Mastozoología Neotropical, 20(2):289-300, Mendoza, 2013
Versión impresa ISSN 0327-9383
Versión on-line ISSN 1666-0536

http://www.sarem.org.ar

Artículo

REVISION OF THE SYSTEMATIC STATUS OF PATAGONIAN AND PAMPEAN GRAY FOXES (CANIDAE: *Lycalopex griseus* AND *L. gymnocercus*) USING 3D GEOMETRIC MORPHOMETRICS

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ABSTRACT. Argentinean "zorros de campo" are currently included in two species: *Lycalopex griseus* and *L. gymnocercus*. *Lycalopex gymnocercus* lives in northern Patagonia and in most of central and northern Argentina. *Lycalopex griseus* is smaller and lives in Patagonia and throughout western Argentina. A previous traditional morphometric study using cranio-dental measurements considered both forms to be the same species, showing clinal reduction in size from northeastern to southwestern Argentina. Here we tested the synonymy of these foxes and the existence of clinal variation using a large sample and geometric morphometric methods. Our results rejected the separation of these foxes in two different species and confirmed, based on cranium and mandible size and shape that they belong to the same species. Also, we show there is a clinal variation in size that has an allometric component in cranial and mandibular shape, which accounts for the differences between these foxes.

RESUMEN. Revisión del estatus sistemático de los zorros grises patagónico y pampeano (Canidae: *Lycalopex griseus* y *L. gymnocercus*) usando morfometría geométrica 3D. Los zorros argentinos de campo son incluidos en dos especies: *Lycalopex griseus* y *L. gymnocercus*. *Lycalopex gymnocercus* habita el norte de Patagonia y gran parte del centro y norte del país, al este de los Andes. *Lycalopex griseus* es una especie de menor tamaño que habita Patagonia, extendiéndose hacia el norte del país bordeando los Andes. Un estudio morfométrico previo, basado en medidas cráneo-dentarias, concluyó que se trataba de una sola especie que presentaba variación clinal, disminuyendo de tamaño desde el NE al SO de Argentina. Este estudio se basó en una muestra limitada y en técnicas estadísticas tradicionales que no permitieron separar la forma del tamaño. Nuestro objetivo es poner a prueba la hipótesis de sinonimia entre estas especies y la variación clinal, estudiando una amplia muestra de especímenes mediante métodos de morfometría geométrica. Los análisis de morfometría geométrica mostraron que no hay diferencias de forma entre ambas, por lo que se apoya la hipótesis de la sinonimia entre las mismas. También se detectó la presencia de una variación clinal en tamaño, la cual incluye un componente alométrico en la forma que básicamente coincide con las supuestas diferencias morfológicas entre estos zorros.

Key words: Geometric morphometrics. *Pseudalopex*. South America. Systematics.

Palabras clave: América del Sur. Morfometría geométrica. *Pseudalopex*. Sistemática.

Recibido 9 junio 2013. Aceptado 31 agosto 2013. Editor asociado: E Lessa

INTRODUCTION

Recent phylogenetic analyses have shown that southern South American foxes (traditionally included in *Dusicyon* Hamilton Smith, 1839 or *Pseudalopex* Burmeister, 1856) form a clade that is independent from other genera (i.e., *Dusicyon*, *Cerdocyon* (Linnaeus, 1766), *Atelocynus* Cabrera, 1940; e.g., Bardeleben et al., 2005; Lindblad-Toh et al., 2005; Slater et al., 2009; Prevosti, 2010; Austin et al., 2013). These findings support the interpretation of Zunino et al. (1995; see also Galliari et al., 1996) who stated that southern South American foxes should be included in the genus *Lycalopex* Burmeister, 1854, this name being the oldest one available for this clade. However, this proposal has not been followed in several subsequent considerations of these foxes (e.g. Novaro, 1997; Sillero Zubiri et al., 2004; Wilson and Mittermeier, 2009; IUCN web site: http://www.iucnredlist.org), and the name *Pseudalopex* is still in use (but see Wilson and Reeder, 2005).

The genus *Lycalopex* includes several species, *L. vetulus* (Martin, 1837), limited to northeastern Brazil; *L. sechurae* Thomas, 1900 of the Sechura desert of Perú and Ecuador; *L. fulvipes* (Martin, 1837) in the Valdivian region of Chile; and *L. culpaeus* (Molina, 1782) along Patagonia and the Andes from southern Chile and Argentina to southern Colombia (Sillero Zubiri et al., 2004; Wilson and Mittermeier, 2009). Two other species included in this genus are gray foxes that also live in open environments of southern South America: *L. gymnocercus* (Fischer, 1814) in grasslands of central and eastern Argentina, southern Brazil, Paraguay (type locality) and Uruguay; and *L. griseus* (Gray, 1837) in dry open environments of Chile and Argentina ranging from western Argentina to Patagonia (type locality; Cost of the Magellan Strait) and most arid environments of Chile (Sillero Zubiri et al., 2004; Wilson and Mittermeier, 2009; **Fig. 1**). These last two species overlap broadly in northern Patagonia and in south-central and northwestern Argentina (Sillero Zubiri et al., 2004; Wilson and Mittermeier, 2009), apparently living in sympatry in several areas (Mares et al., 1989; Barquez et al., 1991; Díaz and Barquez, 2002). However, in these areas of potential sympatry it is difficult to separate *L. gymnocercus* from *L. griseus* using morphological characters, because *L. gymnocercus* is smaller towards the southern and western limits of its distribution. This situation was described by Zunino et al. (1995), who analyzed skull and skins of these species along Argentina and, using traditional morphometrics, found that these species form a geographic cline associated with changes in precipitation, becoming smaller towards the southwest. These authors showed that the cranial characters used to separate them (i.e., development of a sagittal crest, width

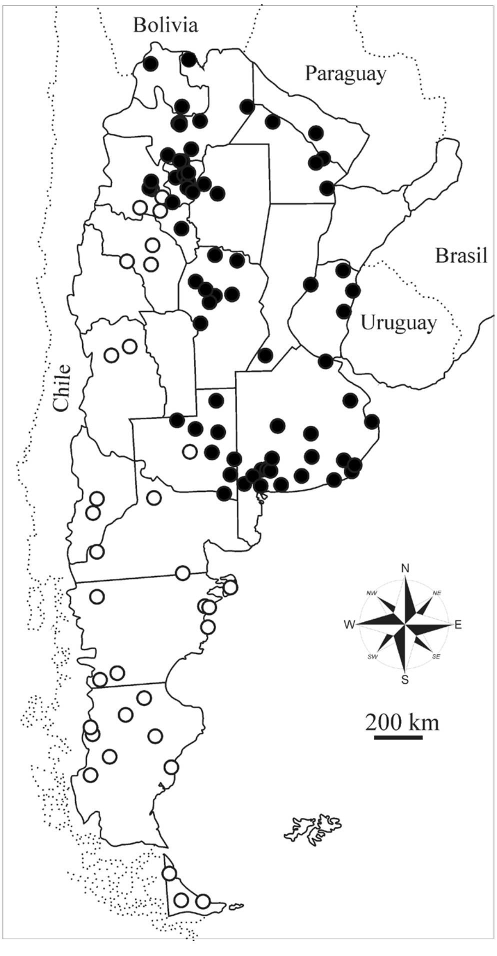

Fig. 1. Map showing the geographical distribution of the studied samples. Open circles: *Lycalopex griseus*; black circles: *Lycalopex gymnocercus*.

of the postorbital constriction, distance from this constriction to the postorbital process; see Kraglievich, 1930; **Fig. 2**) are highly variable, even within populations. They concluded that these foxes belong to the same species and that *L. gymnocercus* should be used as its name. However this taxonomic hypothesis has not always been followed (e.g., Sillero Zubiri et al., 2004; Wilson and Mittermeier, 2009; but see Galliari et al., 1996; Canevari and Vaccaro, 2007; Wilson and Reeder, 2005) or further tested.

The objectives of our work were to evaluate the proposal of Zunino et al. (1995) regarding the synonymy of *L. gymnocercus* and *L. griseus* using a larger sample and broader geographical coverage and geometric morphometric analyses, and to further explore the relationship between cranial shape and size and geography

MATERIALS AND METHODS

Specimens

We examined 479 skulls of *L. griseus* (n = 124) and *L. gymnocercus* (n = 355) from 109 different localities distributed throughout Argentina, Brazil, Bolivia and Uruguay (**Online Supplementary Material 1**). Species assignment were based on size, sagittal crest development, width of postorbital constriction, and distance from this constriction to the postorbital process, characters traditionally used to separate these taxa (see Kraglievich, 1930). We also used the current accepted distribution of these taxa as an additional criterion (Sillero Zubiri et al., 2004; Wilson and Mittermeier, 2009; **Fig. 1** to see their respective localities). Only adult specimens were included in this sample, which were determined by fully erupted permanent dentition. The specimens belong to the mammal collections of the following institutions: American Museum of Natural History (AMNH; New York, USA); Colección Félix de Azara (CFA, Ciudad Autónoma de Buenos Aires, Argentina); Co-lección Mamíferos Lillo (CML, San Miguel de Tucumán, Argentina); Field Museum of Natural History (FMNH, Chicago, USA); Colección Grupo de Ecología Comportamental de Mamíferos (GECM; Bahía Blanca, Argentina); Colección de Mamíferos del Laboratorio de Investigaciones en Evolución y Biodiversidad (LIEB, Esquel, Argentina); Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (MACN, Ciudad Autónoma de Buenos Aires, Argentina); Museo de La Plata (MLP, La Plata, Argentina); National Museum of Natural History, Smithsonian Institution (NMNH, Washington DC, USA).

Fig. 2. Pictures of the dorsal, ventral and lateral views of the cranium, and lateral view of the mandibles of *Lycalopex griseus* (MACN 15265; A, C, E, G) and *Lycalopex gymnocercus* (MACN 15388; B, D, F, H), respectively.

Geometric morphometrics

Thirty-eight cranial and 18 mandibular landmarks were used to describe the skull (**Fig. 3**; **Online Supplementary Material 2**). We used landmarks with clear homology corresponding to types 1 and 2 (sensu Bookstein, 1991), such as tripartite sutures and processes, and semi-landmarks corresponding to type 3 (sensu Bookstein, 1997). The landmarks were digitized in 3Dimensions with a Microscribe MX 6DOF System (GoMeasured3D, Amherst, VA, USA), which has an accuracy of 0.0508 mm and uses optical sensors to measure three-dimensional coordinates, eliminating the problems of nonrandom error found in magnetic digitization systems. Landmarks and semi-landmarks are listed in **Online Supplementary Material 2** and illustrated in **Fig. 3**. We used half the cranium, in order to maximize the sample numbers and to avoid repeating landmarks. To improve visualization and avoid putative Procrustes alignment artifacts, the hemi cranium landmark configuration was reflected in the plane of symmetry defined by sagittal landmarks. To do so, we used the R-function AMP.r written by Annat Haber, University of Chicago (available online at http://life.bio.sunysb.edu/morph/; see also Online Resource 1 of Cassini and Vizcaíno, 2012).

Landmark configurations were superimposed through generalized Procrustes analysis (GPA, Goodall, 1991; Rohlf, 1999), minimizing the sum of squared distances between homologous landmarks by translating, rotating, and scaling them to best fit, using MorphoJ 1.04a (Klingenberg, 2011). This analysis removes the spatial variation that does not correspond to form (Dryden and Mardia, 1998). The semilandmarks, which were taken to characterize the curves of mandible, were resampled and equally-spaced using the software “resample” of NYCEP (Reddy et al., 2007). Procrustes coordinates obtained in the Procrustes superposition are the shape variables, while the centroid size represents the size of cranium and mandibles (Zelditch et al., 2004).

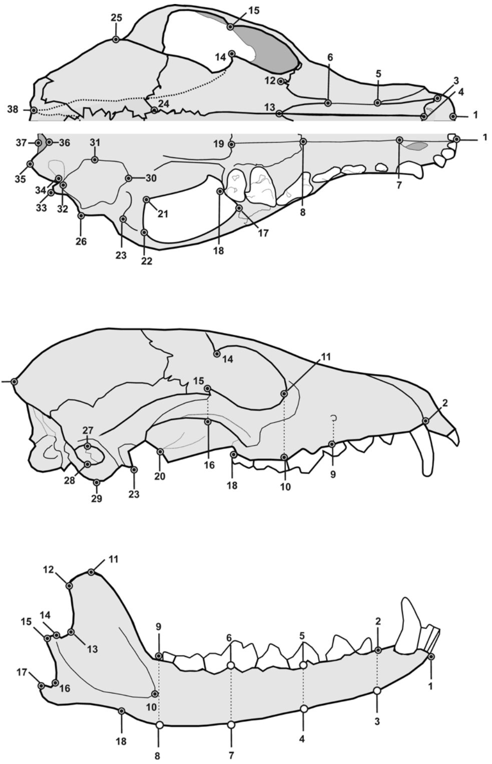

Fig. 3. Placement of the landmarks and semi-landmarks used in the geometric morphometric analyses (see **Online Supplementary Material 2** for more details).

Sexual dimorphism

The sexual size dimorphism was tested by Mann Whitney U test (Zar, 1999), using the centroid size of the cranium and mandible as size proxy with the software R 2.14.1 (R Development Core Team, 2011). The difference in shape between sexes was explored calculating the Procrustes distance between the consensus shape of males and females (using the Procrustes coordinates), and the significance of these differences was established with 10 000 random permutations (Manly, 1997) in the software MorphoJ 1.04a (Klingenberg, 2011). In the last test, it was difficult to understand the size effect on sexual dimorphism. Therefore, we compared the Procrustes distances between the consensus shape of each sex with the mean Procrustes distances of male specimens to the male consensus shape, and of female distances to female consensus shape, using the software R 2.14.1 (R Development Core Team, 2011). When the distance between consensus shapes of the sexes was lower than the mean distance of each sexed specimens to its respective consensus, we considered that sexual dimorphism was irrelevant for our comparisons and analyses. Additionally, we explored the distribution of male and female specimens in the scatterplots of the principal components obtained from the Procrustes coordinates (see below). These analyses were performed independently in the samples assigned to *L. griseus* and *L. gymnocercus*.

L. griseus and *L. gymnocercus* discrimination

Differences in size were evaluated using the mandible and cranium centroid size with the Mann Whitney U test (Zar, 1999). For shape comparisons, we performed principal component analysis (PCA) from the variance-covariance matrix of Procrustes coordinates of cranium and mandible, and discriminant analysis (DA) with cross-validation for posterior correct reclassification estimated, as implemented in MorphoJ 1.04a (Klingenberg, 2011). The Procrustes distance between the consensus shapes of the two species was compared with the distance of each specimen to its respective consensus, as outlined above for sexual dimorphism.

Allometry

Allometry was analyzed with multivariate regression, using Procrustes coordinates as dependent variables and log-centroid size as the independent one, and the significance was established with 10 000 permutations in MorphoJ (Zelditch et al., 2004; Klingenberg, 2011).

Geographic cranial size and shape variation

We used multiple regression analysis between log-transformed centroid size and latitude and longitude (Zar, 1999) with the software R 2.14.1 (R Development Core Team, 2011, lm command). Using the same software, the autocorrelation due to spatial distribution was tested with the Durbin-Watson analysis (Zar, 1999; dwtest command of R). To test for the relationship between shape and latitude/longitude, we used redundancy analysis (RDA, Legendre and Legendre, 1998), and to control for spatial autocorrelation we performed another RDA including a selection of spatial (geographic) filters (Diniz Filho et al., 2005, 2009) obtained with the software SAM 4 (Rangel et al., 2010). Spatial filters were selected using the Akaike information criteria (AIC; see Godínez Domínguez and Freire, 2003; Burnham and Anderson, 2004) with the “step” command of the Vegan R’ module (Oksanen et al., 2013). Because there is some controversy relative to the use of this model selection routine with RDA (see Godínez Domínguez and Freire, 2003; Oksanen et al., 2013), we performed a backward and forward model search to assure that we could find the model with the lower AIC. Examination of partial variation explained by latitude and longitude was also analyzed using redundancy analysis.

RESULTS

Sexual dimorphism

The cranium of *L. griseus* did not present significant size (Mann Whitney U = 30.500, p = 0.568) or shape (Procrustes distance = 0.017, p = 0.263) sexual dimorphism. Contrary to this, the cranium of *L. gymnocercus* presented significant sexual dimorphism in size (Mann Whitney U = 4800.000, p <0.0001) and shape (Procrustes distance = 0.013, p <0.0001). The difference between mean female and male centroid size is only 0.84% of the mean male centroid size, and the Procrustes distance between male and female consensus shape is lower than the mean distance of each sexed specimen and the corresponding size consensus shape (female = 0.053, male = 0.051). PCA did not show any separation between sexes (data not shown). Other studies have also shown that sexual dimorphism in cranial and dental measurements of *L. gymnocercus* is low (Zunino et al., 1995; Prevosti and Lamas, 2006; Luengos Vidal et al., 2009).

Mandibles showed similar results, with significant size and shape differences implying sexual dimorphism only in *L. gymnocercus* (Mann Whitney U = 4325.000, p <0.0001; Procrustes distance = 0.013, p <0.0001). These differences in size between sexes were very small (0.76%), and the Procrustes distance between male and female consensus shapes (0.013) was lower than the mean distance of each sexed specimen to their corresponding size consensus shape (female = 0.062, male = 0.067). PCA did not show separation between sexes (data not shown).

Following these results males and females were pooled together in subsequent analyses.

L. griseus versus *L. gymnocercus*

Lycalopex gymnocercus has a larger cranium centroid size than *L. griseus*, a difference that was highly significant (Mann Whitney U = 3617, p < 0.0001). The PCA (**Fig. 4A**) shows a clear overlap between the two putative species, but *L. griseus* tends to be placed towards positive scores of axis 2, whereas *L. gymnocercus* shows the opposite tendency. The shape analysis shown by axis 2 (7.87% of total variance) indicates that *L. griseus* has shorter, wider, and higher (specially at the braincase) cranium, with larger orbits and tympanic bullae, concave posterior borders of the palate, smaller temporal fossae, wider postorbital constriction, wider pterygoid fossae, and smaller postorbital processes of the frontal. Towards the negative

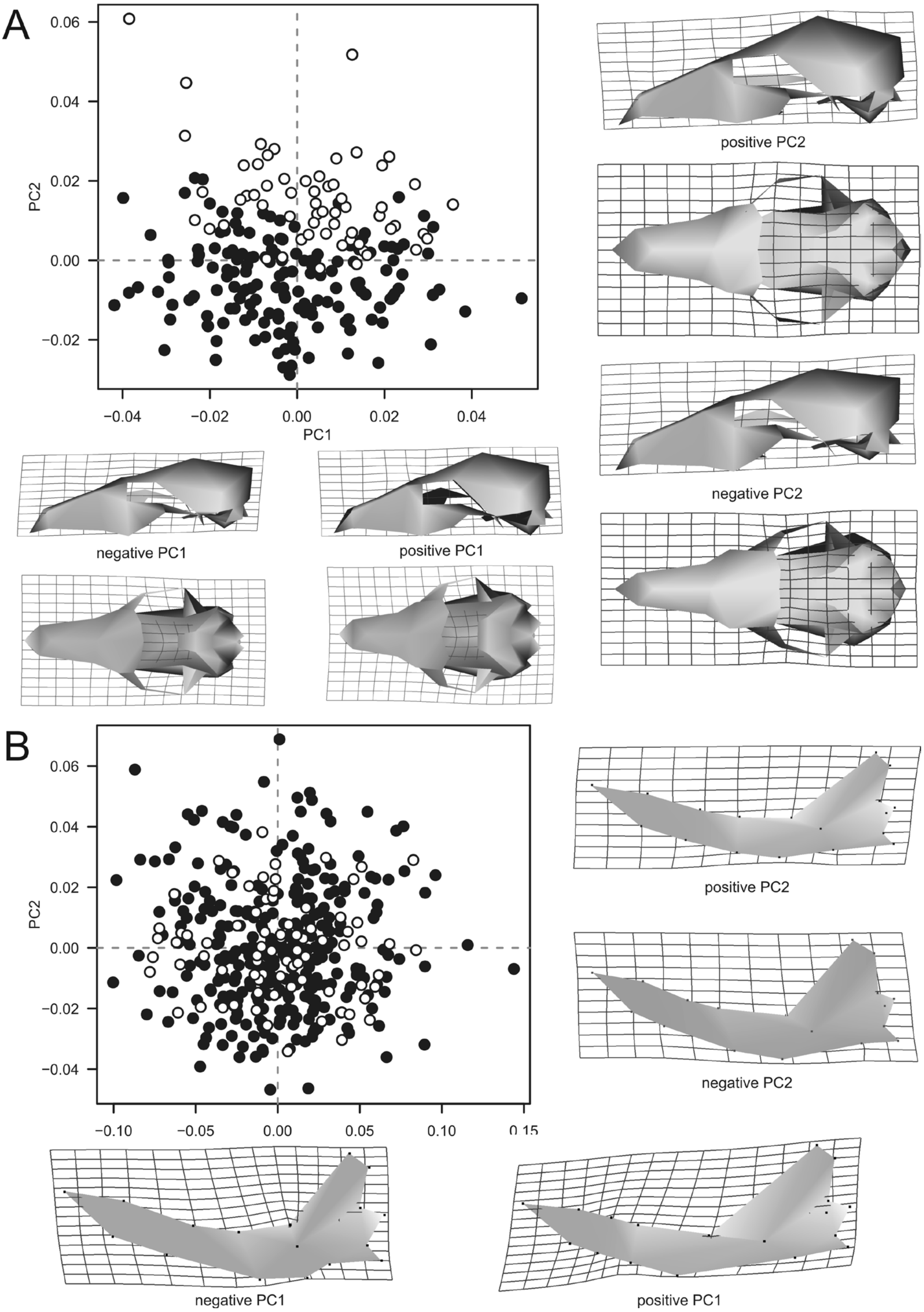

Fig. 4. Bi-variate graph of the first two components of a principal component analysis of the cranium (A) and the mandible (B). Black circles = *L. gymnocercus*; white circles = *L. griseus*.

extreme of axis 2, where only specimens of *L. gymnocercus* are placed, the opposite was observed. Other PCA axes did not show any separation between these taxa.

Discriminant analysis resulted in very good separation between these species, with a significant distance of Procrustes between their shape centroid (0.027, $p < 0.0001$), and a posterior correct reclassification of 95.75%. However the distance between shape centroids (0.027) was less than the mean Procrustes distances between specimens of *L. griseus* (0.057) and *L. gymnocercus* (0.053) to their respective shape consensus.

Size explained 5.62% of shape variance ($p < 0.0001$) and indicated that large specimens have slightly longer cranium with a much flatter braincase, smaller orbits and tympanic bullae, narrower postorbital constrictions and pterygoid fossae, larger postorbital processes of the frontal, stronger zygomatic arches, and shorter palates that are wider at the canines. The opposite tendency is present in small specimens. *L. gymnocercus* tends to have a larger size, and with higher centroid size values separate from those of *L. griseus*, but there is a wide overlapping between these taxa (**Fig. 5**).

The analysis based on mandibles showed the comparable results, but no separation between taxa was found in the PCA (**Fig. 4B**; see **Online Supplementary Material 3**).

Geographic variation patterns

The multiple regression analysis indicates that only longitude is significantly related to cranium size ($t = -7.513$, $p < 0.00001$). The simple regression between longitude and centroid size explained nearly the same percentage of variation ($R^2 = 0.35$) than the multivariate model, and was highly significant ($f = 78.780$,

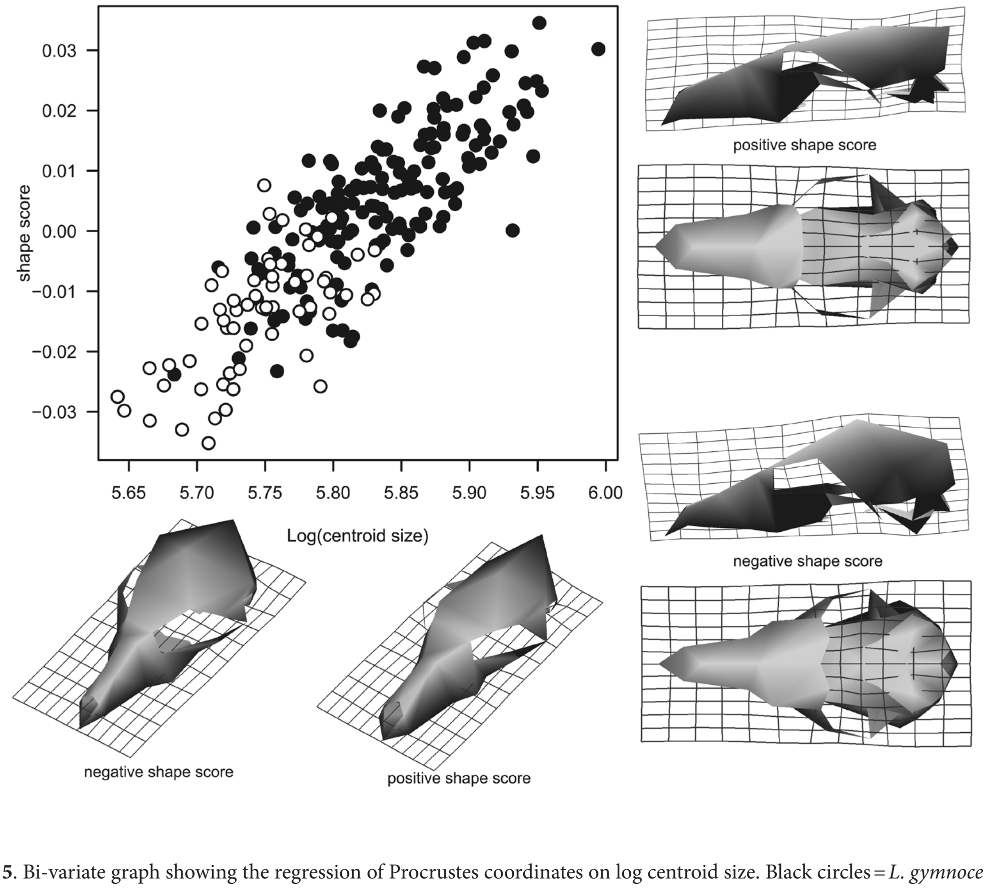

Fig. 5. Bi-variate graph showing the regression of Procrustes coordinates on log centroid size. Black circles = *L. gymnocercus*; white circles = *L. griseus*.

$p < 0.00001$). Results from the Durbin-Watson test were not significant ($d = 1.707$; $p = 0.052$), indicating no autocorrelation in the residuals of this regression.

RDA detected a significant relationship between cranium shape and latitude/longitude (6.9% of explained variation, $p < 0.001$). Axis 1 (Eigenvalue = 9.827e-05) nearly separates *L. griseus* and *L. gymnocercus,* and longitude has the largest loading on this axis (0.999), while the contribution of latitude is lower (0.254) (**Fig. 6**). To the positive extreme of this axis there are specimens with higher cranium, larger orbits and tympanic bullae, shorter and narrower rostrum, more caudally expanded palates, and shorter and wider nasals (**Fig. 6**). Model selection using AIC, kept three geographic filters (axis 1, 2 and 6; AIC = -654.21) of a total of 27 axes, and forward and backward searches converged on the same model. Partial variation explained by latitude and longitude dropped to 3.36% when spatial filters were included, but were still significant ($p < 0.001$). Results from this new model were nearly identical to the previous one, and the scores of axis 1-2 of each RDA were highly correlated ($R^2 = 0.97$ and 0.99, respectively).

DISCUSSION

Our analytic results corroborate the interpretation of Zunino et al. (1995) since we found no relevant morphological differences between *L. gymnocercus* and *L. griseus*, suggesting also that both forms belong to the same species. The main differences between these species was found in size, with a broad overlap between them (**Figs. 4-6**), indicating a continuous variation in body size. Although a continuous variation in cranial shape between these species was found, mandible shape overlapped extensively (**Fig. 4**). Main differences in cranial shape can be explained by the allometric relationship observed between size and shape (**Fig. 5**). *Lycalopex griseus* tends to have wider and taller cranium (especially at the braincase), with larger orbits and tympanic bullae, concave posterior borders of the palate, smaller temporal fossae, narrower postorbital constrictions, wider pterygoid fossae, and smaller postorbital processes of the frontal (**Fig. 4**). These relationships have been detected in several ontogenetic and evolutionary allometric studies performed on carnivores (e.g., Wayne, 1986; Segura and Flores, 2009; Giannini et al., 2010; Prevosti et al., 2010; Segura and Prevosti, 2012; Segura, 2013). The development of crests (e.g., sagittal, occipital) are also positively correlated with size (e.g., Guzmán et al., 2009; Segura and Prevosti, 2012). Thus, the reported shape differences between these nominal species are likely correlates of clinal variation in size.

The significant separation between these foxes for cranial measurements using DA (see above), deserves some clarification. First, the size effect is small relative to variation within taxa, as shown by our analyses of variation in centroid size within and between nominal species. Furthermore, these taxa overlap widely in these traits. More generally, DA is prone to over-represent divergence (e.g., Kovarovic et al., 2011). This could be exacerbated with geometric morphometric studies, because they have a large number of dependent variables (Procrustes coordinates) and it is not possible to meet the dimensional relationship between variables and specimen number (e.g., Kovarovic et al., 2011; Mitteroecker and Bookstein, 2011). Finally, these foxes present a clear clinal variation (Zunino et al., 1995; see above) that affects cranial and mandible size and shape. Consequently, significant differences between geographically distant samples are to be expected. Based on the correlation with latitude and longitude, this variation can be related to climate, especially lower precipitation values towards the southwest (García, 1990; Zunino et al., 1995; see also Schiaffini et al., 2013).

As was illustrated in this paper and in Zunino et al. (1995), there is no evidence in cranium or mandibles to support the separation of *L. gymnocercus* and *L. griseus* as distinct species. Other characters (e.g., skin coloration by Zunino et al. 1995; chromosomes by Gallardo and Formas, 1975; Brum et al., 1980; Vitullo and Zuleta, 1992) showed no differences between these species, which is consistent with the findings reported herein.

Mitochondrial and nuclear genes have mostly been used to resolve phylogenetic relationship

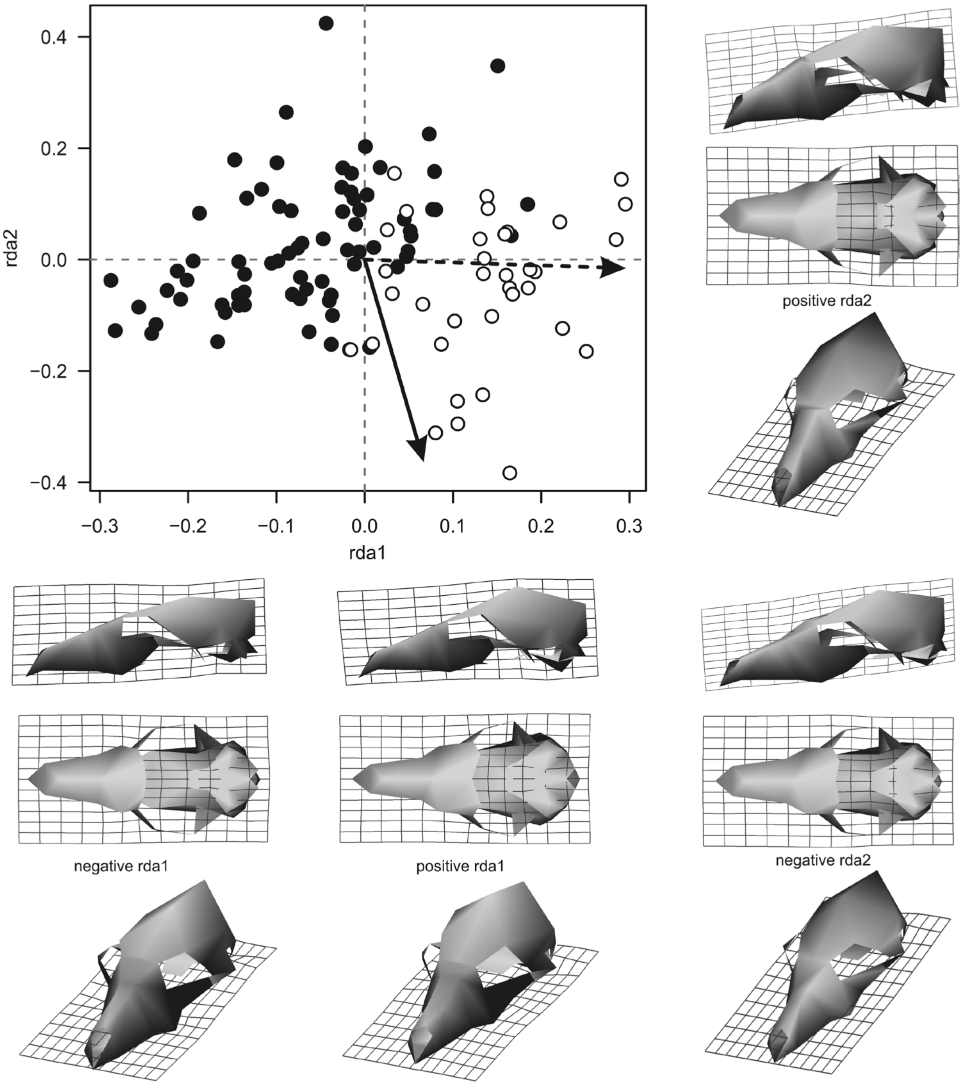

Fig. 6. Graph of the two first axis of a redundancy analysis based on cranial Procrustes coordinates. Black circles = *L. gymnocercus*; white circles = *L. griseus*. Black arrow = latitude; broken black arrow = longitude.

of genera and species of Canidae (e.g., Bardeleben et al., 2005; Lindblad-Toh et al., 2005; Prevosti, 2010), but few studies have been done at lower taxonomic scales with Neotropical canids (see below). Most of these studies have not recovered *L. griseus* and *L. gymnocercus* as a monophyletic group (but see Figs. S24-S25 of Lindblad-Toh et al., 2005, and Fig. 1 of Bardeleben et al., 2005), something that could be interpreted as an opposite evidence to the one presented herein. However, these studies include very few specimens with the potential of obscuring intraspecific variation (see Eizirik, 2012). Moreover, the correct identification of these DNA sequences is impossible to check, since authors have not included a list of analyzed specimens (there is no or limited information about voucher specimens), while some of the sequences assigned to *Lycalopex* spp. that are deposited in GenBank are not correctly identified (M. A. Chemisquy, comm. pers.). This situation illustrates the importance of associating genetic data to voucher specimens deposited in collections.

Unfortunately, no published phylogeographical study on these species is available, and only in two cases molecular data were used to resolve species limits. Yahnke (1995) and Yahnke et al. (1996) used chromatogenic data and mitochondrial DNA, respectively, to test the systematic position of *L. fulvipes*. The first study recovered a clade that included *L. gymnocercus*, *L. griseus* and *L. culpaeus*, but these nominal forms were not reciprocally monophyletic; the latter did not include specimens of *L. gymnocercus*. A recent paper (Ruiz García et al., 2013) included a phylogenetic analysis of mitochondrial data (cytochrome b) with 71 specimens of *L. culpaeus* (from eight localities of Bolivia, Peru and central Chile), 8 specimens of *L. sechurae* from Peru, 4 *L. griseus* from Córdoba Department (Córdoba, Argentina) and 1 specimen of *L. gymnocercus* from Cochabamba (Bolivia). In the most parsimonious tree presented by these authors (Ruiz García et al., 2013; Fig. 2B) *L. griseus* is paraphyletic and the sister taxon of a clade formed by *L. culpaeus* plus the sequence of *L. gymnocercus* from Cochabamba. If the identification of these sequences is correct (something that we cannot state because there is no indication of how they identified the samples), this would indicate that cytochrome b is not a good genetic marker to resolve species limits in this case, especially since there is very strong anatomical and ecological evidence supporting a specific status of *L. culpaeus* relative to the other 2 taxa (e.g., Kraglievich, 1930; Cabrera and Yepes, 1940; Langguth, 1970; Zunino et al., 1995; Novaro, 1997; Sillero Zubiri et al., 2004; Wilson and Mittermeier, 2009). Work in progress including specimens of *L. culpaeus*, *L. gymnocercus* and *L. griseus* with a larger sample and covering most of their geographic distribution using nuclear and mitochondrial genes, also failed to recovered the monophyly of these three taxa (i.e., specimens of *L. gymnocercus* and *L. griseus* are completely mixed, but form a clade that also includes some specimens of *L. culpaeus*; V. Raimondi and A. Chemisquy, comm. pers.).

In sum, available analytical evidence indicates that *L. gymnocercus* and *L. griseus* represent a single species, to which the name *L. gymnocercus* should be applied according to nomenclatural rules, as proposed by Zunino et al. (1995).

ACKNOWLEDGMENTS

For the permission and attention in the study of the material under their care we thank: Robert Voss, Eileen Westwig (AMNH); Sergio Bogan, Yolanda Davies (CFA); Rubén Barquez, Mónica Díaz (CML); Bruce Patterson, Bill Stanley (FMNH); Mauro Lucherini, Estela Luengos (GECM); Mauro Schiaffini, Gabriel Martin (LIEB); David Flores, Sergio Lucero (MACN); Diego Verzi, Itatí Olivares (MLP); Kristofer Helgen, Darrin Lunde (NMNH). Sergio Vizcaíno kindly allowed us to use the Microscribe. Amelia Chemisquy, Mauro Schiaffini, Vanina Raimondi and Pablo Martínez provided valuable comments and corrections. E. Lessa, U. Pardiñas, Noé de la Sancha, and an anonymous reviewer provided additional criticism. This research was partially supported by a Short Term Visitor Fellowship Award from NMNH, and Visiting Scholarship Award from the FMNH to Valentina Segura. This is a contribution to the projects PIP 1054 and PIP 164 of CONICET; CDD 430/09 of UNLu and PICT 2008-1798 and PICT 2011-0309 of ANPCyT.

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ONLINE SUPPLEMENTARY MATERIAL

Supplementary Material 1

Database used in this study, including collection number, a priori taxonomic identification, sex, locality (and geographical coordinates), log centroid size and Procrustes coordinates (ProcCoord).

Supplementary Material 2

Definition of the landmarks and semilandmarks used in this study.

Supplementary Material 3

Complementary analysis based on mandibles.