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IDENTIFYING THE SISTER SPECIES TO THE RAPID CAPUCHINO SEEDEATER RADIATION (*PASSERIFORMES*: *SPOROPHILA*)

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Abstract.—Within the Neotropical genus *Sporophila*, a group of eight species known colloquially as "southern capuchinos" shows remarkable phenotypic variation despite lack of (species level) mitochondrial DNA monophyly and extremely low differentiation in other putatively neutral genetic markers. Previous studies have interpreted this as reflecting recent common ancestry and, perhaps, ongoing hybridization and introgression. A recent taxonomic revision of the only polytypic southern capuchino species, *Sporophila bouvreuil* (with four previously recognized subspecies), prompted the designation of *S. bouvreuil* and *S. pileata* as two distinct species on the basis of plumage color and geographic distribution. We used DNA sequence and microsatellite data to corroborate these new species designations and explored for the first time the relationship between these taxa and the remaining southern capuchinos. Phylogenetic and population genetic analyses showed that *S. bouvreuil* and not *S. minuta*, as was previously thought, is the sister species to the core radiation of which *S. pileata* is part. Our data suggest that the ancestor of the southern capuchinos is derived from northern South America and began to radiate during the lower to middle Pleistocene into at least eight species within the grasslands of northeastern Argentina, eastern Paraguay, and southern Brazil. Consistent with earlier studies, we could not distinguish among southern capuchino species using neutral genetic markers, an expected signature of a rapid and recent radiation. *Received 18 April 2013, accepted 23 July 2013.*

Key words: grassland birds, Neotropics, recent radiation, *Sporophila bouvreuil*, *S. pileata*.

Identificación de la Especie Hermana de la Radiación Rápida de los Capuchinos del Sur (*Sporophila*, Passeriformes)

Resumen.—El género Neotropical *Sporophila* contiene un grupo de ocho especies conocido coloquialmente como los capuchinos del sur. Dicho grupo muestra una marcada variación fenotípica (principalmente en la coloración y el canto de los machos) que contrasta con la falta de monofilia a nivel de especie y bajos niveles de diferenciación en marcadores genéticos neutros. Este patrón ha sido atribuido al origen reciente del grupo y a la hibridación e introgresión entre especies. Una revisión taxonómica reciente de la única especie politípica, *S. bouvreuil* (la cuál contenía cuatro subespecies), designó a *S. bouvreuil* y *S. pileata* como nuevas especies en base a patrones de coloración y distribución geográfica. En el presente estudio utilizamos secuencias de ADN y frecuencias de alelos de ADN microsatélite para corroborar la designación de dichas especies y estudiar por primera vez la relación entre ellas y con respecto a los demás capuchinos del sur. Utilizando análisis filogenéticos y herramientas de genética de poblaciones mostramos que *S. bouvreuil* y no *S. minuta*, como se creía anteriormente, es la especie hermana del grupo, al cual pertenece *S. pileata*. Posiblemente el ancestro de los capuchinos del sur provino del norte de América del Sur y comenzó a radiar durante el Pleistoceno inferior o medio en al menos ocho especies en los pastizales del noreste Argentino, este de Paraguay, y sur de Brasil. Como en estudios previos, no fue posible distinguir entre capuchinos del sur utilizando marcadores genéticos neutros, un resultado esperable en el contexto de una radiación rápida y reciente.

The speciose Neotropical genus *Sporophila* harbors within it a remarkable radiation of 11 granivorous species, colloquially known as *capuchinos* or *caboclinhos* in Spanish and Portuguese, respectively, but lacking a common name in English (Ridgely and Tudor 1989, Rising et al. 2011, Remsen et al. 2013). Eight of the capuchino species are endemic to central and southern South America, where they are predominantly sympatric and often syntopic, and appear to have radiated rapidly during the Pleistocene (Ridgely and Tudor 1989; Lijtmaer et al. 2004; Campagna et al. 2010, 2012). These eight species (*S. cinnamomea*, *S. hypochroma*, *S. hypoxantha*,

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S. melanogaster, *S. nigrorufa*, *S. pileata*, *S. palustris*, and *S. ruficollis*) can show eclipse plumage and gather in mixed flocks when not breeding (Ridgely and Tudor 1989). Evidence from molecular studies shows extremely low neutral genetic differentiation among these taxa, interpreted to be the consequence of recent common ancestry and, perhaps, ongoing hybridization and introgression (Lijtmaer et al. 2004; Campagna et al. 2010, 2012). Phylogenetic affinities among these eight species (hereafter "southern capuchinos") remain unresolved, despite the marked phenotypic differences that exist mainly in male reproductive plumage and vocalizations (Campagna et al. 2012). Southern capuchinos are strikingly sexually dimorphic; females are brown and olive and hard to distinguish among species, whereas males show distinct reproductive plumage patterns that are generally based on the color cinnamon (Ridgely and Tudor 1989). Plumage differences in the UV-portion of the spectrum are found between some female southern capuchinos (Benites et al. 2010). Aside from these phenotypic differences, southern capuchinos are extremely similar in size and shape (Meyer de Schauensee 1952, Ridgely and Tudor 1989, Ouellet 1992).

Recent studies of southern capuchino species have led to the discovery of various alternative color morphs of already recognized species and, in some cases, have prompted taxonomic changes. Traditionally, *S. zelichi* was considered a distinct species, but, primarily on the basis of overall similarity in song and habitat use, it is now regarded as a color morph of *S. palustris* and has been subsumed within the latter taxon (Areta 2008, Remsen et al. 2013). Similarly, a series of alternative adult male color morphs that share song types with different southern capuchinos have been described (for *S. melanogaster*, see Repenning et al. 2010; for *S. hypoxantha*, see Areta and Repenning 2011; for *S. ruficollis*, see Areta et al. 2011). Finally, Machado and Silveira (2010) conducted a detailed taxonomic revision of the four subspecies (*S. bouvreuil bouvreuil*, *S. b. pileata*, *S. b. saturata*, and *S. b. crypta*) from the only polytypic species of the group. They diagnosed only two species based on plumage patterns: *S. bouvreuil* (which now includes *S. b. bouvreuil*, *S. b. saturata*, and *S. b. crypta*) and *S. pileata* (including the former *S. b. pileata*) (Machado and Silveira 2010, Remsen et al. 2013). These newly diagnosed taxa are predominantly allopatric, with a small area of range overlap in southeastern Brazil (Machado and Silveira 2011) where they appear to maintain their phenotypic integrity.

Sporophila bouvreuil is found in open areas from northern South America to central and southern Brazil, whereas *S. pileata* occurs in central and southeastern Brazil, Paraguay, and northeastern Argentina (Machado and Silveira 2011; Fig. 1). Thus, the distribution of *S. bouvreuil* is geographically intermediate to that of other southern capuchino species (including *S. pileata*) and *S. minuta*, the putative sister species of the southern capuchino radiation (Campagna et al. 2010, 2012; Fig. 1). Previous studies also found the former *S. b. bouvreuil* (now included in *S. bouvreuil*) to be more closely allied to the southern capuchino radiation than is *S. minuta* (Campagna et al. 2010, 2012). However, these studies included mostly *S. b. pileata* (now *S. pileata*) and only one individual *S. b. bouvreuil* (now included in *S. bouvreuil*); thus, tests of the monophyly of the latter taxon could not be performed.

Here, we use putatively neutral mitochondrial and nuclear sequences as well as DNA microsatellites to address the following questions. (1) Is there genetic evidence supporting the designation of *S. bouvreuil* and *S. pileata* as separate species? (2) What are the phylogenetic relationships between *S. bouvreuil* and the species that comprise the southern capuchino radiation? Is *S. bouvreuil* part of the radiation, or is *S. bouvreuil* and not *S. minuta* the sister species to the southern capuchinos? If *S. bouvreuil* is the sister lineage, we can then narrow down the geographic and temporal scenarios for the southern capuchino radiation.

Methods

Sampling and data set.—We augmented the data set of Campagna et al. (2012) with genetic data from new individuals of *S. bouvreuil* (n = 19) and *S. pileata* (n = 4). In total, the augmented data set includes the eight southern capuchino species (n = 207 of which 37 belong to *S. pileata*), *S. bouvreuil* (n = 20), *S. minuta* (n = 7), *S. castaneiventris* (n = 5), and 24 individuals from four closely related outgroup species (numbers of samples per southern capuchino species are summarized for each marker in Table 1; for other details, see Table S1 in the online supplementary material). Most tissue samples were from vouchered males in adult plumage (with study skin, skeleton, or specimen in ethanol deposited at the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia," the Museu de Zoologia da Universidade de São Paulo, or another institution; for details, see Table S1). For individuals for which we have blood samples, a picture was taken to serve as a digital voucher before the bird was released and is available upon request.

Genetic markers.—Samples in Campagna et al.'s (2012) data set were genotyped for six previously published microsatellite loci (Escμ6, Hanotte et al. 1994; Mcyμ4, Double et al. 1997; Pdoμ3, Neumann and Wetton 1996; Gf05, Gf08, and Gf12, Petren 1998). This data set also includes DNA sequences from eight markers for most individuals from which fresh tissue samples were available (i.e., not taken from museum study-skin toe pads). These markers included three mitochondrial regions (cytochrome *b* [cyt *b*], 902 base pairs [bp]; cytochrome *c* oxidase I [COI], 694 bp; mitochondrial control region [CR], ~1,050 bp), one autosomal intron (intron 5 of the β-fibrinogen gene: Fib5, ~559 bp), two previously described nuclear sequences of mitochondrial origin (*Numt*2: ~765 bp; *Numt*3: ~679 bp; see Sato et al. 2001), and two Z-linked markers (chromodomain-helicase-DNA binding protein: CHD1Z: ~399 bp; intron 3 of the muscle skeletal receptor tyrosine kinase gene: MUSK, ~697 bp) (for details, see Tables 1 and S1).

For the present study, we genotyped the new *S. bouvreuil* and *S. pileata* samples for the six aforementioned DNA microsatellite loci, and amplified and sequenced COI, cyt *b*, CR, and CHD1Z. DNA extraction, amplification, sequencing, and genotyping followed procedures described by Campagna et al. (2012). We generated additional sequences (29 COI, 29 cyt *b*, and two CR) for some southern capuchinos to assemble a data set of 112 individuals for which most had been sequenced for all three mitochondrial regions. Table S1 provides details and GenBank accession numbers. All sequences were aligned using BIOEDIT, version 7.1.11 (Hall 1999).

Phylogenetic analyses.—The model of nucleotide evolution was selected for each marker using JMODELTEST, version 2.1.1 (Darriba et al. 2012). We used three strategies to explore the phylogenetic affinities among *Sporophila* species. First we obtained a Bayesian gene tree using MRBAYES, version 3.2 (Ronquist et al. 2012), and data from the three mitochondrial markers (COI, cyt *b*, and CR). We placed each marker in a separate unlinked partition, and for each we used the model

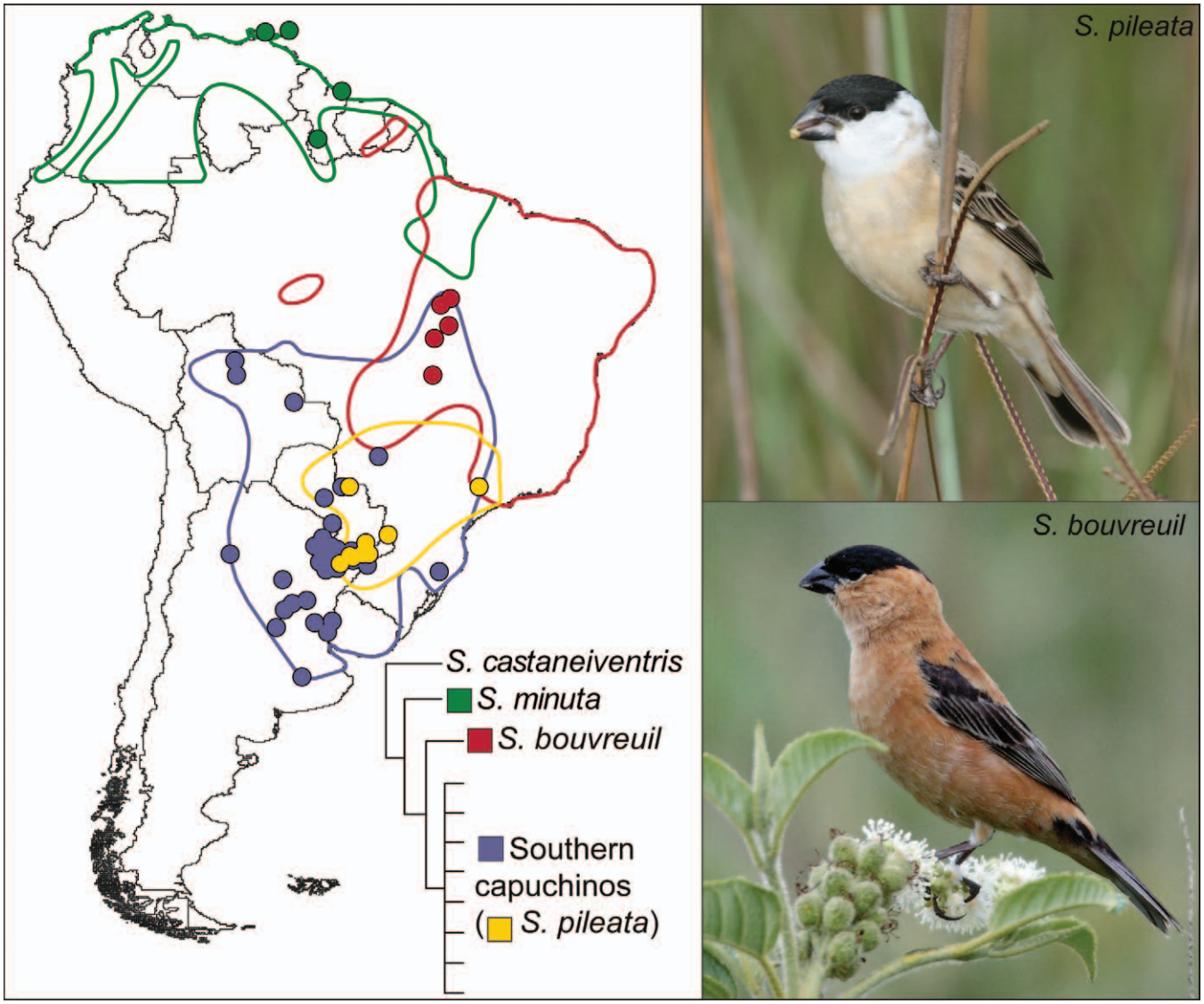

Fig. 1. Approximate range map for *Sporophila minuta* (the distribution of this species outside of South America is not represented here), *S. bouvreuil,* and the eight species that comprise the southern capuchino radiation (the distribution of *S. pileata* is shown separately) following Ridgely and Tudor (1989), Machado and Silveira (2010), and Somenzari et al. (2011). Circles represent sampling locations. Examples of adult male *S. pileata* and *S. bouvreuil* are shown in the right panels, together with a schematic representation of the phylogenetic affinities among capuchinos.

of nucleotide evolution chosen with JMODELTEST (COI and cyt *b*: HKY+I+G, Hasegawa et al. 1985; CR: GTR+I+G, Tavaré 1986). The Bayesian analysis consisted of two independent, simultaneous runs with four chains each (under default priors for all parameters) for 12×10^6 generations, sampling trees every 100 generations. At this point, both runs had reached a stationary state and converged, which was confirmed using the "cumulative" and "compare" functions in the software AWTY (Wilgenbusch et al. 2004). A 50% majority rule consensus was obtained from the combined posterior tree distribution after discarding the initial 25% of trees as burn-in.

The second strategy combined mitochondrial and nuclear data to estimate a species tree using the Bayesian coalescent approach implemented in *BEAST (Heled and Drummond 2010), included in the BEAUTI/BEAST, version 1.7.4, package (Drummond et al. 2012). The concatenated mitochondrial genes (COI, cyt *b*, and CR) and each of the five nuclear markers were placed in different partitions under the model of nucleotide evolution chosen using JMODELTEST (CHD1Z and Fib5: HKY; MUSK: HKY+I; *Numt*3: HKY+G; *Numt*2: GTR+G; and mitochondrial: GTR+I+G). We used a Yule speciation model and a piecewise linear and constant-root population size model. We implemented a relaxed uncorrelated log normal clock and ran the analysis for 1×10^8 generations (sampling every 1,000). We used TRACER, version 1.5 (Rambaut and Drummond 2009), to confirm convergence in parameter estimates and that effective sample sizes exceeded 200. Finally, we used TREE ANNOTATOR, version 1.7.4, from the BEAUTI/BEAST package to summarize the 7.5×10^4 (75%) post-burn-in sampled trees. We obtained a maximum-clade-credibility tree using mean node heights; posterior

Table 1. Sample sizes for each capuchino species and molecular marker (see text) used in this study. Genetic divergence was estimated independently for each marker; the average divergence between the southern capuchinos and *Sporophila bouvreuil* and the highest divergence between two southern capuchino species is shown. For sequence data (mitochondrial and nuclear), we calculated average percent *p*-distances and standard deviations, and for the DNA microsatellite data we computed pairwise F_{ST} values between species.

Species	COI	Cyt *b*	CR	*Numt*2	*Numt*3	Fib5	CHD1Z	MUSK	DNA microsatellites
Sporophila castaneiventris	3	3	3	1	3	–	–	–	5
S. minuta	7	7	7	6	7	–	–	–	7
S. bouvreuil	19	17	19	–	1	–	17	–	20
S. cinnamomea	3	3	3	2	2	3	3	3	14
S. hypochroma	2	2	2	1	1	2	2	1	26
S. hypoxantha	30	30	42	8	33	21	4	5	62
S. melanogaster	7	7	7	4	7	7	7	1	7
S. nigrorufa	1	–	–	–	–	–	–	–	2
S. pileata	10	10	10	6	6	6	8	4	37
S. palustris	10	10	10	4	11	11	5	3	15
S. ruficollis	7	7	7	7	7	7	4	4	44
Southern capuchinos vs. *S. bouvreuil*	1.63 ± 0.51	1.09 ± 0.23	0.89 ± 0.33	–	0.82 ± 0.31	–	0.18 ± 0.17	–	0.041
Maximum among southern capuchino species	0.97 ± 0.44 [a]	0.61 ± 0.24 [b]	0.60 ± 0.31 [c]	0.17 ± 0.18 [d]	1.98 ± 0.09 [e]	0.11 ± 0.23 [f]	0.062 ± 0.12 [g]	0.54 ± 0.16 [e]	0.013 [h]

[a] *S. melanogaster* vs. *S. pileata*.
[b] *S. melanogaster* vs. *S. palustris*.
[c] *S. melanogaster* vs. *S. hypoxantha*.
[d] *S. ruficollis* vs. *S. hypoxantha*.
[e] *S. cinnamomea* vs. *S. hypochroma*.
[f] *S. cinnamomea* vs. *S. ruficollis*.
[g] *S. pileata* vs. *S. hypoxantha*.
[h] *S. melanogaster* vs. *S. cinnamomea*.

probability indicated node support. Finally we used DENSITREE, version 2.1.7 (Bouckaert 2010), to overlay a subsample (2×10^4) of the post-burn-in trees. We also estimated species trees using individual markers to assess the relative contribution of each to the multigene topology.

The third strategy also combined mitochondrial (concatenated COI, cyt *b*, and CR sequences) and nuclear data (CHD1Z, Fib5, MUSK, *Numt*2, and *Numt*3 sequences placed in separate partitions) to perform species delimitation using the Bayesian framework implemented in BP&P, version 2.1b (Rannala and Yang 2003, Yang and Rannala 2010). BP&P generates a posterior distribution of species assignments while accommodating the effects of incomplete lineage sorting on gene trees. We used the topology generated by *BEAST as a guide tree, from which BP&P collapses each internal node while calculating the probabilities of models containing different numbers of species. We used a gamma prior for each population size parameter (θ) and the age of the root in the species tree (τ). For the gamma distributions, we specified a shape parameter ($\alpha = 2$) and a scale parameter ($\beta = 1{,}000$) in both cases. Other divergence time parameters were assigned default Dirichlet priors. We conducted multiple analyses implementing reversible-jump Markov chain Monte Carlo (rjMCMC) algorithms 0 (using $\varepsilon = 2, 5, 10$, or 20) or 1 (trying all combinations of $\alpha = 1, 1.5$, or 2 and $m = 0.5, 1$, or 2) (Yang and Rannala 2010). The analyses were run for 550,000 generations, sampling every 5 and discarding the first 50,000 as burn-in.

F-statistics.—We explored the degree of genetic divergence among *Sporophila* species by calculating all pairwise F_{ST} or Φ_{ST} values with ARLEQUIN, version 3.5 (Excoffier and Lischer 2010); significance was tested using 1,000 random permutations with sequential Bonferroni corrections (Rice 1989). We subsequently displayed these matrices graphically by building neighbor-joining trees with the program NEIGHBOR provided in PHYLIP, version 3.6.9 (Felsenstein 2005). Φ_{ST} and F_{ST} neighbor-joining trees were constructed for both the mitochondrial and the microsatellite data. For these trees, we included only species for which at least five individuals had been sequenced or genotyped. We also calculated F_{ST} based on a single nucleotide polymorphism in CHD1Z, between *S. bouvreuil* and all the southern capuchinos pooled. Finally, analyses of molecular variance (AMOVA) were performed in ARLEQUIN, grouping samples by either species or sampling locality.

Genetic distance and ordination analyses.—We calculated inter-individual genetic distances using either sequence data or the six DNA microsatellite loci. For the former, we estimated uncorrected *p*-distances in MEGA, version 5 (Tamura et al. 2011), whereas for the latter we computed D_{SW} genetic distances (Shriver et al. 1995) in POPULATIONS, version 1.2.32 (Langella 1999). Pairwise matrices of inter-individual genetic distances were displayed using principal coordinate analyses (PCoA) computed in GENALEX, version 6.5 (Peakall and Smouse 2006).

Bayesian clustering analysis.—We used the Bayesian approach implemented in STRUCTURE, version 2.3.4 (Pritchard et al.

2000), and our DNA microsatellite data to assign individuals to different genetic populations (*K*). Before conducting this analysis, for each species we assessed Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using ARLEQUIN and sequential Bonferroni corrections. The following locus–species combinations were not in HWE: Pdoμ6 in *S. palustris* and *S. pileata*, Mcyμ4 in *S. pileata* and *S. bouvreuil*, and Gf08 in *S. bouvreuil*. Mcyμ4 and Gf05 were in LD in *S. cinnamomea*, *S. hypochroma*, *S. hypoxantha*, and *S. ruficollis*. Deviations from HWE and LD had been observed before for some of these loci (Campagna et al. 2012) and could be the product of population-level genetic structure (Wahlund 1928), ongoing hybridization and introgression, or technical difficulties such as undetected allele dropout or null alleles. Thus, our microsatellite data may not fit the model assumed by STRUCTURE, potentially leading to the overestimation of the number of genetic populations. However, the STRUCTURE analyses found very little species-level structure in our data, confirming results from *F*-statistics (see below), which suggests that our results were not spurious.

The STRUCTURE analysis was conducted using the admixture ancestry model, correlated allele frequencies, and, in separate analyses, both with and without locprior (i.e., a prior indicating species identity). We included the eight southern capuchino species and *S. bouvreuil*, exploring values of *K* between 1 and 4 (based on previous results that showed lack of differentiation in these markers among southern capuchino species; Campagna et al. 2012). We performed 10 iterations per value of *K*, each with 2.5×10^6 generations, discarding the initial 25% as burn-in. The most likely value of *K* was determined using Evanno et al.'s (2005) method implemented in STRUCTURE HARVESTER, version 0.6.93 (Earl and vonHoldt 2012).

Divergence time estimations.—We calculated the age of the mitochondrial ancestor (time to most recent common ancestor, TMRCA) between the southern capuchinos and *S. bouvreuil* using BEAUti/BEAST. An estimation of absolute time was reached using cyt *b* data and a calibration of 2.1% divergence per million years (Weir and Schluter 2008). The BEAST analysis was run with a random starting tree for 1×10^8 generations, assumed constant population sizes and a relaxed uncorrelated lognormal clock, and implemented the HKY+I+G model of nucleotide evolution. Results were inspected for convergence and adequate effective samples sizes in TRACER.

Results

The mitochondrial Bayesian tree (based on COI, cyt *b*, and CR sequences; Fig. 2A) suggests that *S. bouvreuil* is phylogenetically distinguishable from *S. pileata* and is indeed the sister species to the southern capuchino radiation. Most *S. bouvreuil* form a highly supported clade that is sister to all individuals involved in the southern capuchino radiation. Using cyt *b* data and a clock calibration of 2.1% divergence Ma^{-1}, we estimated the age of the common ancestor between *S. bouvreuil* and the southern capuchinos to be 0.84 Ma before present (95% high posterior density interval: 0.51–1.19). The clade composed of the southern capuchinos and *S. bouvreuil* is, in turn, sister to *S. minuta* (Fig. 2A). Two of 19 individual *S. bouvreuil* (marked with arrows in Fig. 2A) carry "southern capuchino" mitochondrial haplotypes. Both individuals are male *S. bouvreuil* with adult reproductive plumage (see Fig. S1 in the online supplementary material). Southern capuchino species for which more than one individual was sampled (*S. nigrorufa* being the exception) show lack of species-level monophyly at these loci. However, some southern capuchino species have significant differences in mitochondrial haplotype frequency (measured using Φ_{ST} calculations performed on concatenated COI, cyt *b*, and CR sequences; Fig. 2B; for Φ_{ST} values obtained from individual mitochondrial markers, see Fig. S2 in the online supplementary material). Although Φ_{ST} values between southern capuchino species were generally <0.3, comparisons with *S. bouvreuil* were larger and were in all cases statistically significant (Fig. 2B; for comparisons using genetic distances, see Table 1). The PCoA derived from inter-individual *P*-distances (Fig. 2C) also illustrates the relationship among individual *S. minuta*, *S. bouvreuil*, and southern capuchinos. Southern capuchinos cluster into two groups: individuals that belong to the main clade found within the radiation (Fig. 2A) and a separate cluster comprised of all those remaining. As shown in Figure 2A, one *S. bouvreuil* is found in each southern capuchino cluster. A small percentage of the variation among southern capuchinos is attributable to differentiation among either species or sampling locality (AMOVA: 4.17%, $P = 0.045$ for species; 5.43%, $P = 0.034$ for sampling locality).

Similar results were obtained from the multilocus species-tree analysis (Fig. 3) that included 95 individuals from nine species and data from six unlinked genetic markers. The seven southern capuchino species included in this analysis comprised a highly supported clade that was sister to *S. bouvreuil*. Phylogenetic affinities among southern capuchino species were again uncertain, with low posterior probabilities for all clades within this group (ranging from 0.4 in the clade including *S. hypochroma*, *S. melanogaster*, and *S. cinnamomea* to 0.7 between *S. hypoxantha* and *S. ruficollis*). Alternative topologies to that of the consensus tree (in white) are visible in gray in the cloudogram generated from a subsample of the posterior tree distribution (Fig. 3). Darker shades of gray imply larger numbers of trees with that topology, thus resembling the consensus tree. Trees derived from nuclear data alone did not resolve differences among the southern capuchinos, and Figure 3 is similar to the species tree obtained from mitochondrial DNA alone (for species trees derived from individual markers, see Fig. S3 in the online supplementary material). However, there was a single nucleotide polymorphism in CHD1Z that was close to fixation between individual *S. bouvreuil* and southern capuchinos ($F_{ST} = 0.8$), and the DNA microsatellite data (see below) also support our conclusion that the differentiation between southern capuchinos and *S. bouvreuil* is not solely attributable to mitochondrial markers. Results from the BP&P analysis varied across runs that used different algorithms and combinations of priors but were generally consistent with the results obtained using *BEAST. The data allowed us to distinguish *S. minuta* from the remaining species regardless of the conditions under which the program was run, and in some cases to distinguish between *S. bouvreuil* and the southern capuchinos. However, differences among southern capuchino species were generally not supported.

The F_{ST} neighbor-joining tree based on DNA microsatellite data also shows higher values between southern capuchinos and *S. bouvreuil* than among the former species (the largest inter-capuchino F_{ST} value was 0.013 between *S. cinnamomea*

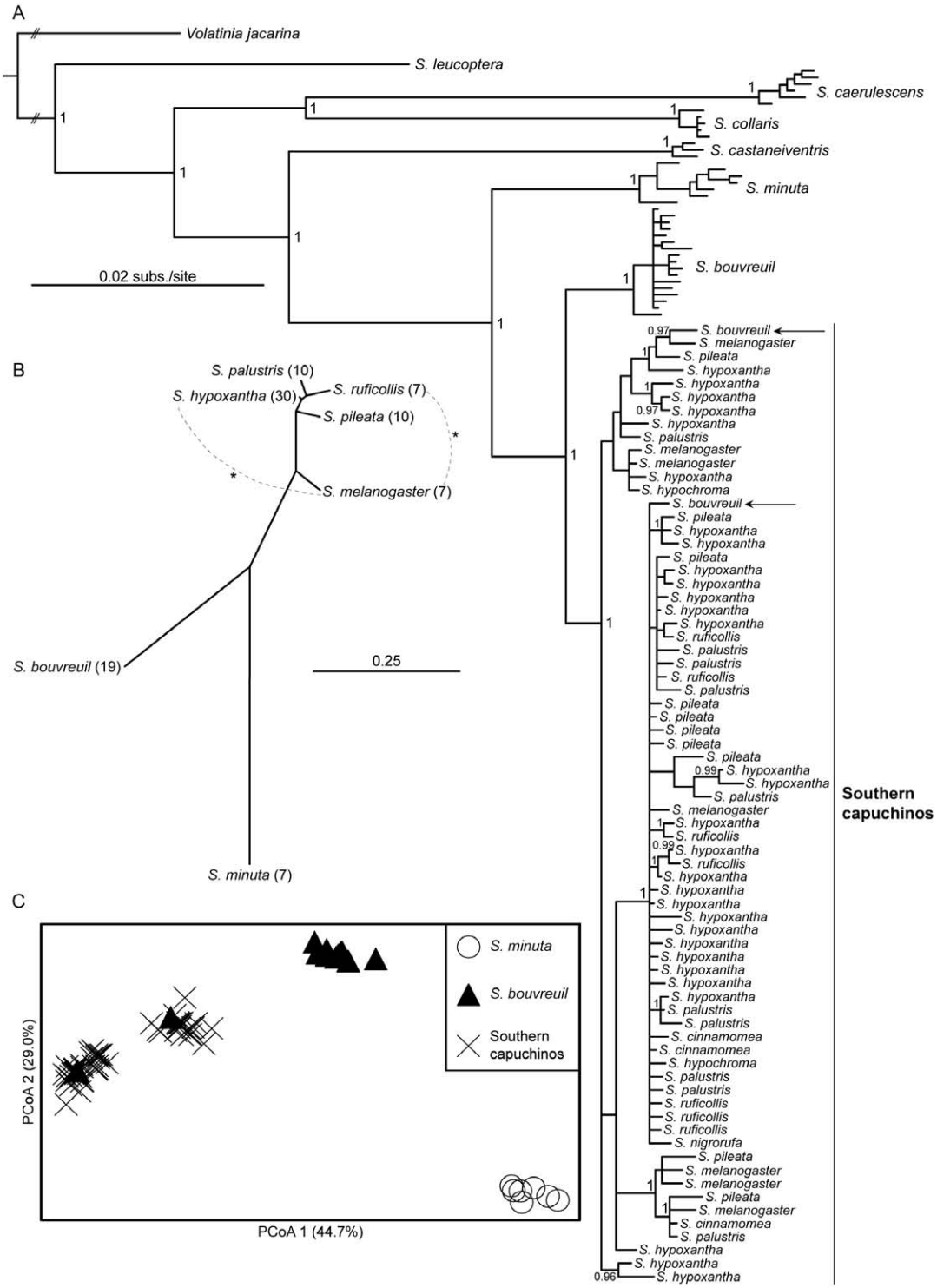

FIG. 2. (A) Bayesian tree for *Sporophila* derived from mitochondrial DNA sequence data (112 individuals; ≈2,650 base pairs from cytochrome *c* oxidase I [COI], cytochrome *b* [cyt *b*], and mitochondrial control region [CR]) with posterior probabilities indicating node support. Arrows indicate two male *S. bouvreuil* that fall within the southern capuchino clade. Posterior probabilities of nodes with low support were omitted for clarity. When individuals belonging to the same species form a clade, species name is mentioned only once. (B) Neighbor-joining trees built using pairwise Φ_{ST} calculations derived from concatenated COI, cyt *b*, and CR data. Comparisons between southern capuchino species that were statistically significant after sequential Bonferroni correction are indicated by asterisks ($\alpha = 0.05$). All comparisons between southern capuchinos and *S. bouvreuil* or *S. minuta* were statistically significant (as was the comparison between *S. bouvreuil* and *S. minuta*). (C) PCoA derived from inter-individual *p*-distances calculated using concatenated COI, cyt *b*, and CR data. Parentheses indicate the percentage of variation explained by each axis.

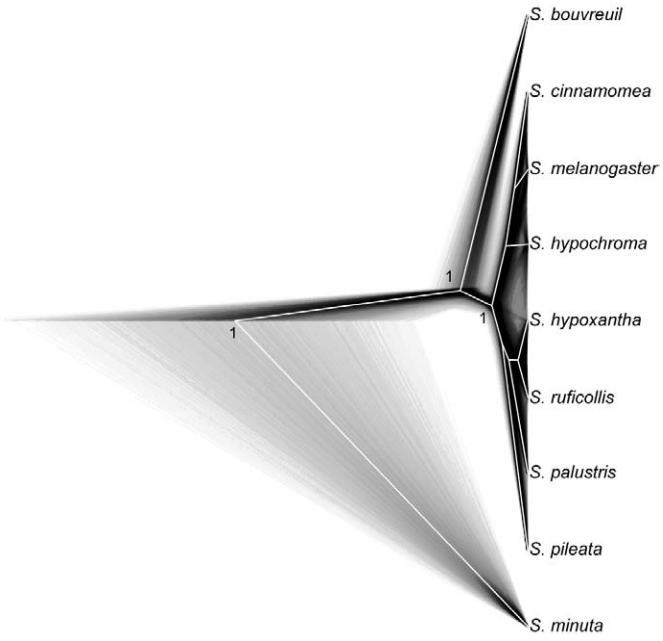

Fig. 3. Species tree for *Sporophila* inferred from mitochondrial (concatenated COI, cyt *b*, and CR) and nuclear (*Numt*2, *Numt*3, CHD1Z, MUSK, and Fib5) sequence data from 95 individuals belonging to nine species. The consensus tree (in white) was superimposed onto a cloudogram derived from 20,000 post-burn-in trees. Each tree is represented in gray, with darker shades implying greater degree of overlap. Node support within the southern capuchino radiation was low (0.4–0.7) and, thus, was omitted for simplicity (see Fig. S3 in the online supplementary material for details).

and *S. melanogaster*; Fig. 4A and Table 1). Only comparisons between southern capuchinos and *S. bouvreuil* were statistically significant. Similar results can be observed in the biplot from a PCoA of inter-individual D_{SW} distances (Fig. 4B). We found no significant differentiation among southern capuchino species (AMOVA: −0.25%, $P = 0.67$), and only a small fraction of the total variation was attributable to sampling locality (AMOVA: 1.16%, $P = 0.006$). For the STRUCTURE analysis using microsatellites, the most likely scenario was $K = 2$ with *S. bouvreuil* assigned to a different cluster than all southern capuchinos (online supplementary material Fig. S4). However, this result was obtained only when species information was used as prior, which suggests that the overall signal in these data is weak ($F_{ST} = 0.04$ between *S. bouvreuil* and all southern capuchinos pooled; Table 1).

Discussion

Our analyses of mitochondrial and nuclear DNA sequence data together with evidence derived from DNA microsatellites support the recent taxonomic change that elevated *S. bouvreuil* and *S. pileata* to species status (Remsen et al. 2013) based on evidence from adult male plumage and geographic distribution of these taxa (Machado and Silveira 2010, 2011). These species can be distinguished using neutral markers and there are, in fact, other southern capuchino species that are closer phylogenetically to *S. pileata* (e.g., *S. palustris*) than is *S. bouvreuil*, despite the similarity in plumage between *S. palustris* and *S. bouvreuil* (see Fig. 1). Our analyses also show that *S. bouvreuil*, and not *S. minuta* as was previously thought (Campagna et al. 2010, 2012), is the sister species to the southern capuchino radiation. The aforementioned studies included only one *S. bouvreuil*, precluding tests for species-level monophyly. Two of the 19 *S. bouvreuil* in the present study have mitochondrial COI, cyt *b*, and CR sequences that place them within the southern capuchino clade and not with the remaining *S. bouvreuil*. These two individuals are adult males that have typical *S. bouvreuil* reproductive plumage and were assigned to the *S. bouvreuil* genetic cluster using DNA microsatellite data. Nuclear DNA sequences alone did not provide sufficient resolution to distinguish among southern capuchinos and *S. bouvreuil*, possibly as a consequence of lower substitution rates, larger effective population size, and incomplete lineage sorting. However, when data from the same individuals were incorporated into the species tree estimation using *BEAST that explicitly models the effects of incomplete lineage sorting and ancestral polymorphism (Heled and Drummond 2010), both the southern capuchino clade and the clade involving these species and *S. bouvreuil* received posterior probability support of 1 (a similar result was obtained from the BP&P analysis). This implies that incomplete lineage sorting could be the reason that a small proportion of *S. bouvreuil* share mitochondrial haplotypes with the southern capuchinos, a pattern that could also have been generated through hybridization. The methodology used to estimate the species tree assumes that admixture (i.e., hybridization and introgression) does not occur among individuals of the different species (Heled and Drummond 2010). Thus, regardless of the results from the species tree analysis, we cannot rule out the possibility of hybridization and introgression between a female of a species belonging to the southern capuchino clade and male *S. bouvreuil*, resulting in mitochondrial introgression from the former into the latter.

Identifying *S. bouvreuil* as the sister species to the southern capuchino radiation allows us to reevaluate the timing and geographic context of the radiation. Using data from cyt *b* and *S. minuta* as the closest species to the southern capuchinos, Campagna et al. (2012) used TMRCA to estimate that the radiation began in the Pleistocene. Here, with nearly double the number of sequences and an improved phylogeny (with *S. bouvreuil* as the sister species to the southern capuchinos), we obtained a similar TMRCA estimate, perhaps as a consequence of the short internode distance between *S. minuta* and *S. bouvreuil*. The 95% high posterior density estimate of the age of the common ancestor between *S. bouvreuil* and the southern capuchinos encompasses the lower to middle Pleistocene. Thus, it is possible that Pleistocene climatic changes and concomitant fluctuations in the distribution of rainforest over open areas (Clapperton 1993, Servant et al. 1993, Ledru et al. 2005) contributed to isolating populations and promoting the southern capuchino radiation. Having excluded *S. bouvreuil* from the core radiation, we can delimit the northern boundaries of the range within which the southern capuchino radiation likely occurred (see Fig. 1). Our data suggest that the ancestor of the southern capuchinos came from northern South America and radiated rapidly into at least eight species within the grasslands of northeastern Argentina, eastern Paraguay, and southern Brazil (the area that shows the highest species concentration; for individual species maps, see Ridgely and Tudor 1989).

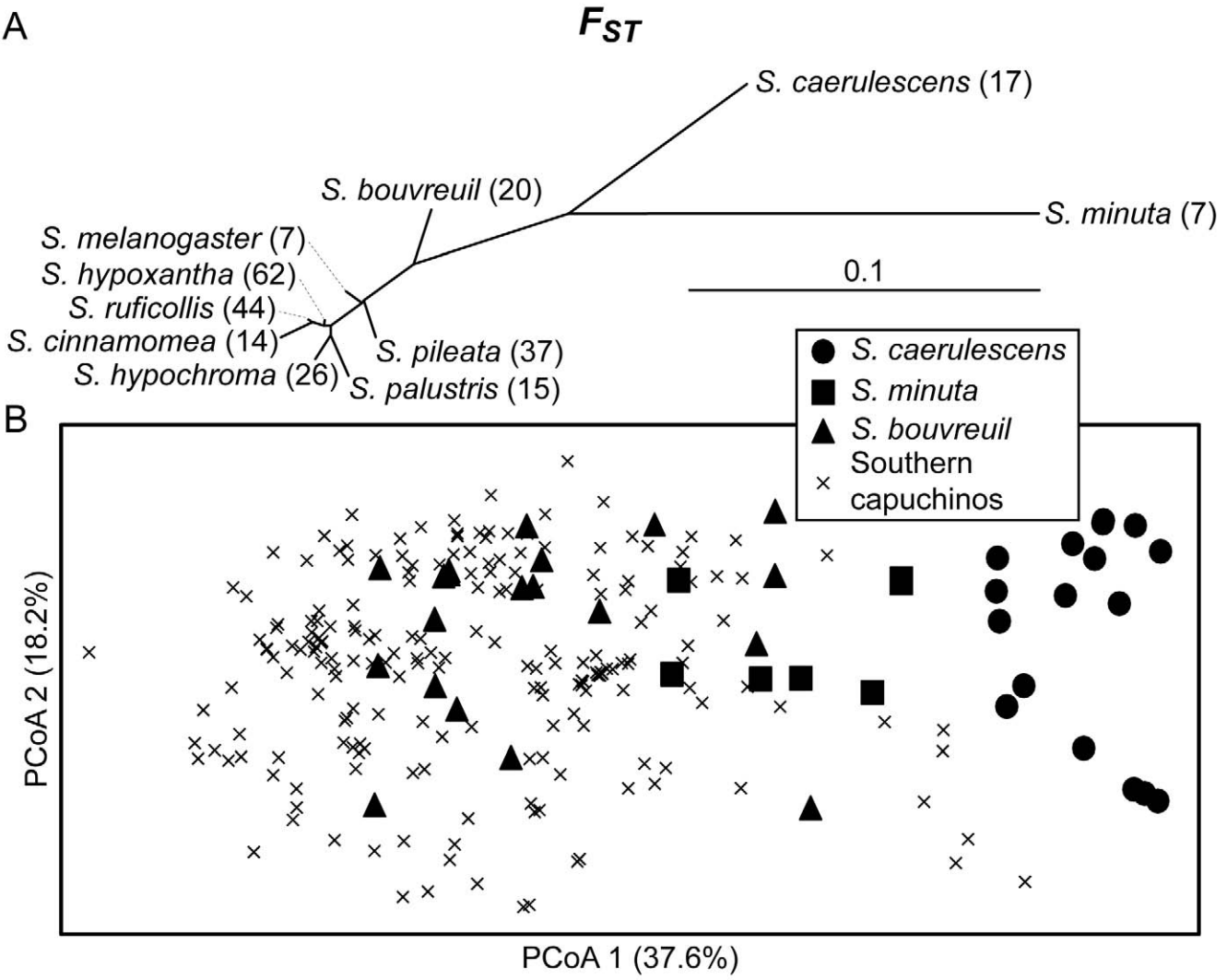

Fig. 4. (A) Neighbor-joining tree for *Sporophila* derived from F_{ST} calculations based on allele frequencies at six DNA microsatellite loci. (B) PCoA displaying an inter-individual pairwise D_{SW} distance matrix (percentage of variation explained by each axis in parenthesis).

Although the diversification of the southern capuchinos is relatively recent compared with other avian radiations (e.g., for warblers in genus *Dendroica*, see Lovette and Bermingham 1999; or for Hawaiian honeycreepers in tribe Drepanidini, see Lerner et al. 2011), the time elapsed since the lower to middle Pleistocene has been sufficient to show striking phylogeographic structure in other Neotropical avian taxa (e.g., for *Zonotrichia capensis*, see Lougheed et al. 2013). In the present study, we did not find species-level monophyly or clear affinities among the eight southern capuchino species, consistent with results from previous studies (Lijtmaer et al. 2004; Campagna et al. 2010, 2012), even in those analyses that account for the effect of ancestral polymorphism and incomplete lineage sorting. This does not mean that southern capuchinos should not be considered good biological species, and various studies have identified differences in male and female plumage and song that are maintained in sympatry (Benites et al. 2010, Areta 2012, Campagna et al. 2012). We interpret this genetic pattern as the consequence of a rapid and ongoing radiation. Using Bayesian simulations that implement the isolation with migration model (IMa2; Hey 2010), Campagna et al. (2012) obtained results consistent with gene flow among southern capuchino species. Thus, ongoing hybridization and introgression since these species split from a common ancestor could preclude us from reconstructing their phylogenetic affinities (both *BEAST and BP&P assume lack of admixture). It is also possible that insufficient time has elapsed for stochastic sorting of neutral genetic markers to have occurred (McKay and Zink 2010); incomplete lineage sorting would be accentuated if the speciation events that led to the eight southern capuchino species occurred rapidly over a short time span.

A similar challenge in reconstructing phylogenetic affinities has been documented for Darwin's ground finches (Freeland and Boag 1999, Sato et al. 1999). In contrast to Darwin's finches (*Geospiza* spp.), southern capuchino seedeaters exhibit similar morphology, including bill dimensions, differing mainly in male plumage and song (Campagna et al. 2012). These differences in key aspects of the avian mate-recognition system (Price 2007) suggest

sexual selection as a driver of speciation in the group (Campagna et al. 2012), and future work should focus on the possible role of these phenotypic differences as mechanisms of reproductive isolation. We expect that identification of genes that underpin phenotypic differences among southern capuchinos will help us understand the relationship among species and provide insight into the mechanisms that promoted speciation in the group. It is worth noting that the same patches and colors, combined into different patterns, are implicated in male plumage differences among southern capuchinos (for representative illustrations, see Ridgely and Tudor 1989; for alternative color morphs within some species, see Areta 2008, Repenning et al. 2010, Areta and Repenning 2011, Areta et al. 2011). Certainly, plumage attributes seem to be evolutionary malleable in the group, possibly meaning that a limited suite of genes and mutations underlie these differences in male coloration. Our future work will focus on employing next-generation sequencing to recover large numbers of loci and search for those loci that show patterns consistent with selection.

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Literature Cited

Areta, J. I. 2008. The Entre Ríos Seedeater (*Sporophila zelichi*): A species that never was. Journal of Field Ornithology 79:352–363.

Areta, J. I. 2012. Winter songs reveal geographic origin of three migratory seedeaters (*Sporophila* spp.) in southern Neotropical grasslands. Wilson Journal of Ornithology 124:688–697.

Areta, J. I., J. I. Noriega, L. Pagano, and I. Roesler. 2011. Unravelling the ecological radiation of the capuchinos: Systematics of Dark-throated Seedeater *Sporophila ruficollis* and description of a new dark-collared form. Bulletin of the British Ornithologists' Club 131:4–23.

Areta, J. I., and M. Repenning. 2011. Systematics of the Tawny-bellied Seedeater (*Sporophila hypoxantha*). II. Taxonomy and evolutionary implications of the existence of a new tawny morph. Condor 113:678–690.

Benites, P., M. D. Eaton, D. A. Lijtmaer, S. C. Lougheed, and P. L. Tubaro. 2010. Analysis from avian visual perspective reveals plumage colour differences among females of capuchino seedeaters (*Sporophila*). Journal of Avian Biology 41:597–602.

Bouckaert, R. R. 2010. DensiTree: Making sense of sets of phylogenetic trees. Bioinformatics 26:1372–1373.

Campagna, L., P. Benites, S. C. Lougheed, D. A. Lijtmaer, A. S. DiGiacomo, M. D. Eaton, and P. L. Tubaro. 2012. Rapid phenotypic evolution during incipient speciation in a continental avian radiation. Proceedings of the Royal Society of London, Series B 279:1847–1856.

Campagna, L., D. A. Lijtmaer, K. C. Kerr, A. S. Barreira, P. D. Hebert, S. C. Lougheed, and P. L. Tubaro. 2010. DNA barcodes provide new evidence of a recent radiation in the genus *Sporophila* (Aves: Passeriformes). Molecular Ecology Resources 10:449–458.

Clapperton, C. M. 1993. Nature of environmental changes in South America at the last glacial maximum. Palaeogeography, Palaeoclimatology, Palaeoecology 101:189–208.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: More models, new heuristics and parallel computing. Nature Methods 9:772.

Double, M. C., D. Dawson, T. Burke, and A. Cockburn. 1997. Finding the fathers in the least faithful bird: A microsatellite-based genotyping system for the Superb Fairy-Wren *Malurus cyaneus*. Molecular Ecology 6:691–693.

Drummond, A. J., M. A. Suchard, D. Xie, and A. Rambaut. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular Biology and Evolution 29:1969–1973.

Earl, D. A., and B. M. vonHoldt. 2012. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359–361.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Molecular Ecology 14:2611–2620.

Excoffier, L., and H. E. L. Lischer. 2010. ARLEQUIN suite, version 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564–567.

Felsenstein, J. 2005. PHYLIP (Phylogeny Inference Package), version 3.6.9. Distributed by the author, Department of Genome Sciences, University of Washington, Seattle.

Freeland, J. R., and P. T. Boag. 1999. The mitochondrial and nuclear genetic homogeneity of the phenotypically diverse Darwin's ground finches. Evolution 53:1553–1563.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95–98.

Hanotte, O., C. Zanon, A. Pugh, C. Greig, A. Dixon, and T. Burke. 1994. Isolation and characterization of microsatellite

loci in a passerine bird: The Reed Bunting *Emberiza schoeniclus*. Molecular Ecology 3:529–530.

Hasegawa, M., H. Kishino, and T. A. Yano. 1985. Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22:160–174.

Heled, J., and A. J. Drummond. 2010. Bayesian inference of species trees from multilocus data. Molecular Biology and Evolution 27:570–580.

Hey, J. 2010. Isolation with migration models for more than two populations. Molecular Biology and Evolution 27:905–920.

Langella, O. 1999. Populations 1.2.30: A population genetic software. CNRS UPR9034. [Online.] Available at bioinformatics.org/~tryphon/populations/.

Ledru, M.-P., D.-D. Rousseau, F. W. Cruz, Jr., C. Riccomini, I. Karmann, and L. Martin. 2005. Paleoclimate changes during the last 100,000 yr from a record in the Brazilian Atlantic rainforest region and interhemispheric comparison. Quaternary Research 64:444–450.

Lerner, H. R. L., M. Meyer, H. F. James, M. Hofreiter, and R. C. Fleischer. 2011. Multilocus resolution of phylogeny and timescale in the extant adaptive radiation of Hawaiian honeycreepers. Current Biology 21:1838–1844.

Lijtmaer, D. A., N. M. Sharpe, P. L. Tubaro, and S. C. Lougheed. 2004. Molecular phylogenetics and diversification of the genus *Sporophila* (Aves: Passeriformes). Molecular Phylogenetics and Evolution 33:562–579.

Lougheed, S. C., L. Campagna, J. A. Dávila, P. L. Tubaro, D. A. Lijtmaer, and P. Handford. 2013. Continental phylogeography of an ecologically and morphologically diverse Neotropical songbird, *Zonotrichia capensis*. BMC Evolutionary Biology 13:58.

Lovette, I. J., and E. Bermingham. 1999. Explosive speciation in the New World *Dendroica* warblers. Proceedings of the Royal Society of London, Series B 266:1629–1636.

Machado, E., and L. F. Silveira. 2010. Geographical and seasonal distributions of the seedeaters *Sporophila bouvreuil* and *Sporophila pileata* (Aves: Emberizidae). Papeis Avulsos de Zoología 50:517–533.

Machado, E., and L. F. Silveira. 2011. Plumage variability and taxonomy of the Capped Seedeater *Sporophila bouvreuil* (Aves: Passeriformes: Emberizidae). Zootaxa 2781:49–62.

McKay, B. D., and R. M. Zink. 2010. The causes of mitochondrial DNA gene tree paraphyly in birds. Molecular Phylogenetics and Evolution 54:647–650.

Meyer de Schauensee, R. M. 1952. A review of the genus *Sporophila*. Proceedings of the Academy of Natural Sciences of Philadelphia 104:153–196.

Neumann, K., and J. H. Wetton. 1996. Highly polymorphic microsatellites in the House Sparrow *Passer domesticus*. Molecular Ecology 5:307–309.

Ouellet, H. 1992. Speciation, zoogeography and taxonomic problems in the Neotropical genus *Sporophila* (Aves: Emberizinae). Bulletin of the British Ornithologists' Club 112:125–135.

Peakall, R., and P. E. Smouse. 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6:288–295.

Petren, K. 1998. Microsatellite primers from *Geospiza fortis* and cross-species amplification in Darwin's finches. Molecular Ecology 7:1782–1784.

Price, T. 2007. Speciation in Birds. Roberts, Englewood, Colorado.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Rambaut, A., and A. J. Drummond. 2009. TRACER, version 1.5. [Online.] Available at beast.bio.ed.ac.uk/Tracer.

Rannala, B., and Z. Yang. 2003. Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. Genetics 164:1645–1656.

Remsen, J. V., Jr., C. D. Cadena, A. Jaramillo, M. Nores, J. F. Pacheco, J. Pérez-Emán, M. B. Robbins, F. G. Stiles, D. F. Stotz, and K. J. Zimmer. 2013. A classification of the bird species of South America. American Ornithologists' Union. [Online.] Available at www.museum.lsu.edu/~Remsen/SACCBaseline.html.

Repenning, M., C. Rovedder, and C. S. Fontana. 2010. Another color morph of *Sporophila* Seedeater from *capuchinos* group (Aves, Emberizidae). Iheringia, Série Zoologia 100:369–378.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223–225.

Ridgely, R. S., and G. Tudor. 1989. The Birds of South America. University of Texas Press, Austin.

Rising, J. D., A. Jaramillo, J. L. Copete, P. G. Ryan, and S. C. Madge. 2011. Family Emberizidae (Buntings and New World Sparrows). Pages 428–683 *in* Handbook of the Birds of the World, vol. 16: Tanagers to New World Blackbirds (J. del Hoyo, A. Elliott, and D. A. Christie, Eds.). Lynx Edicions, Barcelona, Spain.

Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61:539–542.

Sato, A., C. O'hUigin, F. Figueroa, P. R. Grant, B. R. Grant, H. Tichy, and J. Klein. 1999. Phylogeny of Darwin's finches as revealed by mtDNA sequences. Proceedings of the National Academy of Sciences USA 96:5101–5106.

Sato, A., H. Tichy, C. O'hUigin, P. R. Grant, B. R. Grant, and J. Klein. 2001. On the origin of Darwin's finches. Molecular Biology and Evolution 18:299–311.

Servant, M., J. Maley, B. Turcq, M.-L. Absy, P. Brenac, M. Fournier, and M.-P. Ledru. 1993. Tropical forest changes during the Late Quaternary in African and South American lowlands. Global and Planetary Change 7:25–40.

Shriver, M. D., L. Jin, E. Boerwinkle, R. Deka, R. E. Ferrell, and R. Chakraborty. 1995. A novel measure of genetic distance for highly polymorphic tandem repeat loci. Molecular Biology and Evolution 12:914–920.

Somenzari, M., L. F. Silveira, V. Q. Piacentini, M. A. Rego, F. Schunck, and V. Cavarzere. 2011. Birds of an Amazonia-Cerrado ecotone in southern Pará, Brazil, and the efficiency of associating multiple methods in avifaunal inventories. Revista Brasileira de Ornitologia 19:260–275.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28:2731–2739.

Tavaré, S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Pages 57–86 in Some Mathematical Questions in Biology: DNA Sequence Analysis (R. M. Miura, Ed.). American Mathematical Society, Providence, Rhode Island.

Wahlund, S. 1928. Zusammensetzung von Populationen und Korrelationserscheinungen vom Standpunkt der Vererbungslehre aus betrachtet. Hereditas 11:65–106.

Weir, J. T., and D. Schluter. 2008. Calibrating the avian molecular clock. Molecular Ecology 17:2321–2328.

Wilgenbusch, J. C., D. L. Warren, and D. L. Swofford. 2004. AWTY: A system for graphical exploration of MCMC convergence in Bayesian phylogenetic inference. [Online.] Available at ceb.csit.fsu.edu/awty.

Yang, Z., and B. Rannala. 2010. Bayesian species delimitation using multilocus sequence data. Proceedings of the National Academy of Sciences USA 107:9264–9269.

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