Finally, CLUMPP

(Jakobsson and Rosenberg 2007) was used for summarizing the results of all previous runs, and DISTRICT v. 1.1 (Rosenberg 2004) to visualize them.

A Principal component analysis (PCA) of microsatellite data was performed using ADEGENET (Jombart 2008) in R Core Team (R Core Team 2021). We also evaluated the number of clusters with K -means and a Bayesian information criterion (BIC), using the function find.clusters. In ADEGENET, we performed a discriminant analysis of principal components (DAPC) (Jombart et al. 2010). For the sake of clarity, the STRUCTURE analysis, PCA, and DAPC were performed for the entire dataset of both species and the species separately excluding the potential hybrids.

Intraspecific spatial structure and phylogeographical boundaries of *L. culpaeus* and *L. griseus* were evaluated with GENELAND v. 4.0.5 in the R-package (Guillot et al. 2005) for CR and microsatellite data excluding individuals indicated as potential hybrids according to STRUCTURE results. GENELAND was set with 1,000,000 Markov Chain Monte Carlo (MCMC) iterations sampling each 1000 steps and a 20% thinning, using uncorrelated allele frequencies and the spatial model. These parameters were used for five repetitions of K -values (the number of clusters in the data) ranging from 1 to 10. Using the same

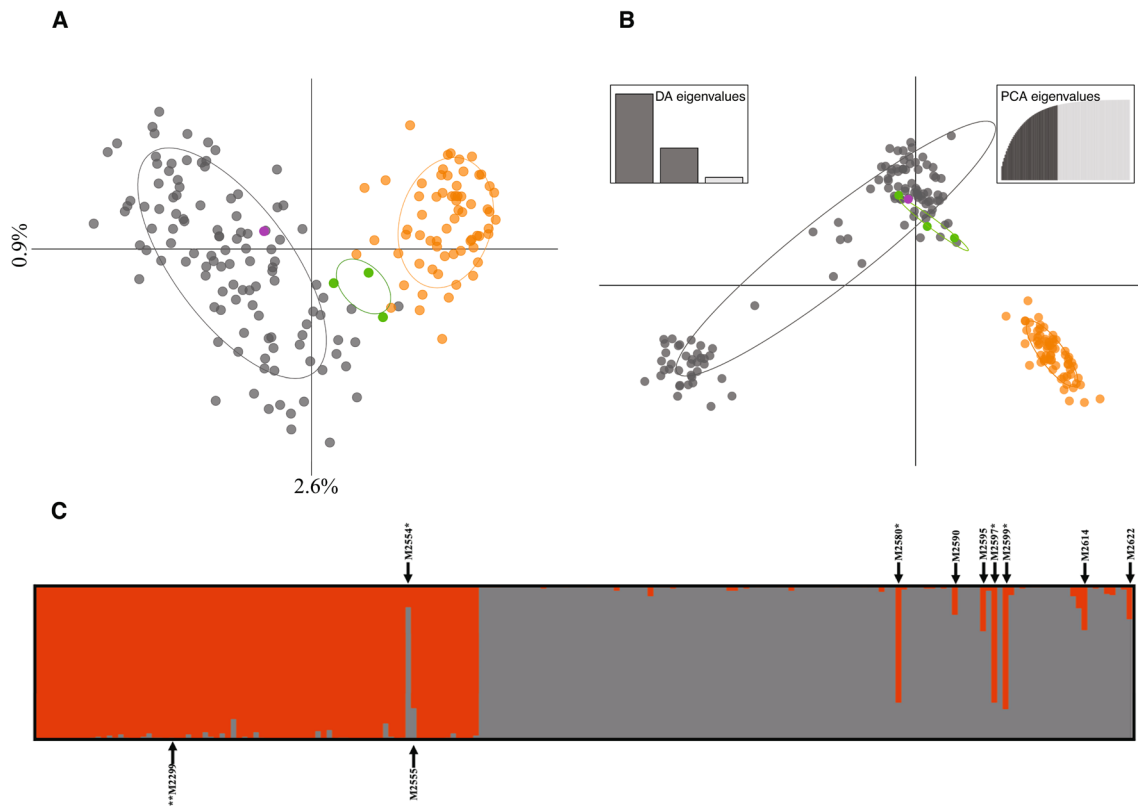


Fig. 4 **A** PCA based on microsatellite data for *Lycopex culpaeus* (orange) and *L. griseus* (gray) and **(B)** DAPC $K=4$. **C** STRUCTURE results for the entire dataset ($K=2$), where each vertical line represents one individual, and the two genetic clusters are represented with two colors: orange for *L. culpaeus* genotype and gray for *L. griseus* genotype. In the PCA and DAPC the three potential hybrids identified

as *L. griseus* are in green and the one identified as *L. culpaeus* in purple. For STRUCTURE, the fraction of each individual color within a bar represents the probability of assignment to species of that color. The nine individuals with a degree of introgression between 20 and 30% are indicated just with an arrow and those higher than 76% with an arrow and an asterisk. **possible misidentified in the field

parameters and the K -values inferred above as a fixed variable, the MCMC algorithm was run 30 times.

The outputs obtained from MJN, STRUCTURE, PCA, DAPC, and GENELAND were considered to define the intraspecific genetic clusters which were used for further population analyses. To assess the population genetic structure between genetic groups and among geographical subgroups, pairwise F_{ST} values were estimated for CR and microsatellite data, and Φ_{ST} only for CR using ARLEQUIN v. 3.5.2.2 (Excoffier and Lischer 2010), using 10,000 permutations and a significance level of 0.05.

Genetic diversity (CR data: S number of polymorphic sites; h number of haplotypes; H_d haplotype diversity, π nucleotide diversity; microsatellite data: H_o observed heterozygosity, H_e expected heterozygosity, A average number of alleles, R allele richness, FIS) was calculated for *L. culpaeus* and *L. griseus*, and for each genetic cluster identified with GENELAND, using ARLEQUIN for CR sequences and the microsatellite loci (Table 1).

Results

Species divergence

A higher diversity was found for *L. culpaeus* at both the CR ($n = 135$, $h = 0.97$, and $\pi = 0.028$ versus $n = 122$, $h = 0.94$, and $\pi = 0.016$; Table 1) and the MT-CYB ($n = 70$, $h = 0.88$, and $\pi = 0.008$ versus $n = 78$; $h = 0.86$; $\pi = 0.0036$) compared to *L. griseus*, since the former includes several divergent lineages (described below). However, *L. culpaeus* ($n = 79$) showed lower nuclear diversity than *L. griseus* with only one FES haplotype as opposed to 16 ($n = 120$; $h = 0.71$; $\pi = 0.004$). Likewise, microsatellite diversity was lower in *L. culpaeus* ($n = 65$, $H_o = 0.57$, $H_e = 0.65$, $A = 7.75$, $R = 4.08$) than in *L. griseus* ($n = 112$, $H_o = 0.57$, $H_e = 0.78$, $A = 11.12$, $R = 6.37$).

The CR tree (Figure S1) and MJNs (Figs. 2 and 3) concordantly supported the reciprocal monophyly and

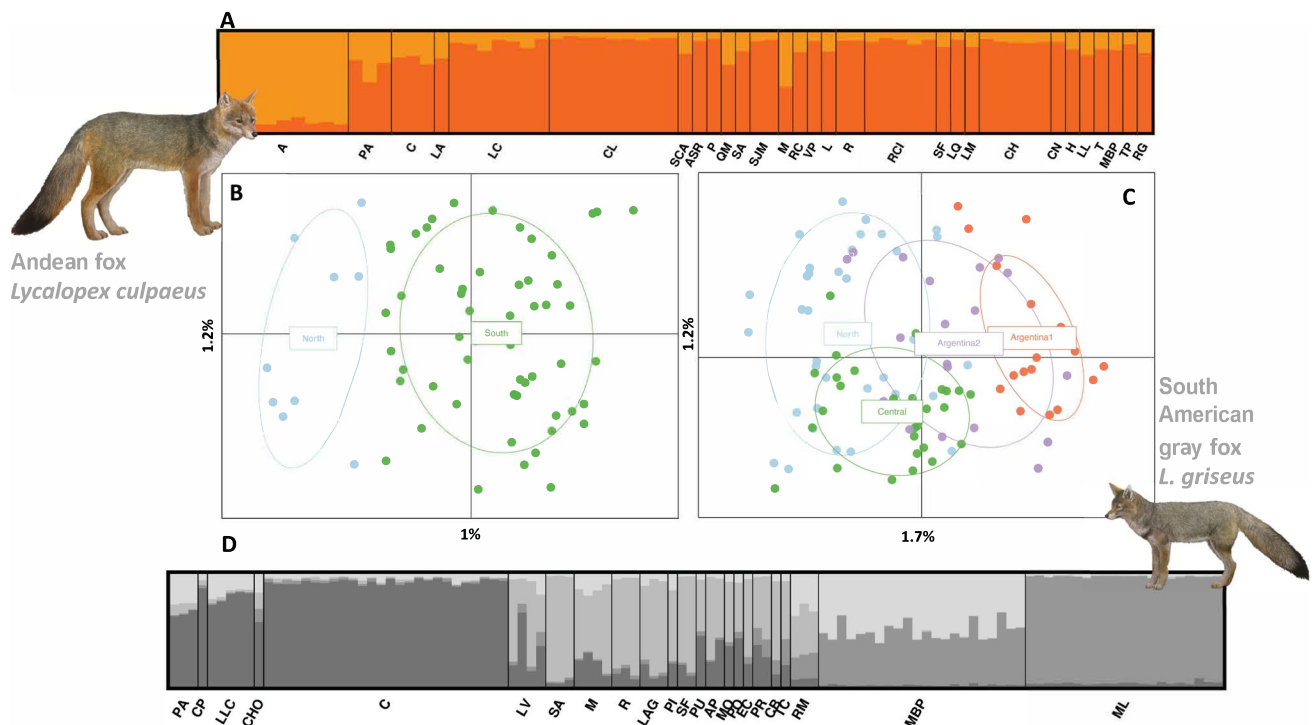


Fig. 5 STRUCTURE of *Lycalopex culpaeus* (A), PCA of *L. culpaeus* microsatellite data (B); PCA of *L. griseus* (C), STRUCTURE of *L. griseus* microsatellite data (D). Each vertical line represents one individual in the STRUCTURE. Location acronyms are included in Table S1 and S2

different genetic clusters for all *Lycalopex* species, including *L. culpaeus* and *L. griseus* (Figs. 2 and 3). However, the phylogeny shows low bootstrap support for branches leading to the main species (Figure S1).

Eight individuals were not assigned to the putative species: one *L. griseus* from Argentina was grouped with *L. gymnocercus* based on CR, and another seven mixed between *L. griseus* and *L. culpaeus*. An individual identified as *L. culpaeus* showed only microsatellites assigned as *L. griseus* (M2554), while another one showed a CR and MT-CYB haplotype belonging to *L. griseus* (M2376). For *L. griseus*, one showed a FES haplotype pertaining to *L. culpaeus* (M2320, Fig. 3 and Table 2), three *L. griseus* individuals had only the microsatellites classifying to the other species (M2580, M2597, M2599), and one *L. griseus* showed all the molecular markers assigned to *L. culpaeus* (M2299), possibly misidentified in the field (Table 2).

CR phylogenetic reconstruction and MJN consistently supported two main clusters for *L. culpaeus* separated by 12 nucleotide differences: (1) northern clade including foxes from Peru, Bolivia, northeastern Chile, and Cordoba in central Argentina; (2) southern clade including foxes from most of Chile and Argentina. Individuals from the northeastern location from Chile (Salar Punta Negra in Antofagasta region) belonged to the two main clusters (Fig. 3). The northern cluster contained three divergent sub-clusters/clades consistent with a geographic structure

without overlap between locations belonging to (1.1) Cordoba; (1.2) Salar Punta Negra (Antofagasta region) in Chile and Oruru, Potosí, Uyuni in Bolivia; and (1.3) Ancash and Junín in Peru.

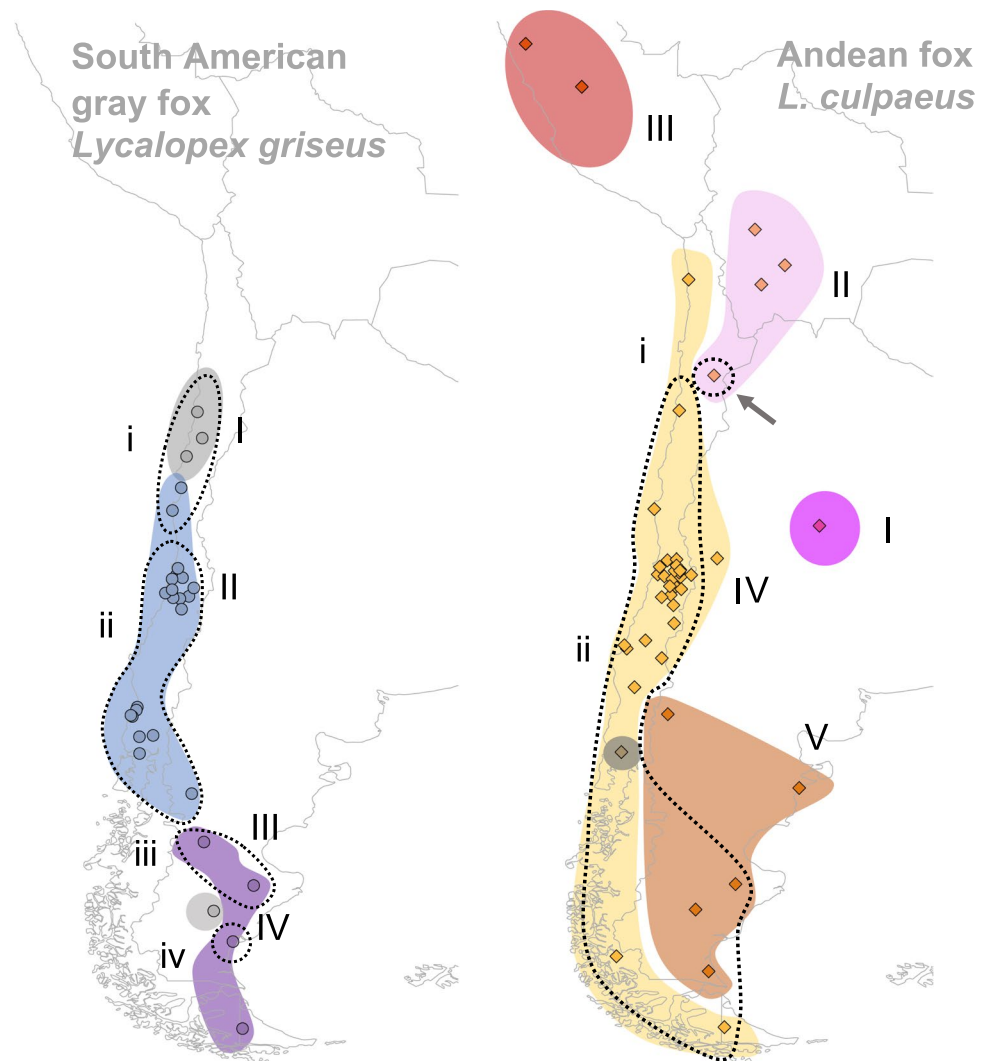
Concerning *L. griseus*, four main CR clusters were identified and they revealed a strong spatial genetic pattern described as follows: (1) most of the locations from Chile; (2) most of locations from northern Argentine Patagonia and a few from northern Chile; (3) Monte León in southern Argentine Patagonia; (4) Tecka in Argentina (Fig. 3).

Species delimitation and population genetics structure

Signatures of genetic structure were detected for both species. The Bayesian clustering analysis for microsatellite data for the entire dataset suggests that the highest posterior mean log-likelihood (mean $\ln P(K)$) and Evanno's method most likely K was 2, clearly separating *L. culpaeus* from *L. griseus* (Figs. 4 and S2).

The microsatellite results also suggest that few individuals could have a signature of introgression or ILS. A total of four individuals, one *L. culpaeus* and three *L. griseus*, were assigned to the other species based on the degree of introgression ($\geq 76\%$), even though the assignment based on mtDNA was consistent with the morphological identification in the field (Table 2). These four individuals, together with

Fig. 6 Map with sample locations representing the results of spatial model in GENELAND for *Lycalopex griseus* (left) and *L. culpaeus* (right). For *L. griseus*, four CR groups were defined (different colors, I to IV) and so was based on microsatellite data (dashed line, i to iv). For *L. culpaeus*, two groups were delimited using microsatellites (i and ii), and five groups were defined using CR (different colors, except the gray circle, I to V); however, one sample from Calbuco was grouped with a northern group (gray circle). The three groups from north and northeast (red, pink, and dark pink) correspond to clade 1 (I to III) from the phylogeny and MJN, and the two groups from south and southwest (yellow and orange) to clade 2 (IV and V). Both clades overlap at Salar Punta Negra, Chile, (indicated by the arrow). GENELAND posterior probabilities of population membership for *L. culpaeus* and *L. griseus* were 0.36–0.38 and 0.55–0.65, respectively, for mtDNA and 0.8–0.9 and 0.45–0.5, respectively, based on microsatellites (Figure S7)



the other two that were assigned to the other species based on mtDNA (M2376, Table 2) or FES (M2320, Table 2), corresponded to 3.2% of the total number of samples analyzed for microsatellite loci and sequences evaluated ($n = 188$). One additional fox (M2299) might represent a case of misidentification since even though first identified as *L. griseus*, genetic evidence univocally labeled it as *L. culpaeus*. These seven individuals were excluded for posterior population analyses within species. Additionally, another five foxes had a small percentage (20–30%) of microsatellite data not assigned to the morphological species, which might be indicative of a lower degree of introgression or ILS (Table 2). PCA clustered the two species with the first principal component explaining only 2.6% of the variance, with the potential hybrids found in an intermediate position along with other individuals. DAPC showed $K = 4$ with the clear distinction of the two species, but also the separation of the two clusters for *L. griseus* and the potential hybrids clustering more closely to *L. griseus* (Fig. 4).

The Bayesian clustering, PCA, and DAPC results also showed population structure within species (Figs. 5 and S2–S6). The highest posterior mean log-likelihood (mean $\text{LnP}(K)$) and the Evanno's method most likely K for *L. culpaeus* ($n = 65$) was $K = 2$: a northern cluster from Antofagasta region (north), and the southern cluster with most samples from Chile and Argentina (south, Figs. 5 and S2). Concordantly, PCA and DAPC using microsatellite data for *L. culpaeus* (Figs. 5 and S5) also revealed two well-differentiated groups discriminated by the first component. Spatial structure inferred at the microsatellite loci showed two clusters (i and ii) with high posterior support (0.8–0.9) for *L. culpaeus*, and similar boundary differentiating the northeastern of Chile from the remaining locations in southern Chile and Argentina (Figs. 6 and S7). The CR spatial structure, which was based on a larger sampling area including the surrounding countries for *L. culpaeus* ($n = 135$), showed 5 groups (posterior support of 0.36–0.38; Figs. 6 and S7) in accordance with the northern boundary identified for

Table 1 Genetic diversity indices calculated for *Lycalopex culpaeus* and *L. griseus* based on CR and microsatellite data. Putative hybrids (Table 2) and individuals with missing information on sampling location (Table S1) were excluded

	Control region				Microsatellites						
	N	S	h	Hd	π	N	Ho	He	A	R	F_{IS}
<i>L. culpaeus</i>	135	76	59	0.97+/-0.006	0.028+/-0.014	65	0.57±0.19	0.65±0.22	7.75±3.28	4.08±1.56	
Northern genetic cluster	35	51	18	0.92+/-0.03	0.026+/-0.013	9	0.56±0.27	0.61±0.27	4.50±2.27	4.00±1.77	0.09 (> 16.22)
Southern genetic cluster	100	48	42	0.95+/-0.01	0.015+/-0.008	56	0.57±0.18	0.64±0.20	6.75±2.71	4.16±1.44	0.11 (> 0.04)
<i>L. griseus</i>	122	67	36	0.94+/-0.01	0.016+/-0.008	112	0.57±0.13	0.78±0.10	11.12±4.55	6.370±2.183	
North	36	16	6	0.69+/-0.06	0.010+/-0.006	36	0.54±0.16	0.67±0.11	6.62±2.39	5.732±2.204	0.22 (> 0.00)
Central	35	15	13	0.88+/-0.03	0.004+/-0.003	30	0.60±0.11	0.75±0.14	7.50±3.81	6.731±3.249	0.24 (> 0.00)
Argentina 1	28	25	9	0.86+/-0.04	0.009+/-0.005	25	0.56±0.17	0.73±0.15	8.25±2.25	7.340±1.893	0.23 (> 0.00)
Argentina 2	23	43	8	0.76+/-0.08	0.015+/-0.008	21	0.64±0.26	0.73±0.09	5.88±1.64	5.674±1.524	0.13 (> 0.34)

N sample size; Control Region mtDNA diversity: S number of polymorphic sites; h number of haplotypes; Hd haplotype diversity; π nucleotide diversity; Microsatellite data: Ho observed heterozygosity, He expected heterozygosity, A average number of alleles, R Allele richness, F_{IS} : *Significant ($p < 0.01$)

Table 2 Putative hybrids between *Lycalopex culpaeus* and *L. griseus* identified by the combination of field identification, mtDNA (CR and MT-CYB), nuclear intron (FES), and Bayesian clustering using microsatellite data

ID	Location	Field Ident	mtDNA	FES	Microsatellite
M2554	Lo Vasquez, CL	CU	CU	CU	CH (0.863)*
M2580	MBP, AR	CH	CH	CH	CU (0.764)*
M2597	MBP, AR	CH	CH	CH	CU (0.762)*
M2599	Monte León, AR	CH	CH	CH	CU (0.806)*
M2376	Calbuco, CL	CU	CH*	CU	CU
M2320	Coquimbo, CL	CH	CH	CU*	CH
M2299**	Coquimbo, CL	CH	CU*	CU*	CU*
M2590	MBP, AR	CH	CH	CH	CH (0.812)***
M2595	MBP, AR	CH	CH	CH	CH (0.705)***
M2614	Monte León, AR	CH	CH	CH	CH (0.711)***
M2622	MBP, AR	CH	CH	CH	CH (0.785)***
M2555	Iquique, CL	CU	CU	CU	CU (0.799)***

ID sample identifier; CL Chile; AR Argentina; CU Andean fox; CH South American gray fox; MBP Parque Nacional Monumento Bosques Petrificados de Jaramillo

*Molecular inconsistency with morphological identification

**Possible misidentified in the field

***Microsatellite assigned to the other species based on 20–30%

microsatellite data. The clades found are similar to those found in the tree and the network (Figs. 3, and S1): the three groups identified by GENELAND from north and northeastern I, II, and III corresponded to the three sub-clades from the main clade 1 (sub-clades 1.1, 1.2, and 1.3, respectively), with the two southern groups both falling within clade 2 (IV and V, Fig. 6).

Structure of *L. griseus* ($n = 112$) showed that the most likely K was 2 using Evanno’s method, and the highest posterior mean log-likelihood (mean $\text{LnP}(K)$) was $K = 8$. For *L. griseus*, the first two groups included all individuals from Chile plus one fox from Tecka in Argentina and all those from the remaining locations from southern Argentina, respectively. The subsequent subdivisions distinguish four groups for *L. griseus*: (i) northern group of Chile (Pan de Azucar to Coquimbo); (ii) north-central Chile with Río Mayo in Argentina; (iii) the Parque Nacional Monumento Bosques Petrificados de Jaramillo (MBP); and (iv) Monte León in Argentina (Fig. 5). PCA for microsatellite data of *L. griseus* showed some overlap between geographic regions with a gradual differentiation from north to south explained

by the first component (Fig. 5). DAPC (Fig. S6) revealed $K=3$, discriminating by the first component the northern individuals from the other locations, with the second component the Central region of Chile from the location from Argentina, finding some overlap between all groups (Fig. S6). Spatial microsatellite structure ($n=112$) supported the same four groups found in STRUCTURE with intermediate posterior support of 0.45–0.5: (i) northern Chile (Pan de Azúcar and Coquimbo); (ii) central Chile including Teka in Argentina; (iii) Río Mayo and MBP both in Argentina; (iv) Monte León in Argentina (Figs. 6 and S7). GENELAND results based on the CR (Figs. 6 and S7) including a larger number of sequences ($n=122$) and geographic locations than for the microsatellite data, showed four genetic groups (I to IV) with similar boundaries with posterior support of 0.55–0.65 (I–IV).

Significant population genetic structure between the two groups (northern and southern) was detected for *L. culpaeus* for CR ($F_{ST}=0.06152$, $\Phi_{ST}=0.06224$, $p<0.0001$) and microsatellite data ($F_{ST}=0.12$, $p<0.0001$). The southern group for *L. culpaeus* showed a higher haplotype diversity but a lower nucleotide diversity than the northern group, which also showed slightly higher values for all microsatellite diversity indexes (Table 1).

A significant genetic differentiation was found among the four mtDNA clusters of *L. griseus* ($F_{ST}=0.13$ – 0.28 ; $\Phi_{ST}=0.13$ – 0.27 , $p<0.0001$) and microsatellite data ($F_{ST}=0.07$ – 0.19 ; $p<0.0001$), with the highest values for both marker systems between northern Chile and Argentina (Fig. S8, Tables S6 and S7). The group from the central region of Chile and Argentina 1 showed higher CR haplotype diversity and lower nucleotide diversity but higher diversity for most of the microsatellite diversity indexes (Table 1).

Discussion

Species delimitation of *L. culpaeus* and *L. griseus* have been under debate due to potentially incomplete lineage sorting, introgression, and/or misidentification. Likewise, morphological studies have shown discrepancies for the subspecies described. This genetic study contributes to assessing these questions and investigates the biogeography of the region in areas with well-established boundaries. Our results support the reciprocal monophyly of *L. culpaeus* and *L. griseus* as recently divergent species, with low signature of introgression or ILS (about 3%). The reciprocal monophyly for both species agrees with Tchaicka et al. (2016) who found a high support (≥ 0.9) using 32 samples of *L. culpaeus* and 28 of *L. griseus*, and Favarini et al. (2022), which used 7 and 8 samples, respectively, restricted geographically, but with greater representativeness of the mitochondrial genome

with 6000 bp sequenced. However, our conclusions do not agree with Yahnke et al. (1996), who did not confirm *L. culpaeus* and *L. griseus* as separate monophyletic clades, possibly because of the small portion of mtDNA (344 bp) and limited sample size (six *L. culpaeus* and 14 *L. griseus*).

Despite both species co-occur in Chile and Argentina across a wide range of habitat types from mountain terrains, steppes, grasslands, scrublands, and open deserts to broad-leaved temperate southern beech forest in the south (González del Solar and Rau 2004; Jiménez and Novaro 2004), *L. culpaeus* tends to prefer mountainous habitats, while *L. griseus* is a lowland species that is rarely seen at high altitudes (Fuentes and Jaksic 1979; Iriarte 2008). Therefore, sympatry mostly occurs at the boundary between the Andes and lowlands, which can limit the degree of introgression between the species. Several cases in which both distributions do overlap have been reported in Chile, north-western Argentina and southern regions of Argentina (Johnson and Franklin 1994; Jiménez et al. 1995, 1996; Zunino et al. 1995; Jayat et al. 1999; Jiménez and Novaro 2004; Novaro et al. 2004). In southern Chile and Argentina, species sympatry is facilitated by the southward altitudinal decrease of the Andes (Fuentes and Jaksic 1979) and body size differences (de Moura Bubadué et al. 2016b). The latter are higher in overlapping areas of distribution, allowing *L. culpaeus* and *L. griseus* to differentiate their feeding strategies and coexist in the same habitat (Rosenzweig 1966; Fuentes and Jaksic 1979; Zapata et al. 2008; Guzmán et al. 2009; de Moura Bubadué et al. 2016a). Other factors that may allow the coexistence of both species are (1) high availability of prey; (2) higher hunting pressures over *L. culpaeus* compared to *L. griseus*, allowing higher densities and expansion of the latter; (3) presence of top predators like the puma (*Puma concolor*), which would keep *L. culpaeus* numbers low (Díaz–Ruiz et al. 2020); and (4) habitat modification, since most of these areas have a homogeneous habitat structure because of high modification made by humans (Novaro et al. 2004).

The challenges of discriminating both species in the field are based on their recent divergence (Tchaicka et al. 2016). *Lycalopex culpaeus* and *L. griseus* are morphologically similar in terms of general appearance, and both share biological and ecological characteristics; however, there is an intraspecific variation along their extensive distribution as well as in terms of life stages (juveniles/adults) which may cause misidentification. These two taxa have similar climatic requirements, with the same optimum tolerance of mean annual temperature of about 8–10 °C, and both inhabit arid regions with an annual rainfall below 300 mm, representing the species with the highest niche overlap inside the genus (Zurano et al. 2017). Moreover, both species are crepuscular, omnivorous, and opportunistic predators with a seasonally and locally variable diet (Atalah et al. 1980; González del Solar and Rau 2004; Jiménez and Novaro 2004; Novaro et al.

2004; Núñez and Bozzolo 2006; Guzmán-Sandoval et al. 2007; Iriarte 2008; Zúñiga et al. 2008; Rubio et al. 2013; Galende and Raffaele 2016; Lagos et al. 2021).

Morphological studies completely support the divergence of *L. culpaeus* and *L. griseus* even in areas of sympatry, with cranial segregation mainly in dental pattern suggesting past selective pressure by intraguild competition dominated by *L. culpaeus* (Zapata et al. 2008, 2014). However, further studies encompassing the entire distribution of both species are required. In the case of *L. griseus* and *L. gymnocercus*, previous studies concluded that both are different forms of the same species (conserving the name *L. gymnocercus*) based on traditional morphometrics using cranio-dental measurements (Zunino et al. 1995) and 3D geometric morphometrics using cranium and mandible size and shape (Prevosti et al. 2013). Here, our molecular analysis suggests that *L. griseus* and *L. gymnocercus* form reciprocally monophyletic clades, thus not supporting the synonymy of these two species. Although we have identified one *L. griseus* from Argentina within the *L. gymnocercus* clade, this could represent a misidentification or a potential event of hybridization between both species. This agrees with Tchaicka et al. (2016) and Favarini et al. (2022); reciprocal monophyly was obtained, but still found some individuals of *L. gymnocercus* in the *L. griseus* clade and individuals of *L. vetulus* in the *L. gymnocercus* clade, respectively, potentially resulting from interspecific hybridization. However, further studies are required to provide convincing evidence in this respect.

Distinguishing phylogenetic signals of species divergences from those of ILS and introgression, and the contribution of each, is limited by the number of loci and samples analyzed. Here, we report six cases of potential events of hybridization and/or ILS between *L. culpaeus* and *L. griseus* inferred from genetic data in sympatric areas of their distribution, supporting interspecific hybridization that commonly occurs in wildlife (O'Brien and Mayr 1991; Hindrikson et al. 2012), sometimes leading to fertile hybrids better adapted to the environment and even favoring speciation (Lehman et al. 1991; Yahnke et al. 1996; Schwartz et al. 2004; Lancaster et al. 2006; Trigo et al. 2008). For the potential hybrids and/or ILS, the visual identification of the species was consistent in most cases with the species assignment of mtDNA haplotype (maternal lineage, 5 out of 6 cases), but discordant with the species assignment according to microsatellites or FES (biparental, 5 out of 6 cases); this suggests a male-mediated introgression. The asymmetric hybridization between sexes is common for other canids (e.g., Hindrikson et al. 2012), and genomic evidence of introgression was recently found within the *Lycalopex* genus (Chavez et al. 2022). However, further studies carried out with population genomic data are required to fully distinguish the contribution of introgression from ILS across the distribution of both species.

Different phylogeographic patterns were found for *L. culpaeus* and *L. griseus* with environmental characteristics and geographical barriers limiting their distribution. For *L. culpaeus*, two main divergent genetic groups were detected: one belongs to the Andean region of Peru, Bolivia, and northern Chile and Argentina; the other one belongs to the remaining southern distribution in Chile and Argentina. This agrees with the subspecies classification described by Guzmán et al. (2009) based on skull morphology. The latter suggests that the genetic clusters found could be associated with adaptive phenotypes for each species, since skull morphology is highly conserved and its variation correlates to a wide range of functions such as foraging behavior (Machado 2020). The clear environmental and geographic delimitation of each group, along with the morphological and genetic differences including reciprocal monophyly, strongly supports two evolutionarily significant units (ESUs) that should be considered for conservation actions. The northern clade includes three clusters (MJN clade 1.1, 1.2 and 1.3, Fig. 3) as the clusters found by Martínez et al. (2018) explained by environmental resistance, probably associated with the Andean Mountains, the desert, and the lack of climatic homogeneity between Córdoba and the Andean Mountains (Martínez et al. 2018).

Initially, six subspecies had been described for *L. culpaeus*, four of them present in Chile (Cabrera 1931): (1) *L. c. reissii* (Hilzheimer, 1906) from the Andes of Ecuador, (2) *L. c. andinus* (Thomas 1914a, b) from the South American Altiplano (high plateau), (3) *L. c. smithersi* (Thomas 1914a, b) from mountains of Córdoba in Argentina, (4) *L. c. culpaeus* (Molina, 1782) from central Chile and the eastern side of the corresponding section of the Andes, (5) *L. c. magellanicus* (Gray, 1836) from Patagonia and southern Chile, and (6) *L. c. lycooides* (Philippi, 1896) from Tierra del Fuego region. However, Guzmán et al. (2009) support only two morphological groups in Chile, one found in northern Chile (Tarapacá and Antofagasta), with a slender skull, and the other one in central and southern Chile, Patagonia, and the austral islands of Tierra del Fuego and Hoste, with a more robust skull. The authors suggested that these two groups would respectively correspond to the subspecies *L. c. andinus* and *L. c. culpaeus*, the last one presenting synonymy with *L. c. smithersi*, *L. c. magellanicus* and *L. c. lycooides*. Therefore, our results (mtDNA sequences and microsatellite loci) are concordant with two main genetic and morphological groups for *L. culpaeus* present in Chile. However, when including CR sequences from other locations in South America, we found that cluster 1 was composed by three divergent monophyletic clades including individuals from (1.1) Córdoba; (1.2) Antofagasta region in Chile and Oruru, Potosí and Uyuni in Bolivia; and (1.3) Ancash and Junín in Peru. These groups could correspond to *L. c. smithersi*, *L. c. andinus*, and *L. c. reissii*, respectively. The latter three

subspecies together with *L. c. culpaeus* support the existence of at least four subspecies. Therefore, *L. c. smitheri* would not be synonymy with *L. c. culpaeus* as suggested by Guzmán et al. (2009).

In *L. griseus*, four subspecies have been described based on morphological traits (Osgood 1943): (1) *L. g. domeykoanus* (from the southern part of the Province of Atacama and southward to the vicinity of Concepción in Central-Southern Chile), (2) *L. g. maullinicus* (Valdivian forest region of south-central Chile), (3) *L. g. gracilis* (western Argentina from Santiago del Estero Province to west Rio Negro Province), and (4) *L. g. griseus* (Argentinean and Chilean Patagonia such as Pampas of western Argentina from the Straits of Magellan northward at least to Chubut; passes into Chile locally along the western valleys of the Andes). Our results show that the clusters identified in the MJN for mtDNA and the concatenated markers do not mirror the geographic distribution of the clusters identified using microsatellite results. Rather, the latter could roughly correspond to the distribution of the subspecies as follows: (i) northern cluster to *L. g. domeykoanus*; (ii) central to *L. g. maullinicus*; (iii) Argentina 1 to the *L. g. gracilis* distributed in the north of Argentina; and (iv) Argentina 2 to the *L. g. griseus* in southern Argentina. However, our microsatellite results show a population structure for the species rather than high divergence supported by reciprocal monophyly of mtDNA and nuclear markers required for subspecies delimitation. Therefore, even though the population genetic structure does not show enough evidence of high genetic divergence, it supports the existence of management units, which should be considered for conservation management actions. Future studies are required to refine the geographic scale at which each of the populations is delimited and to fully understand the evolutionary history of the species.

The boundary between the two main northern clades of *L. culpaeus* coincides with that of several mammal species, such as the subspecies of South American camelids, guanaco (*Lama guanicoe*, Marín et al. 2013) and vicuña (*Vicugna vicugna*, Marín et al. 2007), and small mammals such as lineages of the mountain Degu (*Octodontomys gliroides*, Rivera et al. 2016), or the Andean altiplano mouse (*Abrothrix andinus*) in the Andean Altiplano-pre-Puna region and the olive grass mouse (*A. olivaceus*) in the lowlands of northern Chile (Palma et al. 2005), or the white-bellied fat-tailed mouse opossum (*Thylamys pallidior*) and the elegant fat-tailed mouse opossum (*T. elegans*) in the two environments respectively (Palma et al. 2014). This historical barrier to gene flow for *L. culpaeus* in the Altiplano also applies to other organisms such as birds (*Phrygilus*, Álvarez-Varas et al. 2015), and the diversification of reptiles (*Liolaemus*, Guerrero et al. 2013) or plants (*Chaetanthera*, *Malesherbia*, *Nolana*, Guerrero et al. 2013). This pattern would be related to the presence of the Atacama Desert, which has a severe

climate that promotes this biogeographical break. During the Pleistocene, other important geographic barriers were located in this region. The glacial cycles caused contractions and expansions of several paleolakes in the Altiplano (Placzek et al. 2009). The paleolake Tauca covered a large area in the past on the Bolivian Altiplano with the Chilean border (Nester et al. 2007), which is the exact position between the two main phylogenetic clades identified here for *L. culpaeus*. Toward the south, the Andes decrease in altitude, but can still be an effective barrier for species along with the drastic change of environment from the humid temperate rain forest in the west to dry Pampas in the east. Therefore, they could limit gene flow in the southern region between Chile and Argentina for the groups identified by the different analysis and markers in *L. griseus* and by GENELAND mtDNA in *L. culpaeus*.

Although both species are listed as “least concern” in the IUCN Red List, the combined effects of hunting (for their fur, or retaliation by ranchers who attribute diminutions of their livestock to the foxes), disease spill-overs from dogs (Di Cataldo et al. 2021), and predation by or competition with puma could be generating a decrease in population numbers of both species in some parts of their ranges (González del Solar and Rau 2004; Iriarte 2008; Silva-Rodríguez et al. 2009; Lucherini 2016a, b). In *L. culpaeus*, our results support four monophyletic clades (Fig. S1) that corresponded to the distribution of previously described subspecies. This could be indicative of long-term isolation between these groups, which are independently evolving and should hence be preserved as independent subspecies for *L. culpaeus*; also the clades found in *L. griseus* should be preserved as distinct Management Units (MU: Moritz 1999). The high genetic diversity detected in these two species might be indicative of large effective population sizes. However, given their ecological role and exposure to several threats, we highlight the need to consider the population structure that emerged in this study, and the importance of the distribution boundaries of each genetic group for *L. culpaeus* and *L. griseus* as crucial elements for their conservation. These findings should be considered when establishing management plans (e.g., translocating individuals and releasing rehabilitated individuals) to preserve the ecological role, adaptive variation, and evolutionary processes of such important top predators of the Southern Cone of South America ecosystems.

Our results support the recognition of *L. culpaeus* and *L. griseus* as recently diverged species, with a low signature of introgression or ILS. Past debate about species delimitation is apparently related to the low number of markers and samples/individuals used in previous studies as well as to the external morphological similarities between species in some areas. Population genomic studies could elucidate the degree of introgression between both species or ILS, their

degree of divergence and the underlying local adaptations to the wide range of environments they inhabit.

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Author contribution E.J.P., B.J.K., J.C.M., and J.A.V. obtained the samples from foxes, undertook molecular laboratory and analyses, interpreted the results, wrote the manuscript and prepared the figures. V. M. undertook molecular laboratory. J.A.V. and J.C.M. secured funding support. J.C., G.A.J., N.S.P., D.G.-A., C.B., A.I., A.R., A.T., A.C., J.L.B., J.M., and J.C.M. collected and provided samples for the study. All authors reviewed the full manuscript.

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Data availability All haplotypes found in this study were deposited in GenBank under accession numbers OM169041-OM169067, OM677763 (*L. culpaeus* CR), OM169012-OM169040 (*L. griseus* CR), OM169082-OM169102 (*L. culpaeus* MT-CYB), OM169068-OM169081 (*L. griseus* MT-CYB), OM169113 (*L. culpaeus* FES), and OM169103-OM169112 (*L. griseus* FES).

Declarations

Ethics approval Capture and sampling methods was carried out in strict accordance with the guidelines of the American Society of Mammalogists. The research protocols were approved by all the institutions involved in sample collection in accordance with local regulations from Chile and Argentina as well as and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Competing interests The authors declare no competing interests.

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