Zootaxa 0000: 0–0000 (2012)
www.mapress.com/zootaxa/

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

Article

Taxonomy of the southernmost populations of *Philander* (Didelphimorphia, Didelphidae), with implications for the systematics of the genus

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Abstract

The taxonomic identities of populations of *Philander* Brisson of Argentina are still unclear. *Philander frenatus* (Olfers) is the only species assigned to the country, a conclusion based on incomplete analysis of available material and without a clear taxonomic criterion. The aim of this study was to determine the taxonomic identity of the populations of *Philander* of Argentina. To accomplish this, DNA from eight specimens from Argentina and one specimen from Paraguay was sequenced and compared with sequences published by other authors, using a phylogenetic approach. To complement the molecular information, seven skull measurements were taken from specimens of *P. frenatus* and *P. opossum canus* (Osgood) from Bolivia and Brazil, and compared with the specimens from Argentina and Paraguay using bi- and multivariate analyses. Molecular and morphological results showed that there are two species of *Philander* in Argentina, *P. frenatus* in Misiones province and *P. opossum canus* in Chaco and Formosa provinces. Both species can be morphologically distinguished only by the width of the postorbital constriction. Finally, the phylogenetic analyses and the pairwise genetic distances between the included sequences showed that the taxonomic status of *Philander mcilhennyi*, *P. opossum* and its subspecies should be revisited.

Keywords: *Philander frenatus*, *Philander opossum canus*, cytochrome *b*, morphometric analyses, genetic distances.

Introduction

The genus *Philander* Brisson comprises a group of medium-sized didelphid marsupials commonly known as four-eyed pouched opossums. *Philander* species inhabit tropical and subtropical forests from Tamaulipas and Oaxaca in Mexico to Misiones, Formosa and Chaco provinces in Argentina (Hershkovitz, 1997; Patton and da Silva, 2007). The genus was traditionally considered as monotypic with the sole species, *P. opossum* (Linnaeus), and seven subspecies (*P. opossum andersoni* (Osgood), *P. o. azaricus* (Krumbiegel), *P. o. canus* (Osgood), *P. o. grisescens* (Krumbiegel), *P. o. melanurus* (Thomas), *P. o. opossum* (Linnaeus), and *P. o. quica* (Temminck); Cabrera, 1958), until *P. mcilhennyi* Gardner and Patton was described based on morphological characters from the skull, teeth, and pelage (Gardner and Patton, 1972). The subsequent taxonomic arrangement proposed by Emmons and Feer (1990), Gardner (1993) and Hershkovitz (1997) elevated *P. opossum andersoni* to species status and placed *P. mcilhennyi* as its junior synonym.

Subsequent phylogenetic and phylogeographic studies derived in depth modifications of the systematics and taxonomy of the group (e.g. Patton and da Silva 1997; Patton *et al.*, 2000; Patton and Costa, 2003). These studies conferred specific status to *P. frenatus* (Olfers) (synonym of *P. o. quica*), and considered *P. andersoni* and *P. mcilhennyi* as valid species. However, these authors suggested that some of the subspecies of *P. opossum* might eventually be elevated to species status. New *Philander* species have been recently described based on the morphology of specimens believed to be *P. opossum* (*P. deltae* Lew, Pérez-Hernández and Ventura, and *P. mondolfii* Lew, Pérez-Hernández and Ventura, Lew *et al.* 2006; *P. olrogi* Flores, Díaz and Bárquez, Flores *et al.* 2008), and the current definition of the genus recognizes seven species, with four subspecies for *P. opossum* (Patton and da Silva, 2007).

The taxonomic status of the genus in the southern extreme of its continental distribution (i.e. Atlantic, Paranaense and xerophytic Chacoan forests of Argentina, Brazil and Paraguay) has also been highly discussed.

Accepted by M. Weksler: 2 Aug. 2012; published: ?? Month 2012

Cabrera (1958) recognized the subspecies *P. opossum azaricus* for northeastern Argentina and Paraguay. Hershkovitz (1997) included the southern populations under the subspecies *P. o. quica*, denoting a high degree of morphological uniformity in the populations ranging from southeastern Brazil, northern Argentina, Paraguay, lower parts of Bolivia, eastern Peru, and Ecuador. Patton and da Silva (1997) recognized six subspecies for *P. opossum*, but specimens from its southern extreme (i.e., Chacoan and Atlantic forests of Paraguay and Argentina) were not included in their analysis. In this sense, the identity of the Argentinean populations of *Philander* remains unclear. Most field guides and popular science books use an out-dated definition of the genus and its species and place the four-eyed opossums of Argentina and Paraguay under *P. opossum* (e.g. Olrog and Lucero 1981; Parera 2002; Vaccaro and Canevari 2007). However, Patton and da Silva (2007) compiled the information published by previous authors and mapped the distribution of *P. frenatus* to include the populations of Argentina and Paraguay under this species. According to Flores *et al.* (2007), specimens from northeastern Argentina (Formosa and Misiones provinces) do not differ morphologically from those from southeastern Brazil and Paraguay, for which they recognized *P. frenatus* for northeastern Argentina. This had been previously suggested by Castro-Arellano *et al.* (2000), based on the biogeographic proximity between the Atlantic forest of southeastern Brazil and the Paranaense forests of northeastern Argentina and eastern Paraguay. However, these populations have never been characterized in a molecular or morphometric context, which is important due to the scarce morphological differences reported between *Philander frenatus* and *P. opossum* (Flores *et al.*, 2007), and the variety of environments where *Philander* occurs in Argentina and Paraguay (Castro-Arellano *et al.*, 2000). The aim of this study was to determine the taxonomic identity of the populations of *Philander* present in Argentina (representing the southern extreme of the distribution of this genus in the Neotropics), using molecular information obtained from museum specimens (from the mitochondrial marker cytochrome *b*), and cranial morphometric data.

Materials and methods

Molecular analyses

DNA was extracted on footpads from nine museum specimens of *Philander* (see Table 1 for information on the specimens included). Previous to the extraction, tissues were washed and rehydrated using 1X TNE buffer. Rehydrated tissues were then digested with proteinase K, followed by the extraction with chloroform–isoamyl alcohol and precipitation with ethanol (Sambrook *et al.* 1989).

We sequenced the first 800 base pairs of the mitochondrial gene that codes for cytochrome *b* using the following primers, designed specifically for this study: FP ATGACCAAYMTTCGCAAAACA, RP TGAGGTGGNGKATTKAGGGG, R330 ACTCCAATGTTTCATGTTTCT, F244A TCCACGCTAAKGGAGCATC, R487 GARAAYCCSCCTCARATTCA, F457 ATYCCCTACATYGGMAAYAC, R645 GGATGAAATGGAATTTTRTCT, and F596 TCCTYCAYAAACAGGATCA. 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 94ºC for 5 min, followed by 40 cycles of denaturation at 94 ºC for 45 s, annealing at 48ºC for 1 min, and extension at 72ºC 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. All the sequences (both the new sequences and those downloaded from GenBank) were submitted to BLAST to detect contamination, while functionality (in order to detect pseudogenes) was checked translating the sequences to proteins.

We included 35 sequences of *Philander* downloaded from GenBank, eight unpublished sequences kindly donated by J. Patton, and three sequences of *Didelphis* Linnaeus (*D. albiventris* Lund, *D. aurita* Wied-Neuwied, and *D. virginiana* Allen) to be used as outgroups (Table 1). Sequences were edited and hand-aligned using the BioEdit software (Hall, 1999). Maximum Parsimony (MP) analyses were performed using the software TNT (Goloboff *et al.*, 2008), using 1000 series of random addition 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 10000 pseudoreplicates of jackknife (Farris *et al.*, 1996).

TABLE 1. Specimens and cytochrome B (CytB) sequences used in our study. JP unpublished makes reference to the sequences donated by Dr. Jim Patton.

Species	Locality	Collecion no.	GenBank accession number	Reference
P. andersoni 1	Napo, Ecuador	ROM104030	JQ388602	Nunes *et al*., 2006
P. andersoni 2	Amazonas, Perú	MZV153265	JQ388603	Nunes *et al*., 2006
P. andersoni 3	Amazonas, Brazil	INPA YL139	JQ388604	Nunes *et al*., 2006
P. andersoni 4	Loreto, Perú	KU 144120	JQ388605	Nunes *et al*., 2006
P. frenatus 1	Rio de Janeiro, Brazil	-	U34679	Patton *et al*., 1996
P. frenatus 2	Espírito Santo, Brazil	-	GU112936	Agrizzi *et al*, 2012
P. frenatus 3	Rio de Janeiro, Brazil	-	GU112937	Agrizzi *et al*, 2012
P. frenatus 4	Bahia, Brazil	-	GU112938	Agrizzi *et al*, 2012
P. frenatus 5	Paraná, Brazil	-	GU112939	Agrizzi *et al*, 2012
P. frenatus 6	Espírito Santo, Brazil	-	GU112940	Agrizzi *et al*, 2012
P. frenatus 7	Espírito Santo, Brazil	-	GU112941	Agrizzi *et al*, 2012
*P. frenatus*8	Espírito Santo, Brazil	-	GU112942	Agrizzi *et al*, 2012
P. frenatus 9	Minas Gerais, Brazil	CEG 35	JQ778966	JP, unpublished
P. frenatus 10	Rio de Janeiro, Brazil	LG 39	JQ778970	JP, unpublished
P. frenatus 11	San Pablo, Brazil	MVZ182066	JQ778967	JP, unpublished
P. frenatus 12	San Pablo, Brazil	MZUSP29213	JQ778968	JP, unpublished
P. frenatus 13	Espírito Santo, Brazil	MZUSP29210	JQ388606	Nunes *et al*., 2006
P. frenatus 14	Minas Gerais, Brazil	MZUSP29212	JQ778971	JP, unpublished
P. frenatus 15	Paraná, Brazil	NC 14	JQ778969	JP, unpublished
P. oposum 1	French Guiana	-	AJ628367	Steiner *et al*., 2005
P. oposum 2	French Guiana	-	AJ487009	Steiner and Catzeflis, 2003
P. oposum 3	Guiana	ROM98045	JQ388607	Nunes *et al*., 2006
P. oposum 4	Amazonas, Brazil	INPA JLP 16785	JQ388608	Nunes *et al*., 2006
P. oposum 5	Pará, Brazil	USNM549297	JQ388609	Nunes *et al*., 2006
P. o. fuscogriseus	Panamá	UNSM464248		Nunes *et al*., 2006
P. o. canus 1	Acre, Brazil	MNFS 1031	U34678	Patton *et al*., 1996
P. o. canus 2	Amazonas, Brazil	-	DQ236277	Nunes *et al*., 2006
P. o. canus 3	Amazonas, Brazil	-	DQ236276	Nunes *et al*., 2006
P. o. canus 4	Amazonas, Brazil	-	DQ236275	Nunes *et al*., 2006
P. o. canus 5	Amazonas, Brazil	-	DQ236274	Nunes *et al*., 2006
P. o. canus 6	Amazonas, Brazil	-	DQ236273	Nunes *et al*., 2006
P. o. canus 7	Amazonas, Brazil	-	DQ236272	Nunes *et al*., 2006
P. o. canus 8	Amazonas, Brazil	-	DQ236271	Nunes *et al*., 2006
P. o. canus 9	Amazonas, Brazil	MVZ190343	JQ388610	Nunes *et al*., 2006
P. o. canus 10	Mato Grosso do Sul, Brazil	JLP16968	JQ778972	JP, unpublished
P. o. canus 11	Mato Grosso do Sul, Brazil	LPC597	JQ778973	JP, unpublished
P. mcilhennyi 1	Loreto, Perú	-	AJ628366	Steiner *et al*., 2005
P. mcilhennyi 2	Amazonas, Brazil	-	U34680	Patton *et al*., 1996
P. mcilhennyi 3	Amazonas, Brazil	MVZ190340	JQ388611	Nunes *et al*., 2006
P. mcilhennyi 4	Amazonas, Brazil	MVZ190341	JQ388612	Nunes *et al*., 2006
P. mcilhennyi 5	Amazonas, Brazil	INPA3403	JQ388613	Nunes *et al*., 2006
P. mcilhennyi 6	Acre, Brazil	INPA3397	JQ388614	Nunes *et al*., 2006
Philander sp. 1	Formosa, Argentina	MACN 20742	JQ778957	This work
Philander sp. 2	Chaco, Argentina	MACN 20868	JQ778956	This work
Philander sp. 3	Formosa, Argentina	MACN 20740	JQ778958	This work
Philander sp. 4	Misiones, Argentina	MACN 49.376	JQ778959	This work
Philander sp. 5	Sapucay, Paraguay	MACN 33.172	JQ778960	This work
Philander sp. 6	Misiones, Argentina	MACN 52.19	JQ778961	This work
Philander sp. 7	Chaco, Argentina	MACN 20866	JQ778962	This work
Philander sp. 8	Misiones, Argentina	MACN 51.18	JQ778963	This work
Philander sp. 9	Misiones, Argentina	MACN 51.127	JQ778964	This work
Didelphis aurita	Espírito Santo, Brazil	-	GU112886	Agrizzi *et al*, 2012
Didelphis virginiana	Unknown	-	HM222715	Naidu *et al*., 2012
Didelphis albiventris	French Guiana	-	AJ487004	Steiner and Catzeflis, 2003

For the Bayesian inference (BI) and maximum likelihood (ML) analyses, we first identified the best model of nucleotide evolution using jModeltest (Posada 2008) available online on the server Phylemon (http// phylemon.bioinfo.cipf.es). The general time reversible model including invariant sites (GTR+I) was selected under the Akaike information criterion as the best model. Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Two Markov chains starting with a random tree were run simultaneously for 30 million generations, sampling trees every 1000 generations. The stationary phase was reached when the average standard deviation of split frequencies read 0.001. Other parameters of the run were used following the default options of the software. Trees sampled before the posterior probability of splits stabilized were excluded from consensus as the burn-in phase (corresponding to the first six million generations). Maximum likelihood analyses were conducted using RAxML GUI (Silvestro and Michalak, 2011), a graphical front-end for RAxML-VI-HPC (Randomized Accelerated Maximum Likelihood; Stamatakis, 2006). Maximum likelihood with the thorough bootstrap option was run from a starting random seed to generate 1000 nonparametric bootstrap replicates.

Inter- and intraspecific genetic distances were estimated with the Tamura 3-parameter model (Tamura, 1992) implemented in the software MEGA5 (Tamura *et al.*, 2011). 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 and Meier, 2012). Consequently, the model used for estimating distances was the model that best fit our dataset, as chosen by the software MEGA5.

Morphometric analyses

A total of 45 specimens of *Philander* from Argentina, Bolivia, Brazil and Paraguay were studied (Appendix 1). Seven cranial measures were recorded, modified from Flores *et al.* (2008): occipito-incisive length (OIL), from the anterior tip of the incisive foramina to the posterior-most projection of the occipital condyle; postorbital constriction (PC), the least distance across the cranium measured before the postorbital processes; zygomatic breadth (ZB), the greatest distance across the outer margins of the zygomatic arches; length of nasal (LN), the distance from the posterior border to the anterior border of the nasal; maximum breadth of nasals (BN1), width of the nasals measured from their widest part, at the level of the fronto-maxillar suture; palate length (PL), the distance from the posterior margin of the alveolus of the first incisor to the medial posterior border of the torus; and width across molars (M–M), the distance between the outer margin of the upper last molars (see appendix 2 for a summary of the measurements; the complete data set is available upon request from the authors).

Statistical analyses were performed using the software PAST (Hammer *et al.*, 2001). Measurements were log 10 transformed and used to perform a Principal Component Analysis (PCA) using a variance-covariance matrix, to evaluate the morphological differences between *P. frenatus* and *P. opossum canus*. Statistical differences between both species were assessed using a Discriminant Function Analysis (DFA) and multivariate analysis of variance (MANOVA). In the discriminant analysis, all the groups had the same probability, so a specimen could be assigned to any group independently of the size of the group. Cross validation was performed using the option “leave out”. The percentage of correct posterior classification was used as an indicator of the performance of the function. In the MANOVA, Wilk’s lambda was used to check the significance of pairwise differences (see Cudeck, 2000; Brown and Wicker, 2000; Huberty and Petoskey, 2000; Legendre and Legendre, 1998 for more information on the statistical methods used).

Results

Phylogenetic analyses

The final data set had 151 parsimony informative characters and 218 variable characters. Maximum parsimony analysis recovered 38 trees of 319 steps (CI = 0.687; RI = 0.906). The strict consensus MP tree (Fig. 1a), the ML tree (tree not shown), and the Bayesian tree (Fig. 1b) were highly congruent. All the analyses showed two main

clades inside *Philander*, one grouping the sequences of *P. frenatus* (MP Jackknife = 100/ ML Bootstrap = 100/ Bayesian posterior probability = 1.0) and the other including *P. andersoni*, *P. mcilhennyi*, *P. opossum opossum*, *P. o. fuscogriseus* (J. A. Allen), and *P. o.canus* (99/ 99/ 1.0). The specimens from Misiones (Argentina) and Sapucay (Paraguay) were nested in the *P. frenatus* clade (Fig. 1) while the sequences from Formosa and Chaco provinces (Argentina) were placed in the other clade, together with *P. opossum canus* (98/ 95/ 1.0; Fig. 1).

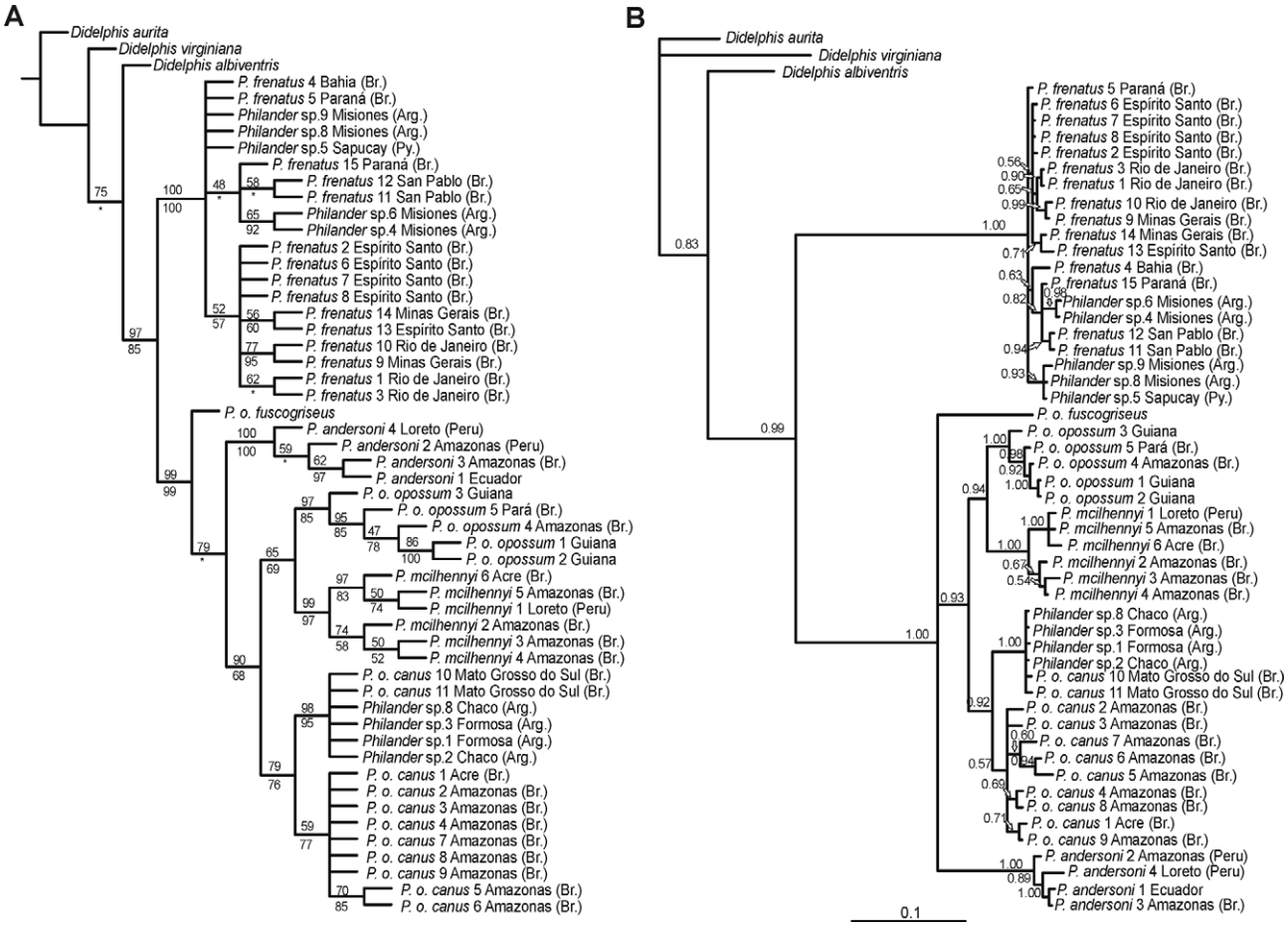

FIGURE 1. A, strict consensus tree of the 151 equally most parsimonious trees obtained. Numbers above the branches indicate MP jackknife support, numbers below the branches indicate ML bootstrap; * indicates a node absent in the ML tree. B, majority-rule consensus resulting from the Bayesian analysis depicting branch lengths. Numbers indicate posterior probability values of the nodes. Species labels are based on morphological or molecular identification, depending on the source of the sequence (see table 1 for references). Br., Brazil. Arg., Argentina. Py., Paraguay.

The clade of *Philander frenatus* showed little resolution, with several polytomies. Two of the specimens from Misiones (MACN 51.127 and MACN 51.18) and the specimen from Paraguay (MACN 33.172) were placed in a polytomy in the MP and ML analyses (Fig. 1a), while they formed a clade in the BI analysis (0.93; Fig. 1b). The remaining specimens from Misiones (MACN 52.19 and MACN 49.376) were grouped together with other specimens from São Paulo and Paraná (Brazil) in the MP and BI analyses (48/0.82).

The second clade showed more structure. In the MP analyses *Philander opossum fuscogriseus* was the sister taxon of the clade formed by *P. andersoni* and *P. opossum* + *P. mcilhennyi* (Fig. 1a), while in the ML and the BI analyses those three clades were grouped in a polytomy (Fig. 1b). The four specimens of *P. andersoni* were grouped in a well-supported clade (100/ 100/ 1.0; Fig. 1). *Philander opossum* as a whole was not monophyletic since the clade of *P. mcilhennyi* was nested inside that species, with the specimens of *P. o. opossum* (65/ 69/ 0.94; Fig. 1). The two subspecies of *P. opossum* (*opossum* and *canus*) turned out to be monophyletic in all the analyses (97/85/1.0 and 79/ 76/ 0.92 respectively; Fig. 1).The specimens from Chaco and Formosa were grouped with specimens from Mato Grosso do Sul (Brazil) (98/ 95/ 1.0; Fig. 1), while the remaining samples of *P. opossum canus*, all of them from Amazonas (Brazil) were placed in a separate sister clade (59/ 77/ 0.57; Fig. 1).

The phylogram obtained in the Bayesian analysis (Fig. 1b) showed a high level of molecular divergence between *Philander frenatus* and the remaining clades. *Philander opossum fuscogriseus* and *P. andersoni* also had high levels of sequence divergence evidenced by the long branches. On the other hand, the two subspecies of *P. opossum* and *P. mcilhennyi* showed short branches, implying low levels of divergence among those taxa (Fig. 1b).The analysis of the genetic distances confirmed these results, since *P. frenatus* had the highest values of divergence when compared to the other species (0.109-0.169; Table 2), while the level of sequence divergence between the three subspecies of *P. opossum* and *P. mcilhennyi* was ten times lower (0.028-0.067; Table 2).

TABLE 2. Range of Tamura 3-parameter distances between taxa for Cytochrome *b* variation of *Philander*. MG, specimens from Mato Grosso (Brazil).

		1	2	3	4	5	6	7	8	9	10	11
1	*P. andersoni*	0.002-0.014										
2	*P. o. canus* MG	0.074-0.076	0.003									
3	*P. o. canus* Amazonas	0.046-0.074	0.020-0.030	0.002-0.014								
4	*P. frenatus*	0.145-0.169	0.136-0.163	0.109-0.151	0-0.020							
5	*P. o. fuscogriseus*	0.080-0.084	0.065	0.058-0.08	0.131-0.151	0						
6	*P. mcilhennyi*	0.072-0.088	0.045-0.056	0.028-0.057	0.126-0.164	0.076-0.080	0.004-0.025					
7	*P. o. opossum*	0.066-0.073	0.041-0.045	0.028-0.054	0.129-0.178	0.059-0.067	0.036-0.054	0.003-0.016				
8	*P.* sp. Misiones	0.156-0.167	0.155-0.154	0.108-0.153	0.005-0.022	0.138-0.146	0.141-0.161	0.146-0.164	0-0.01			
9	*P.* sp. Paraguay	0.151-0.156	0.143-0.149	0.121-0.138	0.008-0.016	0.136	0.142-0.153	0.145-0.160	0.003-0.011	0		
10	*P.* sp. Chaco	0.076-0.081	0.003-0.004	0.020-0.033	0.146-0.164	0.070-0.068	0.052-0.065	0.040-0.048	0.154-0.160	0.155-0.156	0	
11	*P.* sp. Fromosa	0.075-0.081	0-0.002	0.020-0.039	0.146-0.167	0.058-0.066	0.045-0.063	0.039-0.052	0.157-0.161	0.159-0.160	0	0

Morphometric analyses

Bivariate plots of the components I and II resulting from the PCA showed that the specimens assigned to *P. frenatus* and *P. opossum canus* formed two groups, well separated along the first component (88.3% of explained variance; Fig. 2a). The separation between both groups was more noticeable in the plot of components I and III, where the specimens of *P. opossum canus* from Argentina were clearly grouped with the remaining specimens of that subspecies (Fig. 2b).The DFA showed a perfect separation between both species, with all the specimens correctly classified (the same result was obtained using cross validation; data not shown), and the MANOVA was highly significant (Wilk's Lambda = 0.1428; $p < 0.00000001$).

The biplot of the length of the skull (OIL) versus the interorbital width (PC), the variable that better separates both species, showed that *P. frenatus* has a wider interorbital width than *P. opossum canus* (Fig. 3). None of the other variables separated both taxa when plotted against the length of the skull.

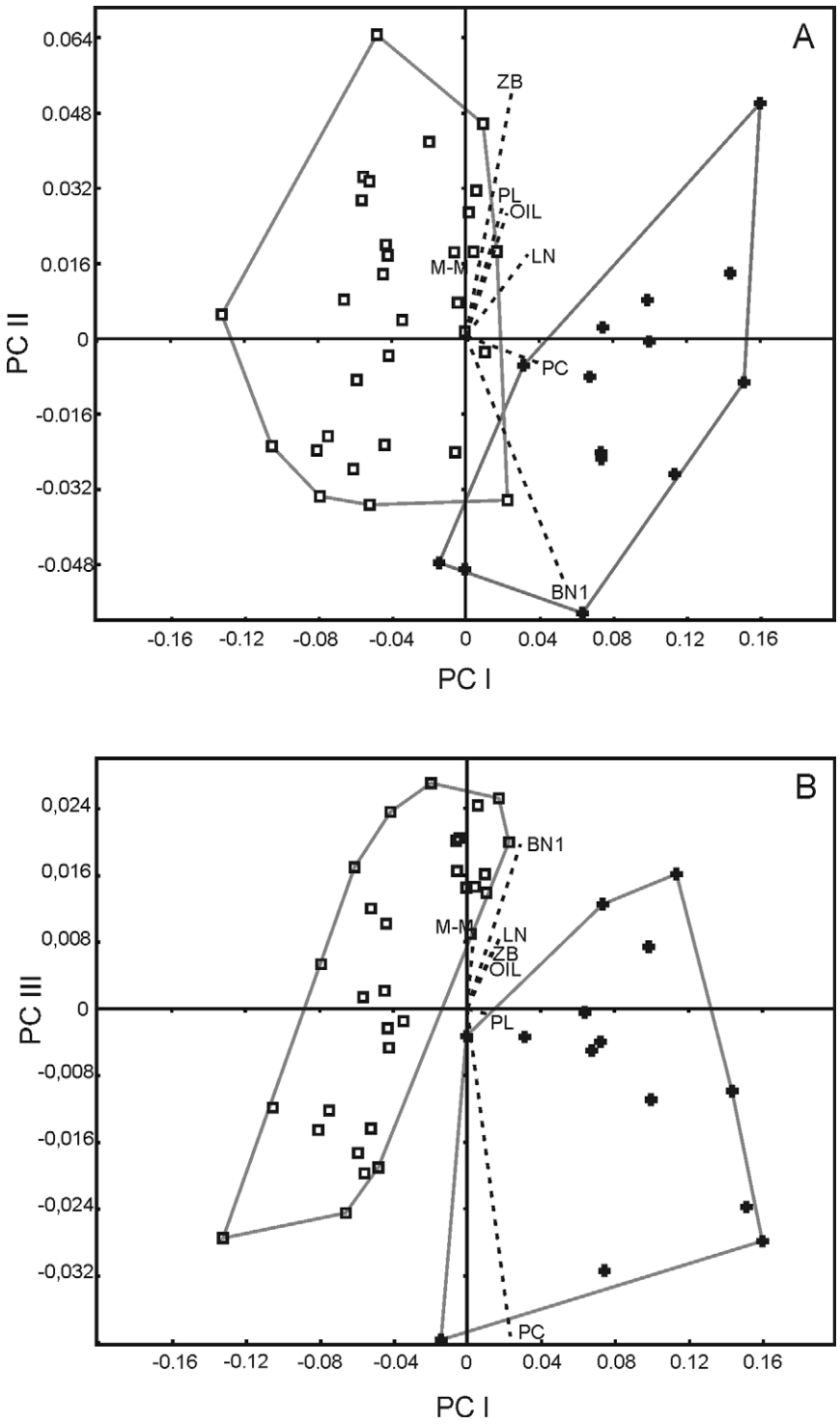

FIGURE 2. Biplots showing the specimen scores of adult individuals of *Philander* for principal components (PC) I and II(A) and I and III (B) extracted from the variance-covariance matrix of 7 craniodental distances and the contribution of each measure to the . White squares, *Philander opossum canus*. Black crosses, *Philander frenatus*.

Discussion

Our results clearly confirm the presence of two species of *Philander* inhabiting Argentina: *P. frenatus* and *P. opossum canus*. The latest bibliography on the group reported only one species for this country, *P. frenatus* (Patton and da Silva 2007; Flores 2006, Flores *et al.*, 2007), based on the inclusion of *P. opossum azaricus* as its synonym

(Patton *et al.* 2000; Patton and da Silva 2007; *P. o. azaricus* was the only species of *Philander* listed in Paraguay and Argentina by Cabrera, 1958), the new combination implied the existence of only *P. frenatus* in both countries. Since *P. frenatus* and *P. opossum* are difficult to separate from each other using morphological characters, which is evident in the key to *Philander* species presented by Patton and da Silva (2007), as well as in our results from the morphometric analysis, the presence of a second species for Argentina could not be tested using only a morphological approach.

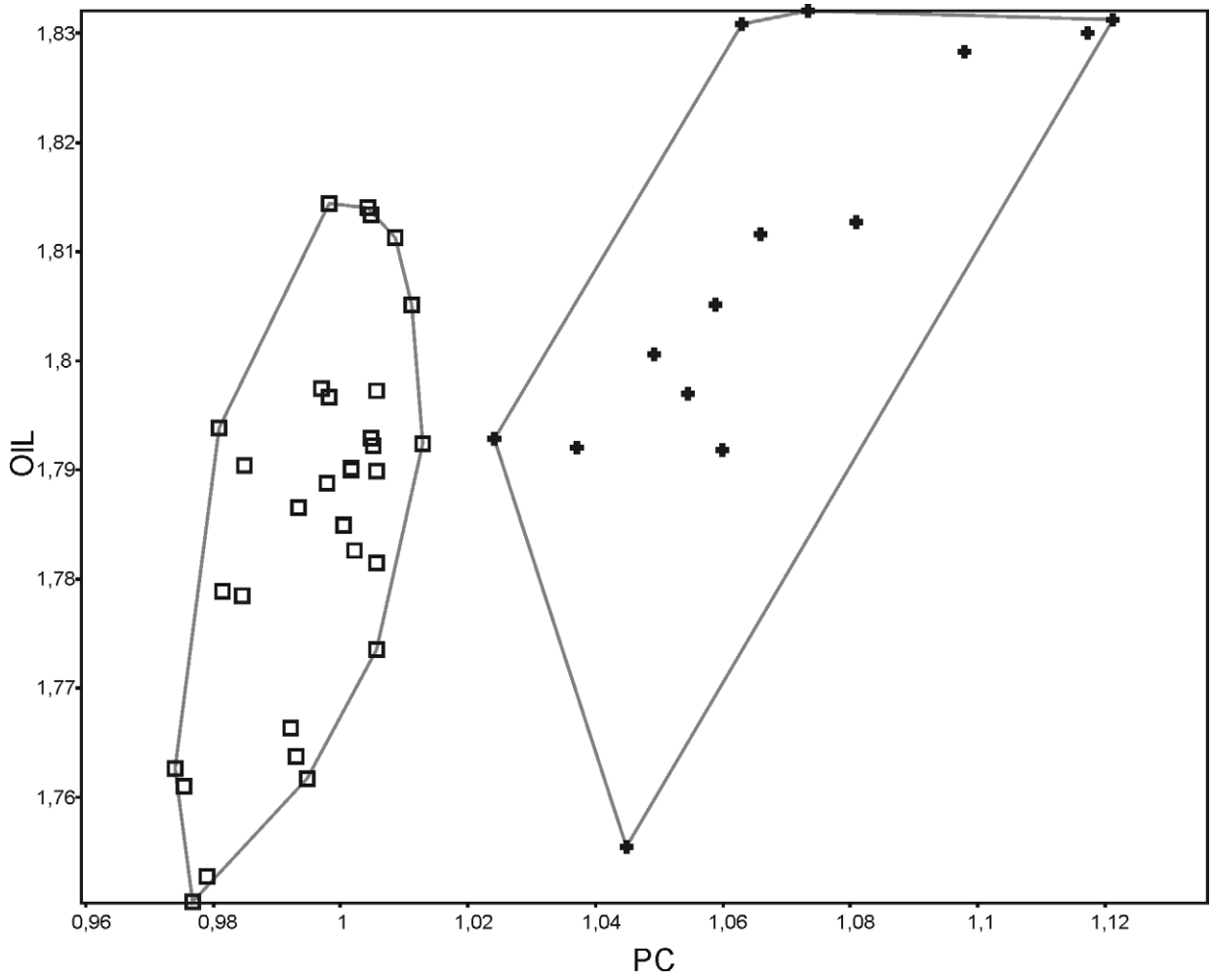

FIGURE 3. Bivariate scatterplots of nasal breadth at the postorbital constriction (PC) on occipito-incisive length (OIL) in populations of *Philander frenatus* (black crosses) and *Philander opossum canus* (white squares).

The importance of defining the geographic extension of *P. frenatus* and *P. opossum canus* has been pointed out by Patton and Costa (2003). The results presented here restrict the distribution of *Philander frenatus* to the Paranaense, Atlantic and Cerrado regions of Argentina, Paraguay and Brazil, and that of *P. o. canus* to the Chacoan region in Argentina, Bolivia and Brazil and the Amazon region of Brazil and Bolivia (Fig. 4). The analysis of populations from the dry forests of western Paraguay is still needed, in order to determine whether both species are present in that country. Up to date, only *P. frenatus* inhabits Paraguay as reported by Patton and da Silva (2007), Smith (2009), and as shown by the only sequence obtained by us (Fig. 1). According to the proposed distribution, the presence of *P. o. canus* in the dry forests of western Paraguay is highly probable, since the Paraguay River apparently acts as a barrier for both species (see below). However, there is no molecular data available for this region yet.

Although there are some recent systematic works published about *Philander* (e.g. Hershkovitz, 1997; Patton *et al.*, 2000; Patton and Costa, 2003; Costa and Patton, 2006; Lew *et al.* 2006; Nunes *et al.* 2006; Flores *et al.* 2008), the genus is in urgent need of a complete systematic revision. Following the present taxonomic arrangement of the genus (Patton and da Silva, 2007), *Philander frenatus* is the only currently recognized species that is monophyletic

and is not nested inside other species in the molecular analyses , while *P. andersoni* and *P. mcilhennyi*, though monophyletic, appeared clustered among the three subspecies of *P. opossum* (Fig. 1), making the later non monophyletic. The genetic distances obtained with our data sets agree with this, since the distance between *P. frenatus* and any other of the species included is 10 times higher than any other inter-species distance. Similar genetic distances for *Philander* have been reported by Patton and Costa (2003) and Costa and Patton (2006), who mentioned the need of analyzing the status of the subspecies of *P. opossum*.

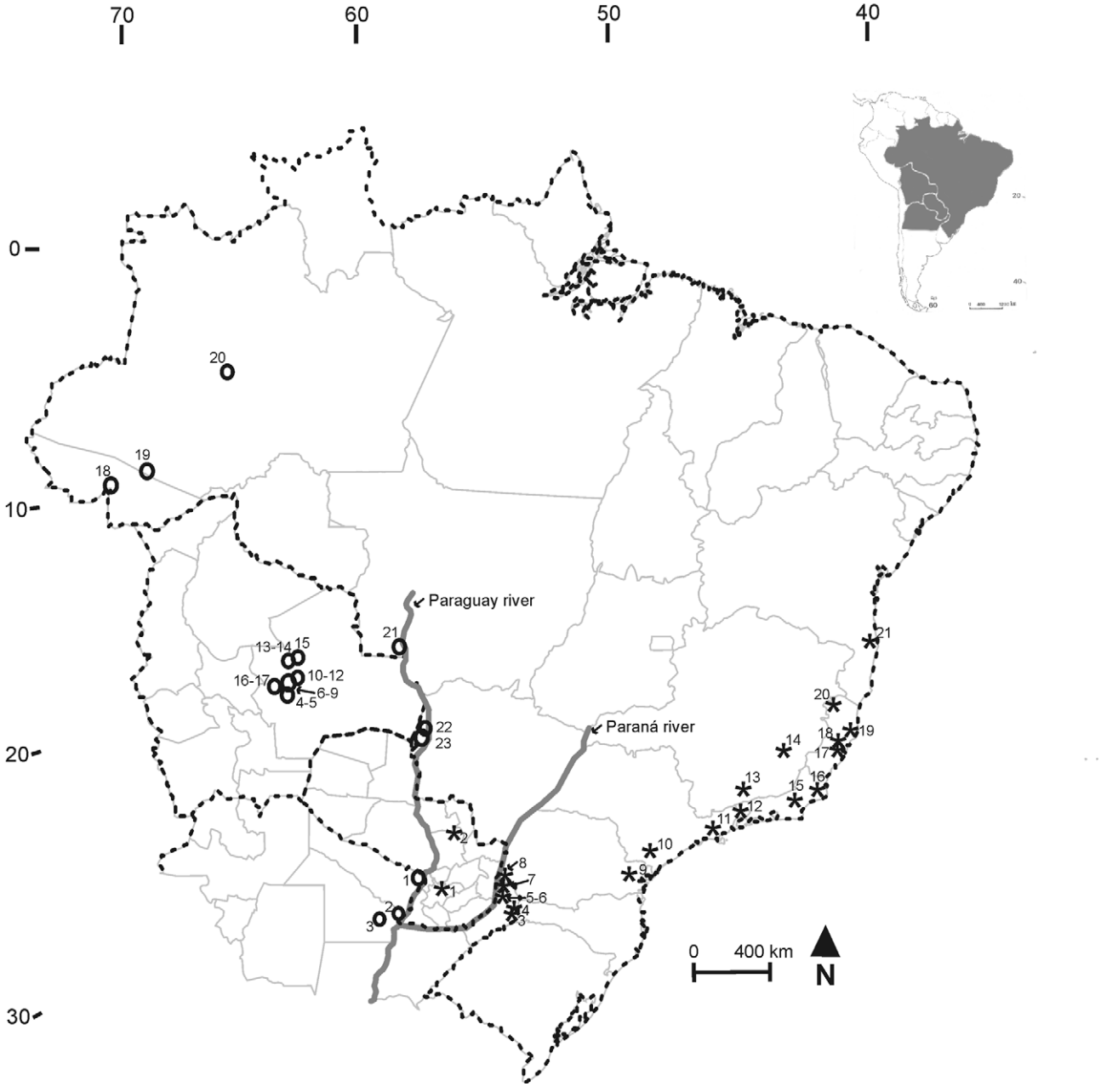

FIGURE 4. Recording localities for the specimens of *Philander frenatus* (asterisks) and *Philander opossum canus* (circles) revised in this work. Numbers indicate the specimens listed on the appendix 1.

The results obtained using cytochrome *b* (both topology and genetic distances) suggest that *Philander andersoni*, *P. mcilhennyi*, *P. opossum fuscogriseus*, *P. o. canus*, and *P. o. opossum* may have the same taxonomic status and could be treated as subspecies of *P. opossum.* This is based on the low inter-taxon distances (less than 9%) between all these clades, as well as in the fact that they all form a well-supported clade (see Fig. 1; Table 2; Patton and Costa, 2003; Costa and Patton, 2006; Nunes *et al.*, 2006; Voss and Jansa, 2009). It is important to mention that each of these groups are monophyletic, so their taxonomic identity would remain, but under a different taxonomic status. Moreover, the subspecies *P. o. canus* could be split in two forms, one including the

specimens from the Mato Grosso and the Chacoan region of Argentina, and the other including the specimens from the upper Amazon, corresponding to the clades south and west reported by Patton and Costa (2003) and Costa and Patton (2006). A different, and less conservative scheme, implies that if the specific status for *P. andersoni* is maintained, we should also support the specific status for *P. o. fuscogriseus*, considering *P. mcilhennyi*, *P. o. opossum* and *P. o. canus* as a subspecies of the monophyletic *P. opossum*, or as independent species, according to the analysis of the genetic distances.

However, interspecific genetic distances of 10-20% have been reported for other genera of opossums such as *Thylamys* Gray, *Marmosa* Gray and *Monodelphis* Burnett (Giarla *et al.*, 2010; Gutiérrez *et al.*, 2010; Carvalho *et al.*, 2011), supporting the idea of keeping only *P. frenatus* and *P. opossum* as species, since they are the only groups with such values of genetic distances. A broader analysis is still needed to solve the systematic relationships among *Philander* species, including a complete morphological analysis of all the species across its geographical range, as well as the inclusion of more molecular markers (both mitochondrial and nuclear). We believe that the inclusion of new sources of molecular information could change the phylogenetic relationships of *Philander* species, defining their limits.

Regarding the two forms present in Argentina, *P. opossum canus* and *P. frenatus*, the main problem is that they are difficult to distinguish using solely morphological characters. The key presented by Patton and da Silva (2007) separates both species using the darkness of the fur, being dark gray in *P. frenatus* and light gray in *P. opossum*, but there are no other conspicuous morphological characters that distinguish them. The morphometric analysis performed in this contribution reflected that similarity between both species, because although we found a good discrimination between *P. o. canus* and *P. frenatus* in the DFA, the PCA showed a slight overlap between both species (Fig. 2). Moreover, only one of the variables measured (inter-orbital width) separated both groups. A similar situation was also observed in the scarce morphological and morphometric evidence to recognize *P. olrogi* as a true species (Flores *et al.*, 2008). Consequently, to reach a correct identification for an unidentified specimen of *Philander* from Argentina there are three options. The easiest way (and least indicated) is to check its geographic precedence, assuming that the specimens coming from the Paranaense forest in Misiones province are identified as *P. frenatus*, whereas the specimens from Chaco or Formosa provinces should be assigned to *P. opossum canus*. Of course, this option is only a first guess, since we do not know for sure the correct range of distribution of both species, so any specimen identified using the geographic precedence should be taken with caution. If the locality is unavailable and the specimen is an adult, a morphological approach could help to a correct identification, because the width of the postorbital constriction could partially discriminate both species, being over 10.5 mm for *P. frenatus* (although it is important to mention that this number could change if more specimens are included in the multivariate analyses). Finally, if a cytochrome *b* sequence can be obtained for the specimen, the identification is easily performed by contrasting the new sequence with the available sequences. This is far from being the ideal way to identify a species, but until further morphological analyses are performed, including dentition, postcranium, pelage and other external features, the options are limited.

The genetic and morphological divergences reported here for the Argentine populations of *Philander*, reveal an increase in the diversity of marsupial fauna in the country. Argentina occupies the southern extreme of the distribution of several didelphid species (Flores, 2006; Flores *et al.*, 2007), that inhabit both humid forests (Yungas and Paranaense forests), and more xeric and pampean environments. In this case, both species of *Philander* reach high latitudes in the Neotropics: *Philander frenatus* is restricted to the humid Atlantic and Paranaense forests in southern Brazil, eastern Paraguay and northeastern Argentina, and *P. opossum canus* inhabits more humid regions in lower latitudes of the Amazonian rainforest, and extends its southern distribution occupying more dry habitats as Chacoan forests in eastern Bolivia, southern Brazil, northern Argentina, and probably western Paraguay, according to the continuous dry forests in the area (Fig. 4). As mentioned above, our topology (Fig. 1) detected some divergence between specimens of *P. o. canus* from humid lower latitudes in Brazil (Amazonas) and Peru, and those from southern dry forests of Brazil (Mato Grosso do Sul) and northern Argentina (Formosa and Chaco Provinces). New efforts to determine the current distribution of both species in their southern distributional extreme is still necessary, especially for Paraguay, where the Paraná-Paraguay river system may be acting as an efficient barrier for both species (Fig. 4). That effect of the Paraná-Paraguay rivers system as a barrier for the distribution of species has been previously reported for other species of opossums, such as *Thylamys citellus* (Thomas) and *T. pusillus* (Desmarest) (Teta *et al*., 2009), and *Marmosa* (*Micoureus*) *constantiae* (Thomas) and *M*. (*M*.) *paraguayana* (Tate) (de la Sancha *et al.*, 2011).

To sum up, this contribution shows that there are two species of *Philander* in Argentina, *P. frenatus* and *P. opossum canus*. Our results are important for delimiting the geographical range of *P. frenatus*, which was believed to be much broader than that shown by our analyses. The analysis of the genetic distances and the topologies obtained also suggest the need of an exhaustive systematic revision of the genus, in order to clarify the number of distinct biological units, either species or subspecies, within the clade *P. andersoni* + *P. mcilhennyi* + *P. opossum*.

Acknowledgments

We thank Vanina Raimondi for helping with the lab protocols, James Patton for kindly donating important unpublished sequences, Pancho Prevosti for reading the manuscript, Guille Cassini for helping with the map, Victoria Eusebi for checking the English, and the two anonymous reviewers for helping improve the manuscript. We also thank Kris Helgen and Darrin Lunde (Smithsonian Institution), Robert Voss and Eileen Westwig (American Museum of Natural History) for permitting access to specimens under their care. This research was partially funded by CONICET PIP 0329.

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APPENDIX 1.

List of specimens included in the morphometric analysis and represented on the map of Fig. 4. Numbers indicate the position on the map. USMN, National Museum of Natural History, Smithsonian Institution, Mammalogy; MACN, MuseoArgentino de CienciasNaturales "Bernardino Rivadavia, ColecciónNacional de Mastozoología; AMNH, American Museum of Natural History, Division of Mastozoology.

Philander frenatus. 1- Sapucay, Paraguay, USMN 212414, USMN 212421, USMN 121412, USMN 121458. 2- Aca Poi, 10 km S Ypané river, Paraguay, USMN 293133. 3- Río Victoria, Guaraní, Misiones, Argentina, MACN 24727. 4- Tobuna, San Pedro, Misiones, Argentina, MACN 52.19. 5- Arroyo Piray Guazú, Eldorado, Misiones, Argentina, MACN 24290. 6- Parque Schwelm, Eldorado, Misiones, Argentina, MACN 24280. 7- Arroyo Urugua-í, Iguazú, Misiones, Argentina MACN 51.127, MACN 49.307, MACN 49.376. 8- Foz do Iguaçu, Parana, Brazil, collection data unknown. 9- Mananciais da Serra, Piraquara, Parana, Brazil, NC 14. 10- Fazenda Intervales, 5.5 km S Capão Bonito, São Paulo, Brazil, MVZ182066. 11- Casa Grande, São Paulo, Brazil, USMN 460503. 12- Praia do Félix, Ubatuba, São Paulo, Brazil, MZUSP29213. 13- Parque Estadual do Ibitipoca, Minas Gerais, Brazil, MZUSP29212. 14- RPPN Belgo Mineira, João Monlevade, Minas Gerais, Brazil, CEG 35. 15- Sítio Xitaca, Nova Friburgo, Rio de Janeiro, Brazil, LG 39. 16- Majé, Garrafão, Rio de Janeiro, Brazil, U34679. 17- Reserva Biologica de Duas Bocas, Cariacica, Espirito Santo, Brazil, GU112942. 18- Santa Teresa, Espirito Santo, Brazil, MZUSP29210. 19- Serra do Caparão,Espirito Santo, Brazil, AMNH 61852, AMNH 139824. 20- Corrego Palmital, Pancas, Espirito Santo, Brazil, GU112941. 21- Fazenda Bolandeira, Bahia, Brazil, GU112938.

Philander opossum canus. 1-Laguna Blanca, Pilcomayo, Formosa, Argentina, MACN 24289. 2- Río de Oro, Bermejo, Chaco, Argentina, MACN 14342. 3- Parque Nacional Chaco, Presidencia de la Plaza, Chaco, Argentina, MACN 20866. 4- 15 km S Santa Cruz, Santa Cruz, Bolivia, AMNH 263966, AMNH 263965. 5- Cordillera Basilia, Santa Cruz, Bolivia, USMN 390565. 6- Tocomenchi, Warnes, Santa Cruz, Bolivia, USMN 390012. 7- Santa Rosita, Warnes, Santa Cruz, Bolivia, USMN 390009, USMN 390006, USMN 390011, USMN 390010, USMN 390007, USMN 390005. 8- El Palmar, Santa Cruz, Bolivia, USMN 390562, USMN 390561. 9- Ibañez, El Palmar, Santa Cruz, Bolivia, USMN 390562. 10- 10 km N of San Ramón, Santa Cruz, Bolivia, AMNH 261278, AMNH261277. 11- Road to Ascensión, Santa Cruz, Bolivia, AMNH 261275. 12- Hamacas, Santa Cruz, Bolivia, AMNH 135887. 13- Sara, 7 km N Santa Rosa, Santa Cruz, Bolivia, AMNH 246441. 14- San Miguel Rincón, Santa Cruz, Bolivia, AMNH 260037. 15- Nuflo de Chavez, Santa Cruz, Bolivia, AMNH 260034. 16- 3 km SE Montero, Santa Cruz, Bolivia, AMNH 263964. 17- Santa Cruz, Bolivia, AMNH 135886, AMNH 210416. 18- Fazenda Santa Fé, Acre, Brazil, MNFS 1031. 19- Nova Empresa, Amazonas, Brazil, collection data unknown. 20- Mamiraua Reserve, Amazonas, Brazil, collection data unknown. 21- Caceres, west side rio Paraguay, Mato Grosso, Brazil, USMN 390014. 22- Urucum, Mato Grosso do Sul, Brazil, AMNH 37063, AMNH 37064, AMNH 37065, AMNH 37066. 23- Corumbá, Mato Grosso do Sul, Brazil, USMN 390013.

APPENDIX 2. Summary (mean and standard deviation) of the skull measurements per province or region. Raw data was represented when there was only one specimen for the region. The number after the locality indicates the number of specimens included. Linear measurements are in millimeters. See Materials and Methods for a description of the measurements.

Species	Locality	OIL	ZB	PC	LN	BN1	PL	M-M
P. frenatus	Sapucay, Paraguay (4)	63.77 ± 2.55	34.58 ± 1.58	11.59 ± 0.68	30.55 ± 1.75	8.02 ±0.41	37.03 ± 1.17	19.30 ± 0.54
P. frenatus	Aca Poi, Paraguay (1)	64.80	34.64	11.64	30.77	8.92	37.00	19.85
P. frenatus	Misiones, Argentina (7)	64.28 ± 4.41	34.19 ± 3.76	11.58 ± 0.91	30.09 ± 1.98	7.94 ± 0.46	36.59 ± 2.26	18.99 ± 1.11
P. frenatus	São Paulo, Brazil (1)	62.65	30.79	11.34	30.50	8.37	35.13	18.80
P. frenatus	Espirito Santo, Brazil (2)	66.29 ± 1.88	35.18 ± 1.99	12.58 ± 0.74	30.64 ± 0.16	8.26 ± 0.9	37.70 ± 0.61	19.23 ± 0.53
P. o. canus	Chaco, Argentina (1)	63.84	33.61	10.26	28.54	7.40	35.35	18.65
P. o. canus	Formosa, Argentina (1)	62.00	31.60	10.30	30.20	7.80	35.05	18.35
P. o. canus	Santa Cruz, Bolivia (23)	60.61 ± 2.42	32.33 ± 1.63	9.85 ± 0.26	27.46 ± 1.65	6.63 ± 0.41	35.00 ± 1.41	18.96 ± 0.35
P. o. canus	Mato Grosso, Brazil (1)	59.36	32.72	10.13	28.97	7.41	33.43	19.04
P. o. canus	Mato Grosso do Sul, Brazil (5)	62.94 ± 2.21	34.13 ± 1.68	10.01 ± 0.07	27.77 ± 1.66	6.93 ± 0.33	35.31 ± 0.65	18.97 ± 0.54