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DNA BARCODING

DNA barcodes provide new evidence of a recent radiation in the genus *Sporophila* (Aves: Passeriformes)

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Abstract

The capuchinos are a group of birds in the genus *Sporophila* that has apparently radiated recently, as evidenced by their lack of mitochondrial genetic diversity. We obtained cytochrome *c* oxidase I (COI) sequences (or DNA barcodes) for the 11 species of the group and various outgroups. We compared the patterns of COI variability of the capuchinos with those of the largest barcode data set from neotropical birds currently available (500 species representing 51% of avian richness in Argentina), and subjected COI sequences to neighbour-joining, maximum parsimony and Bayesian phylogenetic analyses as well as statistical parsimony network analysis. A clade within the capuchinos, the southern capuchinos, showed higher intraspecific and lower interspecific divergence than the remaining Argentine species. As most of the southern capuchinos shared COI haplotypes and pairwise distances within species were in many cases higher than distances between them, the phylogenetic affinities within the group remained unresolved. The observed genetic pattern is consistent with both incomplete lineage sorting and gene flow between species. The southern capuchinos constitute the only large group of species among the neotropical birds barcoded so far that are inseparable when using DNA barcodes, and one of few multispecies avian groups known to lack reciprocal monophyly. Extending the analysis to rapidly evolving nuclear and mitochondrial markers will be crucial to understanding this radiation. Apart from giving insights into the evolution of the capuchinos, this study shows how DNA barcoding can rapidly flag species or groups of species worthy of deeper study.

Keywords: capuchinos, cytochrome *c* oxidase I, DNA barcodes, mitochondrial DNA, *Sporophila*

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Introduction

The avian genus *Sporophila* comprises granivorous species that occur in open and semi-open habitats from the southern USA to southern South America (Meyer de Schauensee 1952). *Sporophila* species are characterized by small size (10–12 cm), stubby bills and strong sexual dimorphism, with colourful and boldly patterned males and drab females (Ridgely & Tudor 1989). The taxonomy of the genus is in flux, with some forms considered distinct species by some researchers, but only subspecies or local variants by others. Moreover, poorly known forms, such as *S. insulata*, *S. melanops* and *S. zelichi*, may simply be hybrids or aberrant individuals of other better known species (Ridgely & Tudor 1989). Consequently, the number of species included in the genus varies between 28 and 32 (Hellmayr 1938; Meyer de Schauensee 1952; Ridgely & Tudor 1989; Sibley & Monroe 1990; Howard & Moore 1991).

The capuchinos include 11 *Sporophila* species that are smaller than the other members of the genus and characterized by cinnamon-based plumage colour patterns (Ridgely & Tudor 1989). The species of this group show

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little differentiation in size and shape, which makes females challenging to identify, whereas males differ considerably in adult plumage and vocalizations. Most capuchino species are highly sympatric with one or more other species of the group and many are rare, having limited ranges with populations in decline due to habitat loss and trapping for the pet trade (BirdLife International 2009, see Table 1). The majority of the capuchinos are seasonal migrants, but little is known about the location of the winter grounds. When not breeding, they are commonly seen in mixed flocks showing similar foraging behaviour (Ridgely & Tudor 1989; Silva 1999). A recent phylogenetic analysis performed by our group (Lijtmaer *et al*. 2004) included 10 of the 11 species (missing *S. nigrorufa*) and suggested that the capuchinos are monophyletic and further divided into two clades: northern capuchinos (*S. castaneiventris* and *S. minuta*) and southern capuchinos (*S. bouvreuil*, *S. cinnamomea*, *S. hypochroma*, *S. hypoxantha*, *S. melanogaster*, *S. palustris*, *S. ruficollis* and *S. zelichi*). These are found predominantly north and south of the Amazon River respectively. The phylogenetic relationships among the southern capuchinos were unresolved, mainly due to the presence of extremely low interspecific sequence divergence and apparent lack of reciprocal monophyly among species.

A short, standardized fragment of the mitochondrial gene cytochrome *c* oxidase I (COI) has recently been proposed as a tool for species identification; a library of COI sequences from taxonomically verified voucher specimens, constituting 'DNA barcodes', serves as species identifiers (Hebert *et al.* 2003). This approach to species identification assumes that intraspecific variation in COI is usually lower than interspecific differences (Hebert *et al.* 2003). COI surveys in several animal groups have already demonstrated high success rates in species identification (e.g. Lepidoptera, Hajibabaei *et al.* 2006; amphibians, Smith *et al.* 2008; fish, Ward *et al.* 2005). Prior studies have shown the effectiveness of COI in identifying bird species as well. In one of the most comprehensive regional analysis performed on vertebrates to date, Kerr *et al.* (2007) showed that around 94% of North American bird species have COI clusters that do not overlap with those of other species, allowing their unequivocal identification. The remaining 6% included a

Table 1 Scientific names, conservation status, estimated number of individuals, breeding habitat and approximate geographic ranges of the species included in the capuchino group (Ridgely & Tudor 1989; BirdLife International 2009)

Scientific name*	Conservation status and estimated number of individuals†	Breeding habitat	Approximate geographic range‡
S. bouvreuil§	LC, unknown¶	Tall grass savannahs	Locally in E and S Brazil; E Paraguay; NE Argentina; S Suriname
S. castaneiventris	LC, unknown¶	Grassy and shrubby clearings, floating vegetation of marshes, lake and river margins	E Colombia; SW Venezuela; E Ecuador; E Peru; N Bolivia; Amazonian Brazil; Guianas
S. cinnamomea	VU, 2500–10 000	Tall grasslands, near marshes	Locally in S Brazil, E Paraguay; NE Argentina; W and extreme SE Uruguay
S. hypochroma	NT, unknown	Tall grasslands near marshes	Very locally in N and E Bolivia; SW Brazil; E and SE Paraguay; NE Argentina; Uruguay
S. hypoxantha	LC, unknown¶	Tall grasslands near marshes	N and E Bolivia; S Brazil; Paraguay; N Argentina
S. melanogaster	NT, unknown	Tall grasslands near marshes and scrub	SE Brazil
S. minuta	LC, 500 000–5 000 000	Tall grass savannahs near water	Colombia; NW Ecuador; Venezuela; Guianas; lower Amazon Brazil; Mexico to Panama
S. nigrorufa	VU, 1000–2500	Tall grasslands near water	E Bolivia; extreme SW Brazil
S. palustris	EN, 1000–2500	Inundated grasslands and marshes	Very locally in S Brazil; SE and central Paraguay; Uruguay; NE Argentina
S. ruficollis	NT, unknown	Grasslands and dry savannah	NE Bolivia; Paraguay; S Brazil; N Uruguay; N Argentina
S. zelichi	CR, 50–250	Tall grass in flooded areas	NE Argentina; S Brazil; E Paraguay; SE Uruguay

*S., *Sporophila*.
†CR, critically endangered; EN, endangered; LC, least concern; NT, near threatened; VU, vulnerable.
‡N, north; S, south; E, east; W, west; NE, north-east; NW, north-west; SE, south-east; SW, south-west.
§This species is polytypic, including *S. bouvrevil bouvreuil* and *S. bouvreuil pileata*. The former is found in the northern portion of the species distribution and is rufous below, while the latter is found in the southern portion and is white below.
¶Although the population has not been quantified it is thought to be larger than 10 000 mature individuals.

few pairs or trios of taxa and one relatively large group of eight species (the large white-headed gulls of the *Larus argentatus–fuscus* species complex) that could not be separated based on COI alone because interspecific variation was indistinguishable from variation within single species. Other studies have shown specifically that DNA barcodes can separate and identify sister or closely related species in diverse avian orders (Vilaça *et al.* 2006; Chaves *et al.* 2008; Tavares & Baker 2008).

As part of an ongoing project to barcode all the birds of Argentina (Kerr *et al.* 2009), we obtained the COI sequences for most species of southern capuchinos and subsequently extended our sampling to include all the members of the capuchino group and various individuals within species. Given the high success of DNA barcodes in species identification in animals in general and birds in particular, the objective of this study was to use COI sequences to separate the capuchino species and to gain further insights into their phylogenetic relationships.

Materials and methods

Data set

Most tissue samples used in this study were either collected on field trips organized by the Ornithology Division of the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN) between 2006 and 2007 or loaned by other institutions (see Table 2). A few samples were obtained from other sources, such as donated specimens or birds confiscated from illegal traders and deposited at the MACN. In most cases, a traditional voucher consisting of a study skin, skeleton or specimen in ethanol is deposited either at the MACN or in another institution. In the case of blood samples, digital pictures were taken of the bird before release, providing an electronic voucher.

All 11 species of capuchinos are represented in our data set, and we used four other sympatric species as outgroups: *S. collaris*, *S. caerulescens*, *S. leucoptera* and *Volatinia jacarina* (all of which lie outside of our ingroup taxa; Lijtmaer *et al.* 2004). When available, we included multiple individuals per species (range 1–9) and from as many localities of their geographic distribution as possible (range 1–5). This is particularly relevant in the case of recently diverged species like the capuchinos, where incomplete lineage sorting is expected (Maddison & Knowles 2006). In total, our data included 38 southern capuchinos, 10 northern capuchinos and 13 outgroup specimens (Table 2). Both colour patterns and geographic range were used to identify capuchino species. The majority of the samples were taken from males in adult plumage which allowed unequivocal identification. In the case of the southern capuchinos, where most females are hard to identify using plumage traits, samples belonging to females or individuals of unknown sex (7 of 38) were clearly identified using information from distribution and the species present in the locality of capture.

DNA extraction, COI amplification and sequencing

DNA sources for this study included frozen pectoral muscle, liver, heart, blood and, in the case of *S. nigrorufa*, toe pads from a museum study skin. Approximately half of the samples were processed at the Canadian Centre for DNA Barcoding (Guelph, ON, Canada) following the extraction procedures described by Kerr *et al.* (2009). The remaining DNA extracts were obtained using the GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich) or following the procedures described by Miller *et al.* (1988). Polymerase chain reactions (PCRs) utilized the primer pair BirdF1 (5′-TTCTCCAACCACAAAGACATTGGCAC-3′) and COIbirdR2 (5′-ACGTGGGAGATAATTCCAAATCCTGG-3′) to obtain 694 base pairs (bp) of the COI. PCRs were run under the following thermal cycle profile: 1 min at 94 °C followed by six cycles of 1 min at 94 °C, 1.5 min at 45 °C and 1.5 min at 72 °C, followed in turn by 35 cycles of 1 min at 94 °C, 1.5 min at 55 °C and 1.5 min at 72 °C, and finally 5 min at 72 °C (Kerr *et al.* 2009). As the *S. nigrorufa* sample was suspected to contain degraded DNA (it was taken from a museum study skin collected in 1885), internal primers were used in conjunction with those above to obtain two shorter, overlapping sequences. These primers were AvMiR1 (5′-ACTGAAGCTCCGGCATGGGC-3′) and AvMiF1 (5′-CCCCCGACATAGCATTCC-3′) (Kerr *et al.* 2009), and the same thermal cycle profile was used. Although the sequence obtained was shorter (462 bp) because only the amplification with the AvMiF1/BirdR1 primer pair was successful, most of the variable sites were recovered. PCR products were visualized on a 2% agarose gel and bi-directionally sequenced on an ABI 3730XL DNA Analyzer (Applied Biosystems).

We deposited all sequences in GenBank (for accession numbers, see Table 2). Approximately half of them were deposited as part of a broader study of the Argentine avifauna (Kerr *et al.* 2009), while the remaining sequences were submitted separately.

Genetic variability and phylogenetic analyses

To compare COI variation patterns of the southern capuchinos with other neotropical birds, we used information from the project 'Birds of Argentina – Phase I' at http://www.barcodinglife.org because this is the largest data set of neotropical birds barcoded thus far (1594 individuals belonging to 500 species; Kerr *et al.* 2009). Kimura 2-parameter (K2P) distances (Kimura 1980) were

Table 2 List of the specimens included in this study

Specimen*	Locality	Sex	Type of sample†	GenBank acc. nos.	Museum collection nos.‡	Haplotype
S. caerulescens 1	Estero Poí, Formosa, Argentina	Male	MLHS	FJ028301	MACN-Or-70897	A1
S. caerulescens 2	Estero Poí, Formosa, Argentina	Male	MLHS	FJ028302	MACN-Or-70916	A2
S. caerulescens 3	Estero Poí, Formosa, Argentina	Male	MLHS	FJ028303	MACN-Or-70937	A3
S. caerulescens 4	San Cayetano, Corrientes, Argentina	Male	MLHS	FJ028298	MACN-Or-69625	A4
S. caerulescens 5	San Cayetano, Corrientes, Argentina	Male	MLHS	FJ028299	MACN-Or-69704	A5
S. caerulescens 6	San Cayetano, Corrientes, Argentina	Male	MLHS	FJ028300	MACN-Or-69771	A6
S. collaris 1	Estero Poí, Formosa, Argentina	Male	MLHS	FJ028307	MACN-Or-70907	B1
S. collaris 2	Estero Poí, Formosa, Argentina	Male	MLHS	FJ028310	MACN-Or-70890	B1
S. collaris 3	Estero Valenzuela, Corrientes, Argentina	Male	MLHS	FJ028306	MACN-Or-69888	B2
S. collaris 4	Estero Valenzuela, Corrientes, Argentina	Male	MLHS	FJ028309	MACN-Or-69889	B1
S. collaris 5	Estero Valenzuela, Corrientes, Argentina	Male	MLHS	FJ028308	MACN-Or-69891	B1
S. leucoptera 1	Estero Poí, Formosa, Argentina	Male	MLHS	FJ028316	MACN-Or-70917	C1
S. castaneiventris 1	Isla Sharamentsa, Pastaza, Ecuador	Unknown	MLHS	GU070583	ZMUC 123784	D1
S. castaneiventris 2	Isla Pasto, Loreto, Peru	Male	MLHS	GU070584	LSUMZ 120308	D2
S. castaneiventris 3	Isla Pasto, Loreto, Peru	Female	MLHS	GU070585	LSUMZ 120303	D3
S. minuta 1	Berbice, Guyana	Male	MLHS	GU070586	USNM 621081	E1
S. minuta 2	Wiwitau Mount., Guyana	Male	MLHS	GU070587	USNM 622227	E2
S. minuta 3	Livestock Research Station, Trinidad	Unknown	BS	GU070588	STRI TR-SMI1	E1
S. minuta 4	Guaraunos, Venezuela	Unknown	BS	GU070589	STRI VE-SMI18	E1
S. minuta 5	Guaraunos, Venezuela	Unknown	BS	GU070590	STRI VE-SMI19	E1
S. minuta 6	Guaraunos, Venezuela	Unknown	BS	GU070591	STRI VE-SMI8	E3
S. minuta 7	Guaraunos, Venezuela	Unknown	BS	GU070592	STRI VE-SMI9	E1
S. bouvreuil 1§**	Unknown	Male	MLHS	GU070593	ZMUC 130533	F1
S. bouvreuil 2	San Luis Nat. Park, Concepción, Paraguay	Male	MLHS	GU070594	KUNHM 88403	F2
S. bouvreuil 3	San Rafael Nat. Park, Itapuá, Paraguay	Male	MLHS	GU070595	KUNHM 91411	F3
S. bouvreuil 4	San Rafael Nat. Park, Itapuá, Paraguay	Male	MLHS	GU070596	KUNHM 3664¶	F4
S. bouvreuil 5	San Rafael Nat. Park, Itapuá, Paraguay	Male	MLHS	GU070597	KUNHM 91403	F5
S. bouvreuil 6	San Rafael Nat. Park, Itapuá, Paraguay	Male	MLHS	GU070598	KUNHM 3691¶	F5
S. bouvreuil 7	San Rafael Nat. Park, Itapuá, Paraguay	Male	MLHS	GU070599	KUNHM 91413	F5
S. cinnamomea 1	Iberá, Corrientes, Argentina	Male	BS	FJ028305	MACN-Or-ct 3121¶	F6
S. cinnamomea 2	Gualeguaychú, Entre Ríos, Argentina	Male	BS	FJ028304	MACN-Or-ct 3122¶	F5
S. hypochroma 1	Gualeguaychú, Entre Ríos, Argentina	Male	BS	FJ028311	MACN-Or-ct 3131¶	F5
S. hypoxantha 1	El Bagual, Formosa, Argentina	Unknown	MLHS	FJ028312	MACN-Or-70846	F5
S. hypoxantha 2	El Bagual, Formosa, Argentina	Male	MLHS	FJ028313	MACN-Or-70957	F7
S. hypoxantha 3	El Bagual, Formosa, Argentina	Male	MLHS	FJ028314	MACN-Or-70958	F5
S. hypoxantha 4	Estero Catalina, Formosa, Argentina	Male	MLHS	FJ028315	MACN-Or-70977	F8
S. hypoxantha 5	El Bagual, Formosa, Argentina	Male	MLHS	GU070600	MACN-Or-70962	F5
S. hypoxantha 6	El Bagual, Formosa, Argentina	Male	MLHS	GU070601	MACN-Or-70963	F5
S. hypoxantha 7	El Bagual, Formosa, Argentina	Male	BS	GU070602	MACN-Or-ct 3097¶	F9
S. hypoxantha 8	El Bagual, Formosa, Argentina	Male	BS	GU070603	MACN-Or-ct 3098¶	F10
S. hypoxantha 9	Velasco, Santa Cruz, Bolivia	Male	MLHS	GU070604	LSUMZ 151408	F10
S. melanogaster 1	Bom Jesus, Río Grande do Sul, Brasil	Female	BS	GU070605	MCP 2072	F11
S. melanogaster 2	Bom Jesus, Río Grande do Sul, Brasil	Female	BS	GU070606	MCP 2073	F12
S. melanogaster 3	Bom Jesus, Río Grande do Sul, Brasil	Male	BS	GU070607	MCP 2074	F10
S. melanogaster 4	Bom Jesus, Río Grande do Sul, Brasil	Male	BS	GU070608	MCP 2075	F13
S. melanogaster 5	Bom Jesus, Río Grande do Sul, Brasil	Male	BS	GU070609	MCP 2076	F14
S. melanogaster 6	Bom Jesus, Río Grande do Sul, Brasil	Female	BS	GU070610	MCP 2077	F15
S. melanogaster 7	Bom Jesus, Río Grande do Sul, Brasil	Male	BS	GU070611	MCP 2078	F16
S. nigrorufa 1	Mato Grosso, Brasil	Female	SS	GU070612	BMNH 1885.2.10.119	F17
S. palustris 1	Gualeguaychú, Entre Ríos, Argentina	Male	BS	FJ028317	MACN-Or-ct 3117¶	F18
S. palustris 2	Gualeguaychú, Entre Ríos, Argentina	Male	BS	FJ028318	MACN-Or-71052	F19
S. palustris 3	Iberá, Corrientes, Argentina	Male	BS	EU906931	MACN-Or-ct 3118¶	F20
S. ruficollis 1	Gualeguaychú, Entre Ríos, Argentina	Male	BS	EU906932	MACN-Or-ct 3128¶	F17
S. ruficollis 2	Gualeguaychú, Entre Ríos, Argentina	Male	BS	EU906933	MACN-Or-ct 3129¶	F5
S. ruficollis 3§	Argentina	Male	BS	FJ028321	MACN-Or-ct 3130¶	F5
S. ruficollis 4	San Cayetano, Corrientes, Argentina	Male	MLHS	FJ028320	MACN-Or-70176	F21

Table 2 Continued

Specimen*	Locality	Sex	Type of sample†	GenBank acc. nos.	Museum collection nos.‡	Haplotype
S. ruficollis 5	Estero Valenzuela, Corrientes, Argentina	Unknown	MLHS	FJ028319	MACN-Or-70178	F5
S. ruficollis 6	San Luis Nat. Park, Concepción, Paraguay	Male	MLHS	GU070613	KUNHM 129¶	F17
S. ruficollis 7	Trapiche, Beni, Bolivia	Unknown	MLHS	GU070614	ZMUC 123280	F5
S. zelichi 1§	Argentina	Male	BS	FJ028322	MACN-Or-ct 3132¶	F22
V. jacarina 1	San Cayetano, Corrientes, Argentina	Male	MLHS	FJ028565	MACN-Or-69796	G1

For each individual, the species to which it belongs, the locality where it was captured, its sex, the type of sample obtained, the museum collection number, the GenBank accession number and its COI haplotype is detailed.
*S., *Sporophila*; *V*., *Volatinia*.
†MLHS, pectoral muscle, liver or heart sample; BS, blood sample; SS, museum study skin.
‡KUNHM, University of Kansas Museum of Natural History; LSUMZ, Louisiana State University Museum of Zoology; NHM, The Natural History Museum; PUCRS, Coleção de Aves do Museu de Ciências e Tecnologia da Pontifícia Universidad Católica do Rio Grande do Sul; STRI, Smithsonian Tropical Research Institute; USNM, Smithsonian National Museum of Natural History; ZMUC, Zoological Museum University of Copenhagen.
§Captive bird.
**This individual belongs to *S. bouvreuil bouvreuil*, while the remaining samples are from *S. bouvreuil pileata*.
¶Tissue numbers provided.

compared using the BOLD Management & Analysis System (Ratnasingham & Hebert 2007) and MEGA4 (Tamura *et al*. 2007). The K2P distance is the best metric when distances have low values (Nei & Kumar 2000), and for this reason this model is used for species-level analysis and identification in DNA barcoding (Hebert *et al*. 2003).

The DNA sequences did not possess any insertions, gaps or stop codons. They were aligned for phylogenetic analyses using BIOEDIT version 7.0.9.0 (Hall 1999). We constructed a neighbour-joining (NJ) tree using K2P distances with MEGA4 (Tamura *et al*. 2007). To assess robustness of the nodes we performed 1000 standard bootstrap pseudoreplicates (Felsenstein 1985). To analyse the sensitivity of topologies to the method of phylogenetic reconstruction we performed Bayesian analyses using MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) and maximum parsimony (MP) analysis using TNT 1.1 (Goloboff *et al*. 2003). For Bayesian analysis, we selected the model of evolution using the hierarchical likelihood ratio test implemented in MODELTEST version 3.7 (Posada & Crandall 1998). The best fit was produced by the HKY two-parameter model (Hasegawa *et al*. 1985) with γ-distributed rate heterogeneity. We ran two independent Bayesian analyses with default priors for 5.0×10^6 generations, at which point the standard deviation of split frequencies was <0.01 indicating that both runs had converged. We sampled trees every 100 generations, discarding the first 12 500 as part of the burn-in period. The Potential Scale Reduction Factor (Gelman & Rubin 1992) was very close to 1 for all parameters, indicating that we had a sufficient sample from the posterior probability. We performed MP heuristic searches consisting of 1000 random addition sequences with the TBR branchswapping algorithm (saving 100 trees per replication). To assess the robustness of the nodes of the resulting phylogenies, we performed 1000 standard bootstrap pseudoreplicates (Felsenstein 1985) consisting of 100 random addition sequences followed by TBR (retaining 10 trees in each pseudoreplicate).

Many COI haplotypes differed by few substitutions and in some instances were shared among species. Thus, a network approach to genealogy might help disentangle relationships. We therefore constructed a statistical parsimony network (Templeton *et al*. 1992) using TCS version 1.21 (Clement *et al*. 2000) to represent the relationship between the COI haplotypes found in the southern capuchinos.

Results

The three methods of phylogenetic reconstruction confirmed with high support that the southern capuchinos are monophyletic in relation to the northern capuchinos and the remaining outgroups. However, none of the phylogenies could distinguish among the southern capuchino species or resolve their phylogenetic affinities. Figure 1a shows the NJ tree based on K2P distances and Fig. 1b shows the virtually identical topology produced using Bayesian analysis. In both trees the northern capuchinos are polyphyletic as *S. minuta* is the sister species of the southern capuchino clade, while *S. castaneiventris* is associated with the outgroup species. Constraining the capuchinos to be monophyletic produced a Bayesian tree with the southern capuchinos forming a monophyletic clade nested within the northern capuchinos. In this topology *S. castaneiventris* was external to the group conformed

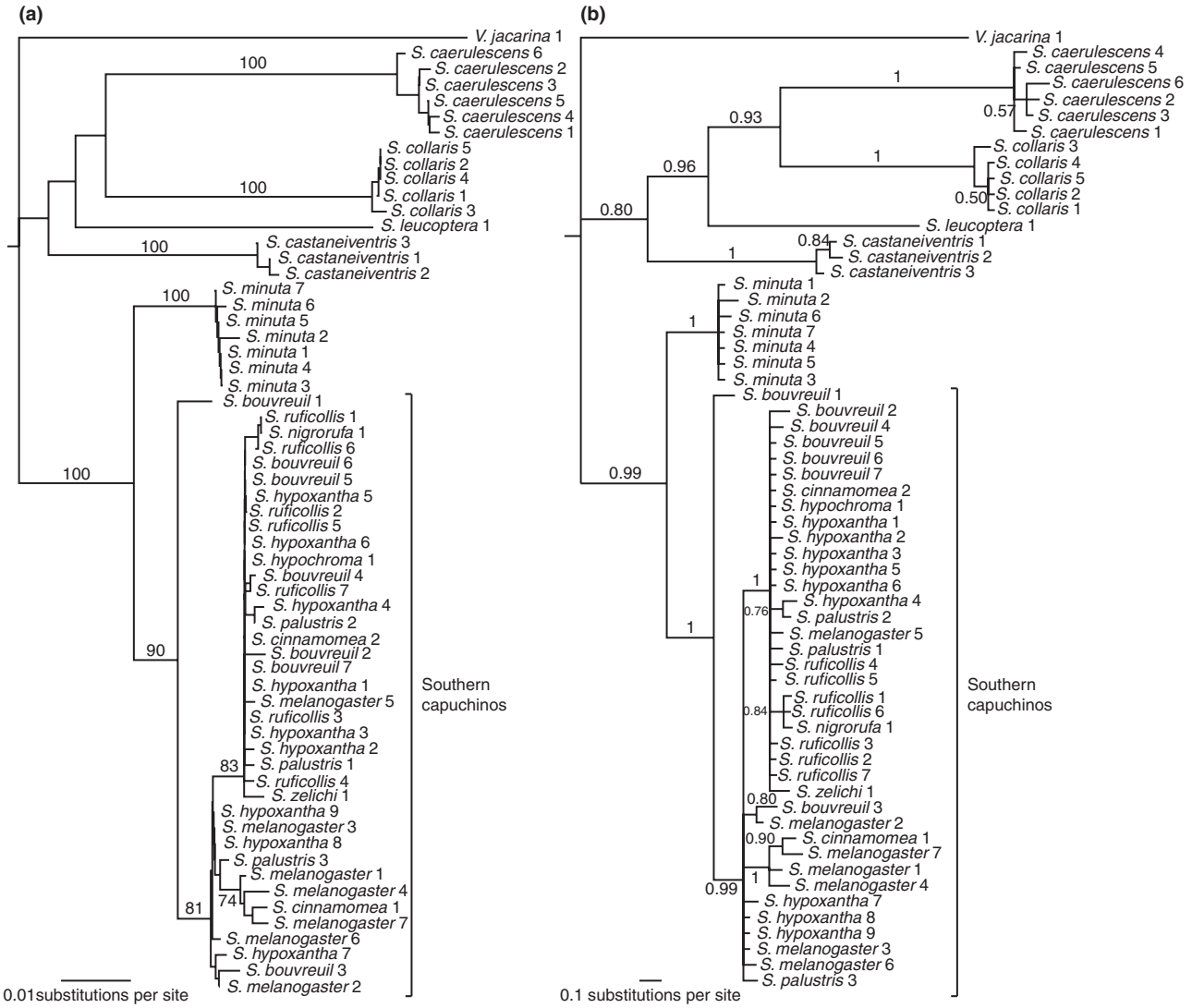

Fig. 1 Phylogenetic analysis based on 694 bp of the COI gene. (a) Neighbour-joining tree generated using K2P distances. Numbers indicate nodes supported in more than 50% of 1000 standard bootstrap pseudoreplicates. Bootstrap values of nodes within species clades as well as most nodes within the southern capuchino clade are omitted for simplicity. (b) Bayesian 50% majority rule consensus tree with posterior probabilities indicating node support.

by *S. minuta* and the southern capuchinos, and therefore the northern capuchinos were paraphyletic. The likelihood of both Bayesian topologies did not differ significantly according to a likelihood ratio test [$2(\ln L_1 - \ln L_0) = 0.2$, d.f. = 1, $P > 0.1$]. The MP analysis (not shown) produced a topology similar to that of the constrained Bayesian analysis, although support values for the monophyly of the capuchinos were low (standard bootstrap value of 36).

The results obtained for the southern capuchinos are not surprising given that haplotypes were shared among species and that some individuals showed higher K2P distance when compared with other individuals of their own species than with representatives of different species of the group. This is apparent in the haplotype network (Fig. 2), where the 22 haplotypes found among the 38 southern capuchino individuals studied are shown. Haplotypes differed in up to 11 mutational steps and the most common one (F5) was present in five of the nine species of southern capuchinos (13 of 38 individuals; Table 2).

This case of nine species that are indistinguishable using DNA barcodes appears unique within the birds of Argentina (based on the analysis of 500 species, which represent 51% of the Argentine avifauna and the only large data set of COI sequences from neotropical

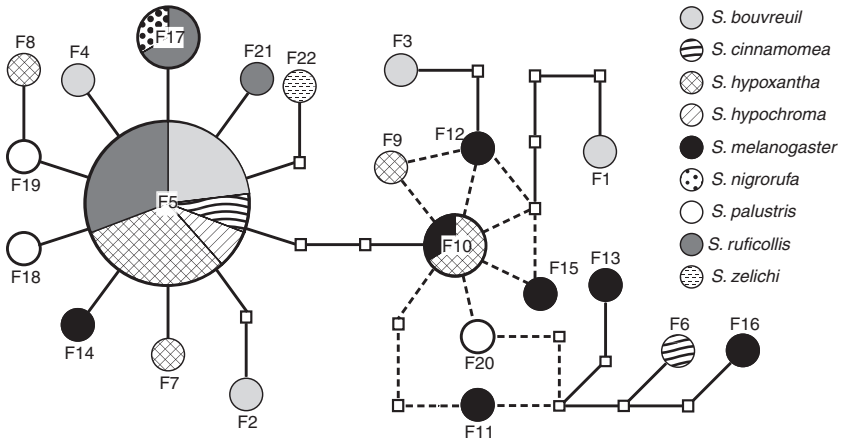

Fig. 2 Unrooted maximum parsimony network showing 95% probability linkages among 22 COI haplotypes obtained from 38 individuals of the nine species of southern capuchinos. Each line represents a single mutational change. Dashed lines show alternative connections that were not unambiguously resolved by the analysis. Empty squares represent un-sampled or extinct haplotypes. Area of circles is proportional to the number of individuals with that haplotype. Haplotype F5 is present in 13 individuals, F10 and F17 in three individuals and the remaining haplotypes are present only in one individual.

birds currently available for comparison; Kerr *et al.* 2009). We thus compared the genetic patterns of the southern capuchinos with those of the rest of the Argentine avifauna (Fig. 3). Both the average of intraspecific and interspecific divergences and its range are similar within the southern capuchinos (K2P; 0.65% vs. 0.60% and 0.14–1.2% vs. 0.07–1.2% respectively). The highest intraspecific divergence (1.9%) was found in *S. bouvreuil*, the only polytypic species of the group, when comparing the rufous morph (*S. bouvreuil bouvreuil*: sample *S. bouvreuil* 1) with the white morph (*S. bouvreuil pileata*: six remaining samples). This finding suggests that further study is needed to clarify the systematics of this species. The average intraspecific distance in the southern capuchinos is higher than that of most Argentine species, an especially striking observation given that many of the species with higher intraspecific distances included in Fig. 3b are now suspected to include more than one lineage deserving species status (Kerr *et al.* 2009; Sanín *et al.* 2009). By contrast, the average interspecific divergence among the southern capuchinos is in the lowest extreme of the distribution of the rest of the congeneric comparisons (Fig. 3a). Therefore, the interspecific divergence within the southern capuchinos is closer to the average intraspecific divergence than to that of the average congeneric divergence for the Argentine avifauna (a similar result is obtained if compared exclusively with the rest of the Argentine passerines). The remaining *Sporophila* species analysed, including the northern capuchinos, showed a marked difference between average intraspecific and interspecific divergence (K2P; 0.21%, range 0.12–0.37% vs. 8.2%, range 6.5–9.3%).

Discussion

In this study we sequenced 694 bp of the COI gene from the 11 species of capuchinos and several outgroups. Consistent with Lijtmaer *et al.* (2004), we found that the southern capuchinos are monophyletic in relation to the northern capuchinos and the remaining outgroups and that they have extremely low levels of interspecific divergence with most species sharing haplotypes. This explains why the COI gene neither separated the species nor resolved the phylogenetic relationships within the group. Different processes could cause shared COI

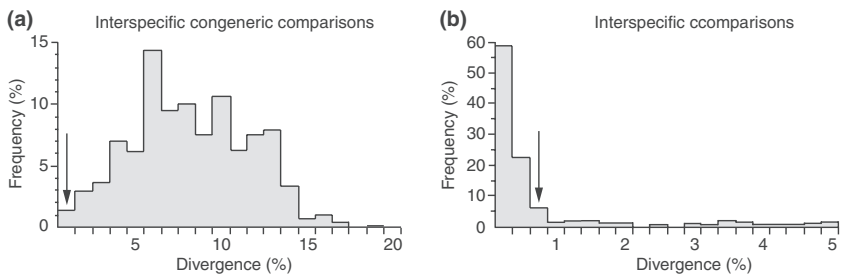

Fig. 3 Average interspecific congeneric and intraspecific K2P distances of the southern capuchinos compared with that of species present in the Argentine data set (excluding the southern capuchinos). (a) Frequency distribution of interspecific congeneric comparisons. (b) Frequency distribution of intraspecific comparisons. The arrowhead shows the position of the southern capuchinos relative to the species in the Argentine data set.

haplotypes among species and lack of reciprocal monophyly between them.

First, some cases could represent single taxa erroneously divided into more than one species (Johnston 1961). For example, it has been recently suggested, on the basis of similarities in song and habitat preference, that *S. zelichi* should be considered conspecific with *S. palustris* (Areta 2008). However, this is unlikely to be the case for most species in the group as all males differ considerably in coloration patterns and recent results show that some females can also be distinguished between species when coloration is objectively assessed with a spectrophotometer and an avian visual model is used to evaluate the results (P. Benites, unpublished data). Moreover, most southern capuchino males can be distinguished by song, showing significant differences in parameters related to time, frequency and complexity of vocalizations (L. Campagna, unpublished data) and implying some degree of reproductive isolation.

A second possibility is incomplete lineage sorting, which occurs when recently diverged taxa have not yet accumulated sequence differences in the locus analysed (Funk & Omland 2003). This option appears to be the most common in cases of avian species that lack reciprocal monophyly (Funk & Omland 2003). In the early stages of divergence, when lineage sorting is incomplete, common haplotypes are shared between species (Omland *et al.* 2006). Some COI haplotypes, notably haplotype F5, were widely shared among species of southern capuchinos, suggesting that lineage sorting is still incomplete in this group.

Alternatively, these taxa may share mtDNA because of introgressive hybridization. Around 9% of all bird species are known to have hybridized in nature (Grant & Grant 1992) and there are records of hybridization in *Sporophila* (Sick 1963; Ouellet 1992; Stiles 1996). Recurrent hybridization could explain the genetic pattern observed in the southern capuchinos as extensive gene flow among multiple species can make it difficult to infer patterns of genetic exchange and strongly affect mitochondrial tree topology (Funk & Omland 2003). The southern capuchino species showed higher average intraspecific and lower interspecific genetic distances than other Argentine species and we found numerous divergent haplotypes that differed by up to 10 mutational steps within single capuchino species. In this sense, incomplete lineage sorting is less likely to involve divergent allelic lineages than is introgression (Funk & Omland 2003), suggesting that in addition to a lack of lineage sorting, introgression of haplotypes via hybridization could also be responsible for the genetic pattern observed in the southern capuchinos.

We found no evidence for monophyly of the northern capuchinos. Instead our data support either paraphyly or polyphyly. Lijtmaer *et al.* (2004) suggested that the northern capuchinos were monophyletic, although this result was equivocal because a possibly misidentified previously published sequence form *S. castaneiventris* (obtained from GenBank) was far removed from the remaining representatives of the species and therefore excluded from the conclusions. More work is needed to distinguish between these possibilities and to define whether *S. castaneiventris* should be included in the capuchinos.

Our study significantly augments the main findings of Lijtmaer *et al.* (2004) in relation to the southern capuchinos. We used a more comprehensive sampling, both in relation to the species of southern capuchinos and the geographic distribution of each of them, including all species and more than twice as many individuals. We used fresh tissue samples (except for *S. nigrorufa*) instead of museum study skins or previously published sequences and therefore minimized the risk of cross-contamination or species misidentification. Finally, the previous study was also done with mitochondrial DNA (498 bp corresponding to 303 bp of the cytochrome *b* gene and 195 bp of part of the cytochrome oxidase subunit II, the complete lysine transfer RNA and part of the ATP synthase subunit 8); however, an advantage of using COI in the present study is that this gene has been shown to be successful in separating sister species pairs of birds differing by as little as 0.6–0.9% sequence divergence (Baker *et al.* 2009). Moreover, COI has produced similar results compared with multigene approaches (Baker *et al.* 2009). A further advantage of DNA barcodes is that there is a quality-assured COI database of many species to which new data can be compared.

This study flags the southern capuchinos as an exceptional radiation of birds. They are the only multispecies group that cannot be identified or separated by DNA barcodes among the neotropical birds barcoded so far (Vilaça *et al.* 2006; Chaves *et al.* 2008; Kerr *et al.* 2009). Moreover, very few cases were identified showing average intraspecific distances <1% and these always involved a pair or trio of species that do not share COI haplotypes and exhibit diagnostic differences in their COI sequences (Kerr *et al.* 2009). The southern capuchinos are comparable only with the large white-headed gulls (*Larus argentatus–fuscus* species complex), the only similar case encountered among North American birds (Kerr *et al.* 2007). As in the southern capuchinos, the large white-headed gulls have very similar COI barcodes and show similarly low divergence at other loci (Hebert *et al.* 2004). These gulls are thought to have diverged less than 10 000 years ago, and hybridization is common among them (Crochet *et al.* 2002, 2003). For bird genetic studies generally, where a variety of loci are analysed, the only other large group of species with genetic divergences as

low as in the cases mentioned above are Darwin's finches (Freeland & Boag 1999; Sato *et al.* 1999, 2001), the dark-eyed junco (*Junco hyemalis*) species complex (although the number of species in this complex remains a matter for debate; Milá *et al.* 2007) and possibly the crossbills (*Loxia* spp.; Edelaar *et al.* 2003), three groups of recent origin known to hybridize. For capuchinos, extending our analysis to rapidly evolving nuclear (e.g. intronic SNPs and microsatellites) and mitochondrial markers (e.g. control region) will be crucial to understanding the radiation of the southern capuchinos.

Apart from giving insights into the evolution of the capuchinos, the present study clearly shows how a standardized mitochondrial survey, like DNA barcoding, rapidly flags species or groups of species worthy of deeper study. Detecting evidence of gene flow may lead to studies of hybrid zones, mechanisms of reproductive isolation and re-examination of species limits leading to more stable classifications. Cases of incomplete lineage sorting can motivate studies in demography and speciation rates and finally high levels of intraspecific divergence may help discover cryptic species (Funk & Omland 2003). As the project to barcode the birds of the world advances, many other cases of interest to evolutionary biologists will undoubtedly be revealed.

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