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Artículo

HOW MANY SPECIES OF GREY FOXES (CANIDAE, CARNIVORA) ARE THERE IN SOUTHERN SOUTH AMERICA?

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ABSTRACT. Two species of grey foxes are recognized in the Southern Cone of America: *Lycalopex griseus*, and *L. gymnocercus,* which were traditionally separated by size and some cranial differences. Morphometric analyses of the skull showed that both species could be the same and that they show clinal variation, while DNA studies based on one mitochondrial marker suggested that they belong to different species. Our objective is to evaluate the systematic status of these foxes using three mitochondrial markers (cytochrome B, cytochrome oxidase I, and control region), and a large sample covering a wide geographic range. The results indicate that there are two clades, that are not sister taxa, a finding that is more congruent with the hypothesis of two species, but their geographic distribution is not coincident with the accepted distribution of *L. griseus* and *L. gymnocercus.* Consequently, the distribution of *L. griseus* is extended eastern including north and center of Argentina, towards the west and south of the Paraná, Paraguay and Río de la Plata rivers. On the other hand, the clade that probably represents *L. gymnocercus* is restricted to the east of those rivers, except for a few specimens collected in Santa Fe, close to the Paraná river. However, an analysis of a wider sample using nuclear DNA is needed to confirm the taxonomic identity of these species of grey foxes.

RESUMEN. **¿Cuántas especies de zorros grises (Canidae, Carnivora) hay en el sur de Sudamérica?** Dos especies de zorros grises se reconocen en el Cono Sur de América: *Lycalopex griseus* y *L. gymnocercus*, que tradicionalmente estaban separadas por tamaño y algunas diferencias craneales. Análisis morfométricos del cráneo mostraron que estas especies podrían ser una sola que muestra variación clinal, mientras que estudios de ADN basados en un marcador mitocondrial sugirieron que pertenecen a especies diferentes. Nuestro objetivo es evaluar el estado sistemático de estos zorros utilizando tres marcadores mitocondriales (citocromo B, citocromo oxidasa

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I y región control) y una muestra grande que cubre un amplio rango geográfico. Los resultados indican que hay dos clados, que no son taxones hermanos, un hallazgo que es más congruente con la hipótesis de dos especies, pero su distribución geográfica no es coincidente con la distribución aceptada de *L. griseus* y *L. gymnocercus*. Consecuentemente, la distribución de *L. griseus* se extiende hacia el este, incluyendo el norte y centro de la Argentina, hacia el este y el sur de los ríos Paraná, Paraguay y Río de la Plata. Por el otro lado, el clado que probablemente representa a *L. gymnocercus* está restringido hacia el este de esos ríos, excepto por algunos especímenes colectados en Santa Fe, cerca del río Paraná. Sin embargo, se necesita un análisis de una muestra más amplia que utilice ADN nuclear para confirmar la identidad taxonómica de estas especies de zorros grises.

Key words: foxes, mitochondrial DNA, species delimitation, systematics

Palabras clave: ADN mitocondrial, delimitación de especies, sistemática, zorros

INTRODUCTION

Knowledge of the taxonomic limits and distribution of species is important not only for their conservation, but also for the study of their evolutionary history (e.g., Kutschera et al. 2014; vonHoldt et al. 2016). Recently in Carnivora, molecular studies have been changing these boundaries, both splitting species (e.g., Trigo et al. 2013; Helgen et al. 2013), as well as merging different taxa (e.g., Schiaffini et al. 2013). Also, molecular studies have been revealing complex patterns of evolution, such as hybridization and introgression, which also lead to a redefinition of species boundaries (e.g., Kutschera et al. 2014; vonHoldt et al. 2016). In this context, there are still some doubts regarding the identity and the species boundaries of South American canids, which have not been thoroughly evaluated using molecular data (e.g., Zunino et al. 1995).

Foxes of the Southern Cone of America include two genera, *Cerdocyon* that inhabits different kind of forested areas in the northern part of this area, and *Lycalopex* that covers the whole area with the exception of the Paranean and Atlantic forests (Macdonald & Sillero Zubiri 2004; Sillero Zubiri et al. 2004; Wilson & Mittermeier 2009). *Lycalopex* include several species: the culpeo fox (*Lycalopex culpaeus*) that is a large fox (ca. 10 kg) with a diet mostly composed of small mammals, and with an Andean and Patagonian distribution; the grey foxes that were commonly assigned to *Lycalopex griseus* and *Lycalopex gymnocercus*; and Darwin's fox (*Lycalopex fulvipes*) (Macdonald & Sillero Zubiri 2004; Sillero Zubiri et al. 2004; Wilson & Mittermeier 2009). These last three species are smaller (ca. 3-7 kg) and have a more omnivorous diet and a wider distribution, that virtually covers the whole area, with the exception of *L. fulvipes* that is limited to the northwestern Chilean Patagonia (Macdonald & Sillero Zubiri 2004; Sillero Zubiri et al. 2004; Wilson & Mittermeier 2009). *Lycalopex griseus*, as currently defined, is distributed in dry habitats of Patagonia, western Argentina, central and northern Chile, and western Bolivia, while *L. gymnocercus* is present in wetter areas of central, northern and eastern Argentina, eastern Bolivia, Paraguay, southern Brasil and Uruguay (Zunino et al. 1995; Macdonald & Sillero Zubiri 2004; Sillero Zubiri et al. 2004; Wilson & Mittermeier 2009; Prevosti et al. 2013; **Fig. 1**). The distribution of these species is apparently overlapping along western parts of Argentina, and in northwestern Argentina, where supposedly they live in sympatry (Mares et al. 1989; Barquez et al. 1991; Díaz & Barquez 2002). *Lycalopex fulvipes* occurs in the Chiloé island and continental areas of Chile with Valdivian forests, in the regions of Biobío, La Araucanía and Los Lagos (Macdonald & Sillero Zubiri 2004; Sillero Zubiri et al. 2004; Wilson & Mittermeier 2009; Farías et al. 2016).

While the status of *L. culpaeus*, *L. sechurae* and *L. fulvipes* as valid species is clear, the separation of the other two species is disputed

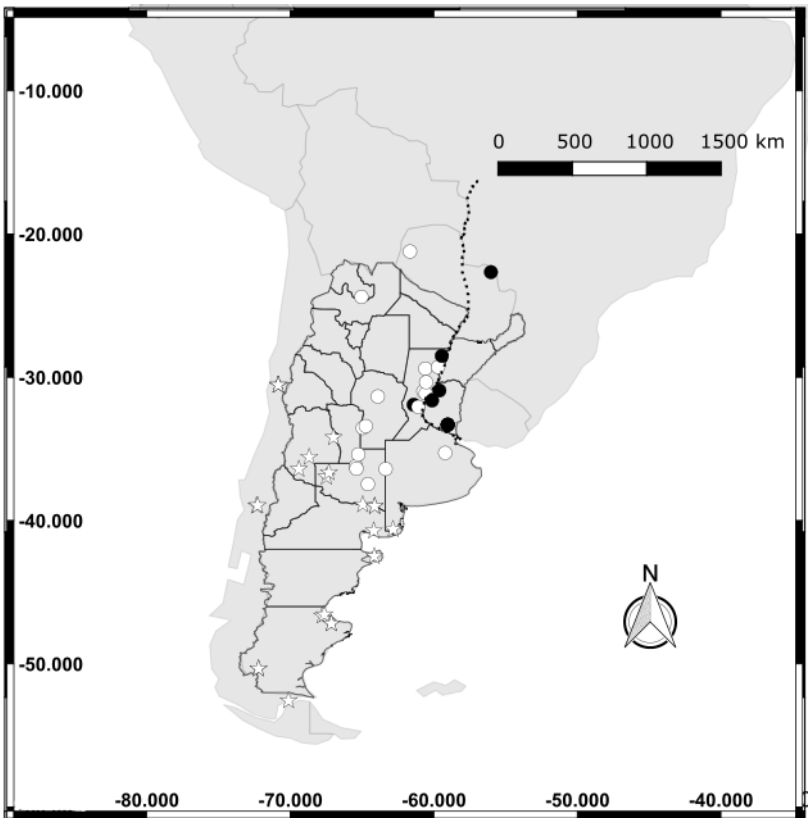

Fig. 1. Geographical distribution of the samples analyzed. Stars: *Lycalopex griseus*; circles: *L. gymnocercus*. White circles: Clade B, black circles: Clade A. Dotted line indicates the Paraguay-Paraná river.

(Zunino et al. 1995; Prevosti et al. 2013). Some molecular, and "total evidence" phylogenetic analysis of canids showed that *L. griseus* is the sister taxon of *L. culpaeus* instead of *L. gymnocercus* (Bardeleben et al. 2005; Lindblad-Toh et al. 2005; Prevosti 2010; Austin et al. 2013; Tchaicka et al. 2016), something that could be interpreted as evidence of the separation of both species of grey foxes. Ruiz García et al. (2013), published a phylogenetic analysis based on CytB mitochondrial gene that includes a large sample of *L. culpaeus,* four specimens of *L. griseus,* and one of *L. gymnocercus,* plus other species of the genus, that indicates that *L. griseus* is paraphyletic (in the maximum parsimony analysis) and the sister of a clade of *L. culpaeus* that contains the only specimen of *L. gymnocercus*. A recent study based on DNA control region (Tchaicka et al. 2016), which included a larger sample for both species of grey foxes (although geographically limited, including one locality in Argentina and Bolivia, seven localities from central and northern Chile, and four from southern Brazil), found that *L. gymnocercus* and *L. griseus* were not reciprocally monophyletic, since mitcochondrial DNA (mtDNA) lineages from three specimens originally determined as *L. gymnocercus* were nested in the *L. griseus* clade. The authors concluded that both species are valid, and proposed that secondary hybridization and mtDNA introgression are the best explanation for the position of the *L. gymnocercus* samples within the *L. griseus* clade (Tchaicka et al. 2016).

On the other hand, morphological studies based on cranial and skin characters, using a morphometric approach (both traditional and 3D geometric morphometry), and the analysis of qualitative traits, failed to separate *L. griseus* from *L. gymnocercus*, supporting the hypothesis

that these species form a cline from the north-east to the south-west of Argentina, in which *L. griseus* inhabits drier areas and has a smaller size (Zunino et al. 1995; Prevosti et al. 2013). These studies indicate that specimens were assigned to each species based on their geographic provenance, something that could introduce logic circularity. Moreover, the contradiction between morphological and DNA studies could be an artifact, because most of the studies based on DNA are focused in resolving the relationships of the Canidae family, and in consequence include few specimens for each species. On the other hand there is no way to corroborate the taxonomic assignation of the sequences due to the lack of voucher information, and even the assignation of some Genbank public sequences is wrong (see Prevosti et al. 2013).

The main objective of this article was to reassess the species limits between *L. griseus* and *L. gymnocercus* analyzing molecular data from three mitochondrial markers (CytB, COI, CR), from a large, widely distributed sample of specimens of both species and others of the genus *Lycalopex*.

MATERIALS AND METHODS

Biological samples and molecular methods

The sample comprised sequences from 25 specimens identified as *L. griseus*, 30 specimens identified as *L. gymnocercus*, and 21 specimens of the following species used as outgroups: *L. culpaeus* (5), *L. fulvipes* (1), *L. vetulus* (1), *L. sechurae* (1), *Cerdocyon thous* (6), *Speothos venaticus*, *Chrysocyon brachyurus* (**Table 1**). Samples were obtained from road-killed animals. Most of the sequences were generated for these analyses, but we also included sequences from GenBank (see **Table 1)**. Phylogenetic trees were rooted using *Canis lupus*. Since several papers showed that there is no way to separate *L. griseus* and *L. gymnocercus* using skin or osteological characters (e.g., Zunino et al. 1995; Prevosti et al. 2013) we assigned each of these species using their current accepted distribution (e.g., Wilson & Mittermeier 2009).

DNA extractions from fresh tissue (muscle) were performed using a SDS-proteinase K-ClNa protocol (modified from Miller et al. 1988). Three different fragments were amplified by the polymerase chain reaction (PCR): (i) the complete cytochrome b gene (cytB) using primers CytBDF1 and CytBDR1 (Tchaika et al. 2006); (ii) the cytochrome c oxidase I (COI) in two fragments using universal primers LCO1490 and HCO2198 (COX; Folmer et al. 1994), and primers L6569 and H7227 (COI; Wayne et al. 1997); (iii) the 5′ portion of the mtDNA control region (CR), containing the first hypervariable segment (HVS-I), was amplified using primers MTLPRO2 and CCR-DR1 (Tchaika et al. 2006). Polymerase chain reactions (PCR) were performed in a final volume of 15 µl. Each reaction contained between 50 and 100 ng of DNA, 1.5 units of Taq polymerase, 1x PCR Buffer, 5 mM MgCl2, 0.2 µM of each primer and 0.025 mM dNTP each. BSA 0.4% was included as additive and enhancing agent to increase the yield of PCR reactions. PCR amplifications were carried out as follows: a first denaturation period at 94 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 45 s, annealing at 50-56 ºC for 1 min, and extension at 72 ºC for 1 min. Final extension at 72 ºC for 6 min terminated the reactions. A negative control with no template was included for each series of amplifications to test for contamination. PCR products were electrophoresed on a 1% TBE agarose gel stained with ethidium bromide. Sequencing was performed in MACROGEN (Korea).

Phylogenetic relationships

Sequences were edited and hand-aligned (since the alignments were trivial) using the software BioEdit (Hall 1999). Maximum Parsimony (MP) analyses were performed using the software TNT (Goloboff et al. 2008), using 1000 series of random addition of sequences (RAS), swapping the trees with tree bisection-reconnection (TBR), plus an additional rearrangement of all the most parsimonious trees found using TBR. A strict consensus was calculated using all the most parsimonious trees found. Branch support was evaluated with 10 000 pseudoreplicates of jackknife (JK; Farris et al. 1996). Maximum likelihood analyses were conducted using RAxML GUI (Silvestro & Michalak 2012), a graphical front-end for RAxML-VI-HPC (Randomized Accelerated Maximum Likelihood; Stamatakis 2006). Maximum likelihood with the thorough bootstrap (BS) option was run from a starting random seed to generate 1000 nonparametric bootstrap replicates. Analyses were performed for each marker separately and also combining the three markers in a single matrix. In the combined matrix, we excluded specimens that only had information for one marker. The analyses were performed using a GTR+G+I model as selected by using jModeltest (Posada 2008) available online on the server Phylemon (http//phylemon.bioinfo.cipf.es).

Table 1

Specimens and sequences used in our study. COX and COI represent two fragments of Cytochrome Oxidase I (see **Materials and Methods**). GB indicate sequences obtained from GenBank.Voucher number corresponds to the tissue collection of the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina (MACN-Ma-CT). Specimens without voucher correspond to tissues from personal collections of some of the authors (VR and PM).

Species	Sequence code / voucher number	Origin	Coordinates	GenBank Accsession codes			
				COX	COI	CytB	CR
Canis lupus	GB			KJ637137	AF028189	JF489119	AB605576
Cerdocyon thous	02/478	Arg, Corrientes, Mercedes	-58.13 W;-28.82 S	MK321407	MK321358		MK321538
	132/457	Arg, Santa Fe				MK321481	MK321549
	355/458	Arg, Entre Ríos, Paraná		MK321404	MK321347	MK321482	MK321523
	356/456	Arg, Entre Ríos				MK321483	
	368	Arg, Santa Fe, Vera		MK321413		MK321460	MK321552
	140	Arg			MK321348	MK321477	
	491	Arg, Entre Ríos, La Paz					MK321559
	GB			JQ601062	AF028193	EF106989	EF107031
Chrysocyon brachyurus	189	Arg, Corrientes, Mburucuyá	-58.13 W; -28.04 S	MK321444	MK321385	MK321497	MK321570
Lycalopex culpaeus	16/405	Arg, Chubut, Cholila	-71.56 W; -42.52 S	MK321434	MK321360	MK321489	MK321554
	18/463	Arg, Santa Cruz		MK321438	MK321381	MK321494	MK321537
	GB				AF028199	AF028175	
	590	Chile, Coquimbo		MK321445	MK321401	MK321499	
	95	Arg, Santa Cruz		MK321417	MK321351	MK321479	
	12	Arg, Santa Cruz		MK321418	KF701565	MK321476	
Lycalopex fulvipes	604	Chile, Araucanía, Gorbea		MK321446	MK321398	MK321516	MK321569
Lycalopex griseus	GB				AF028200	AF028152	
	05/376	Arg, Río Negro, 40 km W Viedma	-62.83 W; -40.60 S	MK321421	MK321353	MK321467	MK321540

(Table 1 cont.)

Species	Sequence code / voucher number	Origin	Coordinates	GenBank Accsession codes			
				COX	COI	CytB	CR
	06/377	Arg, Río Negro, RN 3 Between Viedma and San Antonio Oeste	-64.17 W; -40.70 S	MK321422	MK321354		MK321541
	08/379	Arg, Chubut, Península de Valdés	-64.14 W; -42.48 S	MK321423	MK321379	MK321468	MK321527
	10/451	Arg, Mendoza, Payunia, Arroyo Los Leones	-69.40 W; -36.40 S	MK321424	MK321355	MK321484	MK321518
	11/450	Arg., Mendoza, Malargüe, El Nevado	-68.68 W; -35.56 S	MK321419		MK321485	MK321542
	13	Arg, Santa Cruz, Jaramillo	-67.13 W; -47.19 S	MK321440	MK321372	MK321517	MK321555
	15/460	Arg, Mendoza, RP 173 near Monte Lomón	-66.98 W; -34.18 S	MK321420		MK321488	MK321529
	17/406	Arg, La Pampa, Algarrobo del Aguila	-67.48 W; -36.94 S		MK321361	MK321490	MK321544
	18/407	Arg, La Pampa, Algarrobo del Aguila	-67.30 W; -36.64 S	MK321435	MK321362	MK321491	MK321530
	40	Arg, Santa Cruz, Calafate	-72.21 W; -50.33 S	MK321426	MK321373	MK321469	MK321519
	43	Arg, Santa Cruz, Calafate	-72.21 W; -50.33 S	MK321427	MK321374	MK321495	MK321534
	59	Arg, Santa Cruz, Cañadón Seco	-67.62 W; -46.55 S	MK321428	MK321375	MK321474	MK321546
	117	Arg, Río Negro, Río Colorado	-64.11 W; -38.98 S	MK321429	MK321380		MK321547
	123	Arg, Río Negro, Río Colorado	-64.95 W; -38.87 S	MK321410	MK321376	MK321475	MK321535
	124	Arg, Río Negro, Río Colorado	-64.11 W; -38.98 S	MK321406	MK321377		MK321548
	458/481	Chile, Magallanes	-70.11 W; -52.56 S	MK321459	MK321366	MK321466	MK321524

(Table 1 cont.)

Species	Sequence code / voucher number	Origin	Coordinates	GenBank Accsession codes			
				COX	COI	CytB	CR
	594	Arg, Santa Cruz, Jaramillo	-67.14 W; -47.18 S	MK321416	MK321350	MK321478	
	595	Chile, Araucanía, near La Orilla	-72.27 W; -38.93 S	MK321449	MK321387	MK321498	MK321566
	596	Chile, Araucanía, near La Orilla	-72.35 W; -38.96 S	MK321450	MK321399	MK321510	MK321568
	597	Chile, Coquimbo	-70.83 W; -30.53 S	MK321451		MK321509	
	598	Chile, Coquimbo	-70.83 W; -30.53 S	MK321452	MK321386	MK321508	MK321567
	599	Chile, Coquimbo	-70.83 W; -30.53 S	MK321453	MK321396	MK321507	MK321565
	600	Chile, Coquimbo	-70.83 W; -30.53 S	MK321454	MK321400	MK321506	MK321576
	1013	Arg, Santa Cruz, Caleta Olivia	-67.77 W; -46.64 S	MK321415	MK321349	MK321480	
Lycalopex gymnocercus	GB1						EF107034
	GB2						EF107036
	GB3						EF107037
	GB4				AF028201	AF028153	
	01/476	Arg, Buenos Aires, Pellegrini	-63.35 W; -36.39 S	MK321405	MK321382	MK321472	MK321525
	04/474	Arg, Buenos Aires, Lobos	-59.20 W; -35.26 S	MK321412	MK321383	MK321473	MK321526
	12/468	Arg, La Pampa, Loventué RP 13 near RN 143	-65.39 W; -36.37 S	MK321425	MK321356	MK321486	MK321528
	13/469	Arg., La Pampa, RN 152, 8 km south General Acha	-64.59 W; -37.45 S	MK321439	MK321359	MK321487	MK321543
	20/409	Arg, La Pampa, Victorica	-59.03 W; -33.30 S	MK321436	MK321363		MK321531
	21/410	Arg, San Luis, Buena Esperanza	-65.25 W; -35.37 S	MK321437	MK321364	MK321515	MK321545
	23/412	Arg, Córdoba, Sampacho	-64.73 W; -33.39 S		MK321357	MK321492	MK321532

(Table 1 cont.)

Species	Sequence code / voucher number	Origin	Coordinates	GenBank Accsession codes			
				COX	COI	CytB	CR
	24/413	Arg, Córdoba, Sampacho	-64.94 W; -33.52 S		MK321365	MK321493	MK321533
	134/452	Arg, Santa Fe, near Reconquista	-59.70 W; -29.27 S	MK321430	MK321367	MK321460	MK321520
	136/454	Arg, Santa Fe, Vera, RN 11	-60.53 W; -30.33 S	MK321431	MK321368	MK321461	MK321521
	137/455	Arg, Córdoba, Río Segundo, RN 19	-63.90 W; -31.33 S	MK321432	MK321378	MK321470	MK321536
	181	Arg, Santa Fe, RP 80 between Galvez and Arocena	-61.07 W; -32.05 S	MK321408	MK321369	MK321462	MK321522
	344/453	Arg, Santa Fe, Colonia Belgrano	-61.40 W; -31.91 S	MK321433	MK321370		MK321550
	357	Arg, Santa Fe, near Villa Ocampo	-59.43 W; -28.49 S	MK321411	MK321352	MK321463	MK321551
	378	Arg, Santa Fe, Nelson	-60.72 W; -30.98 S	MK321409	MK321371	MK321471	MK321539
	382	Arg, Santa Fe, El Bonete	-60.58 W; -29.38 S	MK321414		MK321465	MK321553
	467	Arg, Santa Fe, RN 1 near Cayastacito	-60.56 W; -31.12 S		MK321397	MK321514	MK321575
	490	Arg., Entre Ríos, Paraná, RN 12	-60.12 W; -31.61 S	MK321447	MK321389	MK321513	MK321560
	492	Arg., Entre Ríos, La Paz, RN 12	-59.61 W; -30.91 S	MK321448	MK321388	MK321512	MK321558
	587	Arg, Jujuy, RN 66 near San Juancito	-65.03 W; -24.37 S		MK321395	MK321511	MK321572
	621	Py, Boquerón, PN Teniente Enciso	-61.65 W; -21.20 S				MK321574
	623	Py, Amambay, PN Cerro Corá	-56.01 W; -22.64 S		MK321403		MK321573
	626	Arg., Entre Ríos	-59.03 W; -33.30 S	MK321442	MK321390	MK321505	MK321557

(Table 1 cont.)

Species	Sequence code / voucher number	Origin	Coordinates	GenBank Accession codes			
				COX	COI	CytB	CR
	627	Arg., Entre Ríos	-59.03 W; -33.30 S	MK321455	MK321393	MK321504	MK321556
	628	Arg., Entre Ríos	-59.03 W; -33.30 S	MK321456	MK321394	MK321503	MK321562
	629	Arg., Entre Ríos	-59.03 W; -33.30 S	MK321457	MK321391	MK321502	MK321563
Lycalopex sechurae	GB	GB			AF028202	AF028154AF028178	
	583	Perú		MK321441	MK321402	MK321501	MK321564
Lycalopex vetulus	GB				AF028196	AF028148 AF028172	EF107035
	633			MK321458	MK321392	MK321500	MK321561
Speothos venaticus	620	Brasil, Lagoa da Pedra, Januária,		MK321443	MK321384	MK321496	MK321571

Inter- and intraspecific genetic distances were estimated with the Tamura 3-parameter model (Tamura 1992) implemented in the software MEGA6 (Tamura et al. 2013) for each marker separately. The variation rate among sites was modeled with a gamma distribution (shape parameter = 1). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. Other parameters were used following the default option of the software. The Kimura 2-parameter model is frequently used without justification, but recent analyses showed that is not always the best model for the data being analyzed (Srivathsan & Meier 2012). Consequently, the model used for estimating distances was the model that best fit our dataset, as chosen by the software MEGA6.

For the grey foxes, analyzing each marker separately, a Mantel test was performed between genetic distances and geographic (Euclidean) distances using the package ade4 for R (Dray & Dufour 2007; R Development Core Team 2016). For each analysis, 9999 replicates were performed.

RESULTS

In **Table 2** there is a summary of the characteristics of the sequences and data sets analyzed in this article. All the new sequences were deposited in GenBank (codes in **Table 1**). The MP analysis of the combined dataset (three mitochondrial markers) resulted in 3726 trees of 1424 steps. The strict consensus tree showed *Lycalopex* as a well-supported monophyletic group (JK 99), sister to *Cerdocyon* (**Fig. 2**). *Lycalopex sechurae* is the sister taxon of the other species of *Lycalopex*, which are in a polytomy that include well to moderately-supported clades including the remaining species of *Lycalopex*: *L. fulvipes*, *L. vetulus* (JK 98), *L. culpaeus* (JK 87), and the specimens of *L. gymnocercus* and *L. griseus* grouped in two clades (A and B) that include haplotypes coming from individuals of both species (i.e., the traditional concept of these species is not monophyletic). Clade A (JK 85) includes haplotypes from specimens of *L. gymnocercus* from the northeast of Argentina (Entre Ríos and Santa Fe Provinces), northeastern Paraguay (Amambay) and southern Brazil. Clade B (JK 73) includes sequences of specimens of *L. gymnocercus* from Buenos Aires, Santa Fe and Córdoba (Argentina) and western Para-

Table 2
Characteristics of the datasets analyzed

Dataset	Number of sequences (own/GB)	Length of the sequences (bp)*	Number of variable characters	Number of parsimony informative characters
CytB	65 (58/7)	1024	288	173
COX	59 (57/2)	658	159	96
COI	65 (58/7)	570	154	104
CR	65 (58/7)	601	167	115
Combined data sets	78	2867	762	480

* some sequences have a shorter length due to sequencing problems

guay (Boquerón), and of specimens assigned to *L. griseus,* from Coquimbo, Araucanía and Magallanes (Chile), and Southern Argentina (La Pampa, Mendoza, Río Negro, Chubut and Santa Cruz). None of the clades that group specimens of *L. gymnocercus* and *L. griseus* show any phylogeographic pattern in the arrangement of the specimens.

The ML analysis of the combined dataset resulted in a tree (likelihood -11538.03797; **Fig. 3**) with more resolution inside the *Lycalopex* clade. In this case, *L. vetulus* is the most basal species, and all the clades that were collapsed in a polytomy in the MP tree show clearer relationships (although with low support; **Fig. 3**). Clades A and B are also present, with moderate to high support, but in this analysis, *L. culpaeus* is the sister taxon of Clade B (BS of Clade B + *L. culpaeus*: 51; **Fig. 3**), while Clade A is placed as sister clade of *L. fulvipes* + Clade B + *L. culpaeus* (BS 61; **Fig. 3**).

The analysis of each marker separately resulted in trees that were inconclusive, with little resolution and low support. The results of these analyses are presented as supplementary data (**Supplement 1**).

Distance between Clades A and B was 0.039, which is similar (or even larger) than the distances between other species of *Lycalopex,* and larger than the intra-clade distance (**Table 3**). The Mantel test showed that there is no correlation between the genetic and the geographic matrix for any of the matrices (R -0.1012; -0.0036; 0.0393 for CR, cytB and COI respectively, $p > 0.1$ in all the cases), which is congruent with the lack of correlation between geography and topology obtained in the phylogenetic trees. The results did not change when analyzing Clade B separately.

DISCUSSION

Our results based on three mitochondrial genes show that South American grey foxes (*L. griseus* and *L. gymnocercus*) do not form a monophyletic group, and instead could be separated in two clades (A and B), with moderate node support (**Figs. 2-3**), that are not always recovered in individual gene analyses (**Supplement 1**). Also it should be noted that the relationship of Clades A and B to other *Lycalopex* species (*L. culpaeus* and *L. fulvipes*), is ambiguous since in the MP analysis the four species are in a politomy, while in the ML analysis the nodes that support the relationships among them are poorly supported (**Figs. 2-3**). This lack of resolution in the phylogenetic placement of grey foxes should be taken into consideration, since the distribution of both species is not ideally sampled, mainly in the area where they are in contact as traditionally considered, and also in the area where clades A and B are in contact (at least as evidenced from our sample; see **Fig. 1**). This apparent recognition of two clades that do not form a monophyletic group is congruent with previous phylogenetic works (Wayne et al. 1997; Zrzavý & Řičánková 2004; Bardeleben et al. 2005; Prevosti 2006, 2010; Perini et al. 2010; Fuentes González & Muñoz Durán 2012; Austin et al. 2013; Tchai-

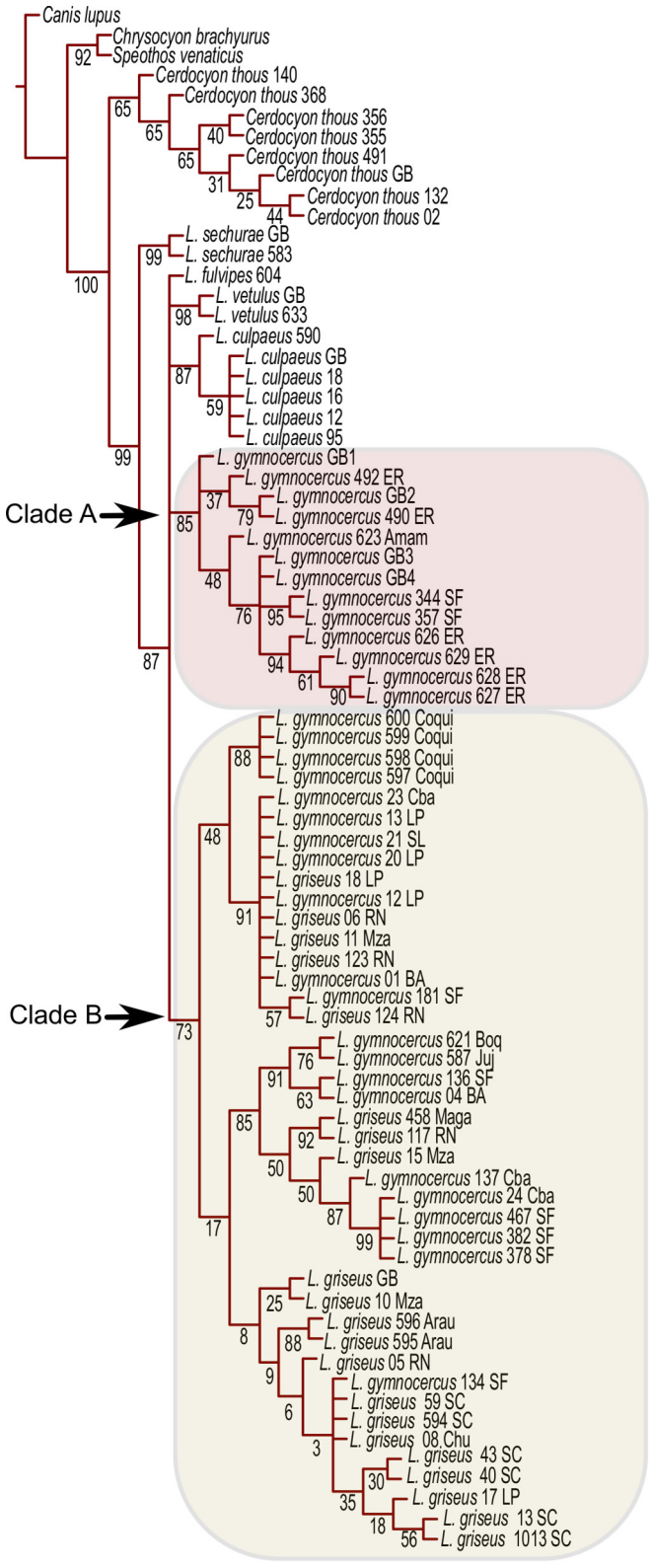

Fig. 2. Strict consensus tree obtained from the maximum parsimony analysis of the three mitochondrial markers. Numbers below the branches indicate jackknife support values. Numbers associated to taxon names refer to sequence codes (see **Table 1**). Amam: Amambay Department (Paraguay); Arau: Araucanía Region (Chile); BA: Buenos Aires Province (Argentina); Boq: Boquerón Department (Paraguay); Cba: Córdoba Province (Argentina); Chu: Chubut Province (Argentina); Coqui: Coquimbo Region (Chile); ER: Entre Ríos Province (Argentina); Juj: Jujuy Province (Argentina); LP: La Pampa Province (Argentina); Maga: Magallanes Region (Chile); Mza: Mendoza Province (Argentina); RN: Río Negro Province (Argentina); SC: Santa Cruz Province (Argentina); SF: Santa Fe Province (Argentina); SL: San Luis Province (Argentina).

cka et al. 2016) that found that *L. griseus* and *L. gymnocercus* do not form a monophyletic group. However, in other analyses based on nuclear genes (Bardeleben et al. 2005; Lindblad-Toh et al. 2006) or in some combining nuclear and mitochondrial genes (Bardeleben et al. 2006) both species form a single clade, suggesting that the non-monophyletic relationship between *L. griseus* and *L. gymnocercus* is a signal that comes from mitochondrial data. It should be noted that some level of incongruence between nuclear and mitochondrial genes in the canid phylogeny was detected by Prevosti (2010) using different topological measurements. In this context it is interesting that the relationships found with nuclear data are more in agreement with morphological studies, which in the case of these species failed to find differences between *L. griseus* and *L. gymnocercus* (Zunino et al. 1995; Prevosti et al. 2013).

The geographic distribution of Clades A and B do not agree with

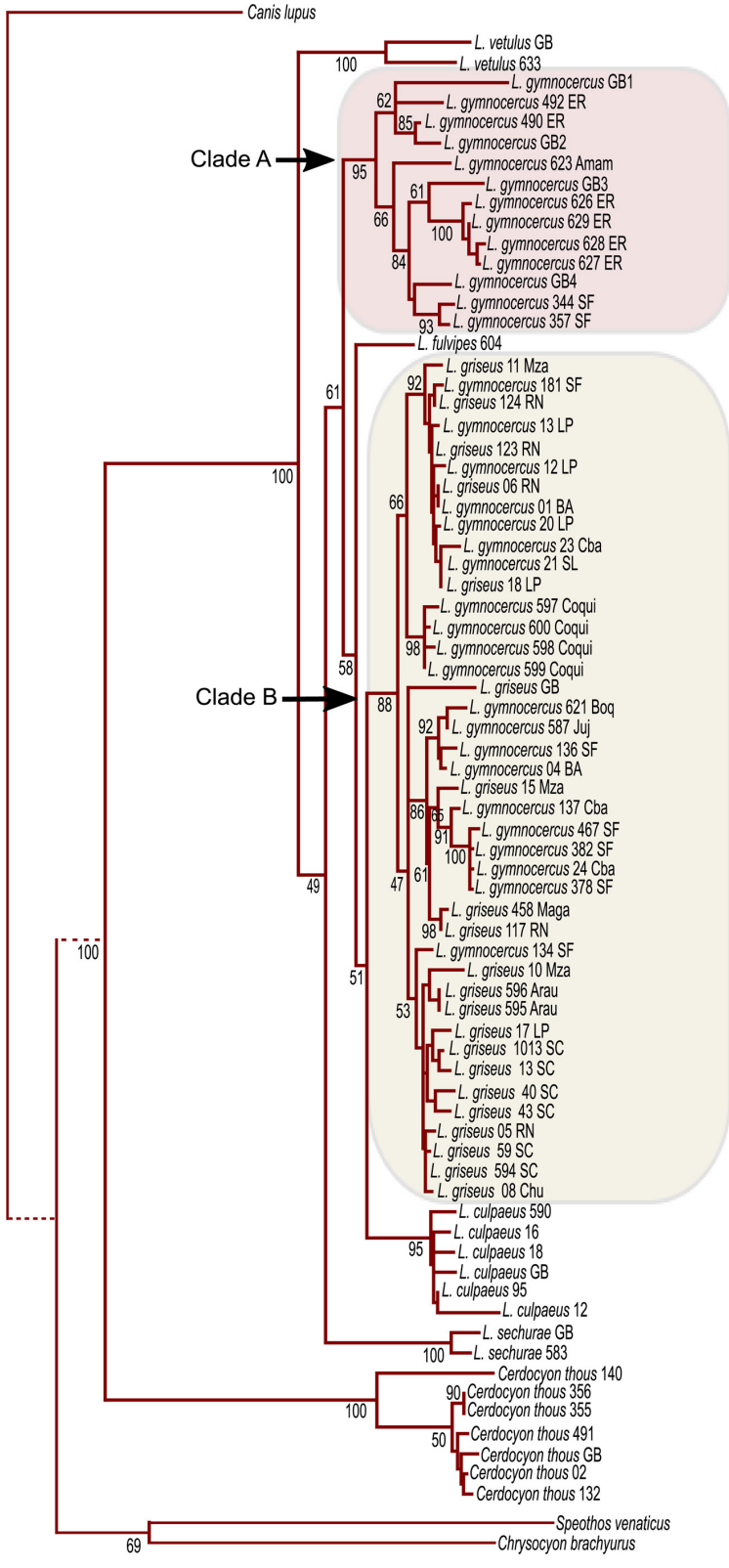

Fig. 3. Maximum likelihood phylogram based on the analysis of the three mitochondrial markers. Numbers below the branches indicate bootstrap support values. Numbers associated to taxon names refer to sequence codes (see **Table 1**). Amam: Amambay Department (Paraguay); Arau: Araucanía Region (Chile); BA: Buenos Aires Province (Argentina); Boq: Boquerón Department (Paraguay); Cba: Córdoba Province (Argentina); Chu: Chubut Province (Argentina); Coqui: Coquimbo Region (Chile); ER: Entre Ríos Province (Argentina); Juj: Jujuy Province (Argentina); LP: La Pampa Province (Argentina); Maga: Magallanes Region (Chile); Mza: Mendoza Province (Argentina); RN: Río Negro Province (Argentina); SC: Santa Cruz Province (Argentina); SF: Santa Fe Province (Argentina); SL: San Luis Province (Argentina).

the traditionally accepted distribution of *L. griseus* and *L. gymnocercus* (the first in Chile, Patagonian region and west part of Argentina and Bolivia, and the second on central, northern and eastern Argentina, part of Bolivia, Paraguay, Uruguay and southern Brazil; Zunino et al. 1995; Macdonald & Sillero Zubiri 2004; Sillero Zubiri et al. 2004; Wilson & Mittermeier 2009). In this sense, our results are more congruent with Tchaicka et al.'s (2016) recent work, that although it has a limited geographic sample, mainly in central areas where both species are in contact, found that grey foxes do not form a

Table 3

Estimates of evolutionary divergence over sequence pairs between groups. The number of base substitutions per site from averaging over all sequence pairs between groups are shown.

	C. thous	Clade B	Clade A	*L. culpaeus*	*L. sechurae*	*L. fulvipes*
C. thous	0.012					
Clade B	0.092	0.017				
Clade A	0.092	0.039	0.025			
L. culpaeus	0.09	0.021	0.030	0.007		
L. sechurae	0.09	0.035	0.041	0.035	0.008	
L. fulvipes	0.089	0.023	0.035	0.020	0.035	n.a.

monophyletic group and could be separated in clades that are similar to our clades A and B. It must be noted that the traditional interpretation about the distribution of these supposed valid species (i.e., *L. griseus* and *L. gymnocercus*) is arbitrary, since there is no clear way to separate them on the basis of morphological features (Zunino et al. 1995; Prevosti et al. 2013). This arbitrary taxonomic assignation becomes apparent in the different geographic distribution of both species established by different authors. For example, Kraglievich (1930) considered that the foxes from the La Pampa province, in central Argentina, belong to *L. griseus*, while Cabrera (1957) considered that they belong to *L. gymnocercus*. Based on all these evidences, the most recent list of mammals of Argentina consider both species as synonyms under *Lycalopex gymnocercus* (Teta et al. 2018).

Regarding nomenclatural issues, Clade B includes one specimen from the topotypic area of *L. griseus,* that is the coast of the Magellan Strait (Cabrera 1957), but we do not have access to samples from the type locality of *L. gymnocercus* (around Asunción, Paraguay; Cabrera 1957). The absence of samples from the type locality of *L. gymnocercus* prevents us to confirm the assignation of Clade A to *L. gymnocercus.* Moreover, the two specimens sampled from Paraguay are nested in the different clades, and none is geographically close to Asunción to make any inference (see **Fig. 1**). In fact, the clade that is assigned by Tchaicka et al. (2016), to *L. gymnocercus*, cannot be confirmed as belonging to this species due to the same issue.

Although the distribution of these mitochondrial clades do not match with the traditionally delimited distribution of *L. griseus* and *L. gymnocercus*, they present an interesting pattern, since they are separated by the Paraguay and Paraná rivers (**Fig. 1**), Clade A being limited to northeastern Argentina and Paraguay, and southern Brazil, while Clade B includes specimens from the rest of Argentina, Chile and the western Paraguay (**Figs. 2**-**3**). A similar pattern of species separation has been already described for other animals and plants (e.g., Parodi 1934; Ringuelet 1961; Cabrera & Willink 1980; Myers 1982; Pennington et al. 2000; Giraudo & Arzamendia 2004; Arzamendia & Giraudo 2009, 2012; De la Sancha et al. 2011; Chemisquy & Flores 2012). Athough the rivers themselves do not strictly limit the distribution of Clade A to the west of Paraguay and Paraná rivers (**Fig. 1**), specimens are restricted to an area close to these rivers, as happens with other mammals, vertebrates and plants (Myers 1982; Pennington et al. 2000; Giraudo & Arzamendia 2004; Arzamendia & Giraudo 2009, 2012). In fact, due to historical (e.g., geologic, edaphic) and ecological (environmental conditions) factors, these rivers appear to act as a dispersal root and the generator of special environments that facilitate the spread of some species that are distributed to their east (Myers 1982; Giraudo & Arzamendia 2004; Arzamendia & Giraudo 2009, 2012). The annual precipitation also di-

minishes from east to west in this area (Parodi 1934; García 1991; Cabrera & Willink 1980; Pennington et al. 2000), and in the Santa Fe province (Argentina), where Clade A is found west of the Paraná river; it is found in an area that from a geologic point of view belongs to the zone of influence of the Paraná river (Iriondo 2010). Thus historical and ecological factors related to this major river could explain why Clade B is limited to the south and west of the Paraná and Paraguay rivers, while Clade A is distributed to the east of these rivers or very close to them.

It should be noted that specimens of Clades A and B are very closely distributed and even overlap in the Santa Fe province (Argentina; **Fig. 1**), where they are probably in sympatry, since specimens of each clade came from localities located less than 40 km apart from each other, and are in similar environments (areas transformed in agroecosystems) without any barrier between them (**Fig. 1**). This potential sympatric distribution in this area could be a recent phenomenon, generated by the strong environmental modifications caused by humans since the nineteenth century, where large areas where transformed in crop fields or occupied by livestock farming. Also, this area could be considered a hybrid zone, which could be facilitated by the fact that both species were separated recently, as suggested by molecular dating analysis (~500 000 ybp; Tchaicka et al. 2016).

We think that two competing hypotheses could be discussed regarding the biological meaning of our results, and previously published evidence using morphology and nuclear DNA: 1) grey foxes belong to two different species, as is traditionally supported, and as suggested by recent works (e.g., Tchaicka et al. 2016); 2) they belong to the same species as morphology (Zunino et al. 1995; Prevosti et al. 2013), and the limited published nuclear data (Lindblad-Toh et al. 2005) indicate. The first hypothesis is the best supported from our results, although not all the analyses recovered Clades A and B (**Supplement 1**), and when present, they showed moderate branch support values (**Figs. 2**, **3**). Consequently, one can consider that *L. griseus* (= Clade B) is widely distributed in Argentina, excluding the Mesopotamia (i.e., Entre Ríos, Corrientes and Misiones) and apparently is present western to the Paraguay river in Paraguay. "*Lycalopex gymnocercus*" (= Clade A), on the other hand, is present eastern to the Paraná-Paraguay river, with some specimens also present in Santa Fe. It is important to note that until a specimen from the type locality of the species is included in the analysis, we cannot be sure that Clade A is assignable to *L. gymnocercus*, and that is why we are using inverted commas in the name of the species.

FUTURE CONSIDERATIONS

A limitation of the interpretation that clades A and B represent two different species is that our results are based only on mitochondrial genes, and since they are inherited only from the mother they only tell part of the genetic history of these foxes (Funk & Omland 2003), and could be interpreted as a linkage-group tree because the three genes we used are not independent (Moore 1995; Giannasi et al. 2001). Moreover, in the last years several papers were published showing a difference in species delimitation based on nuclear and mitochondrial genes. Analyses published in bears (Hailer et al. 2012; Cronin et al. 2014; Cahill et al. 2013; Kutschera et al. 2014), monkeys (Zinner & Ross 2014), cricetids (Cañón et al. 2014) and goats (Ropiquet & Hassanin 2006) showed that morphology is more in agreement with nuclear DNA than with mitochondrial genes, which has important implications in species delimitation (Cahill et al. 2013; Cañón et al. 2014; Zinner & Ross 2014; vonHoldt et al. 2016). These studies showed the relevance of hybridization, introgression and incomplete lineage sorting, and that multiple independently inherited loci are needed to resolve complex evolutionary patterns (Kutschera et al. 2014; vonHoldt et al. 2016). In this context, and without information from other lines of DNA evidence (i.e., nuclear markers) that could discard the presence of introgression or incomplete lineage sorting, we must consider that our results can change with future evidence, and that both species could end up being synonymized, as morphology suggests.

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SUPPLEMENTARY ONLINE MATERIAL

Supplement 1

https://www.sarem.org.ar/wp-content/uploads/2019/07/SAREM_MastNeotrop_26-1_Chemisquy-sup1.pdf

Phylogenetic analysis of the individual markers. The COI analyses (both MP and ML) were the ones that best resemble the combined analyses, recovering Clades A and B (Supp. **Fig. S1 a**, **b**). The MP analysis of cytB failed to recover Clade A (Supp. Fig. S1 c), and although that clade is present in the ML analysis, its support is extremely low (BS 25; Supp. Fig. S1 d). Moreover, in the ML analysis *L. culpaeus* is nested inside Clade B (**Fig. S1 d**). Finally, the MP analysis of the CR dataset did not recover Clade B, and most specimens of that clade are in a large polytomy (individually or in small clades) that also include Clade A, and the remaining species of *Lycalopex* (Supp. **Fig. S1 e**). In the ML analysis of CR, clade B was recovered (but with low support; BS 45), and all the internal nodes that separate the different *Lycalopex* species have almost no support (BS > 30; Supp. **Fig. S1 f**).

Fig. S1. Schematic representation of the results of the maximum parsimony (MP) and maximum likelihood (ML) analyses of each marker separately. a, MP analysis of COI; b, ML analysis of COI; c, MP analysis of cytB; d, ML analysis of cytB; e, MP analysis of CR; f, ML analysis of CR. Numbers above the branches represent jackknife and bootstrap values respectively.