

Unmasking hidden genetic, vocal, and size variation in the Masked Flowerpiercer along the Andes supports two species separated by Northern Peruvian Low

Silvia C. Martínez-Gómez,^{1,a} Carlos Esteban Lara,² J. V. Remsen, Jr.,³ Robb T. Brumfield,³ and Andrés M. Cuervo^{1,3,*}

¹Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, Colombia

²Dirección Académica, Universidad Nacional de Colombia Sede de La Paz, La Paz, Colombia

³Museum of Natural Science, Louisiana State University, Baton Rouge, Louisiana, USA

^aCurrent address: Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” — CONICET, Buenos Aires, Argentina

*Corresponding author: amcuervom@unal.edu.co

ABSTRACT

Genetic divergence among isolated populations is not always reflected in phenotypic differentiation. We investigated the genetic and phenotypic differentiation in *Diglossa cyanea* (Thraupidae; Masked Flowerpiercer), a widely distributed species in the tropical Andes. We found strong evidence for 2 main lineages separated by the Marañón River valley in the Northern Peruvian Low (NPL). These 2 lineages show a deep sequence divergence in mitochondrial DNA (mtDNA; ~6.7% uncorrected *p*-distance, *n* = 122), spectral frequency and song structure (with exclusive final whistles in southern populations, *n* = 88), and wing length (the northern populations are smaller, *n* = 364). The 2 divergent *D. cyanea* mitochondrial lineages were not sister to each other, suggesting a possible paraphyly with respect to *D. caerulescens* (Bluish Flowerpiercer) that remains to be tested with nuclear genomic data. No genetic variation, size difference, or song structure was observed within the extensive range of the southern group (from the NPL to central Bolivia) or within all sampled northern populations (from the NPL to Venezuela). These vocal differences appear to have consequences for song discrimination, and species recognition, according to a previously published playback experiment study. We propose that the southern taxon be elevated to species rank as *D. melanopsis*, a monotypic species (with the proposed name Whistling Masked-Flowerpiercer). In turn, we provide a redefinition of *D. cyanea* (Warbling Masked-Flowerpiercer), which is now restricted to the northern half of the tropical Andes as a polytypic species with 3 subspecies (*tovarensis*, *obscura*, and *cyanea*). Based on our results, the subspecies *dispar* should be treated as a junior synonym of *cyanea*. Our study highlights the need to continue amassing complementary data sets from field observations, experiments, and collection-based assessments to better characterize the evolutionary history, biogeography, bioacoustics, and taxonomy of Neotropical montane birds.

Keywords: Andes, biogeography, biological collections, *Diglossopsis*, speciation, species delimitation, vocalizations, wing length

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LAY SUMMARY

- We analyzed variation in mitochondrial DNA, songs, and body size among populations of a bird species of the Andean mountains (Masked Flowerpiercer) that shows relatively little plumage variation across its 3,800-km-long distribution from northern Venezuela to central Bolivia.
- We found that the Masked Flowerpiercer consists of two divergent populations separated by a deep geographic depression known as the Northern Peruvian Low (NPL).
- The degree of mitochondrial sequence divergence between them is as great as between either of them and another species, the Bluish Flowerpiercer.
- The two populations also have distinct songs, with the song of the southern group (*melanopsis*) ending with clear whistles that are completely absent in the northern group (*cyanea*). The southern group also tend to have longer wings than do populations north of the NPL.
- The southern population should be treated as a separate species, *Diglossa melanopsis* (Whistling Masked Flowerpiercer), whereas the northern group *D. cyanea*, with three recognized subspecies (*obscura*, *tovarensis*, *cyanea*).

Desenmascarar la variación genética, vocal y de tamaño del pinchaflores enmascarado a lo largo de los Andes demuestra dos especies separadas por la Depresión del Norte del Perú

RESUMEN

La divergencia genética entre poblaciones aisladas no siempre se refleja en diferencias fenotípicas. Investigamos la diferenciación genética y fenotípica en *Diglossa cyanea* (Thraupidae), una especie común de los Andes tropicales. Encontramos dos linajes principales separados por el cañón del río Marañón en la Depresión del Norte del Perú (DNP). Estos dos linajes tienen una profunda divergencia en ADN mitocondrial (~6.7% de distancia p no corregida, $n = 122$), en el rango de frecuencia y estructura del canto (con silbidos finales exclusivos en las poblaciones del sur, $n = 88$), y en la longitud del ala (las poblaciones del norte son más pequeñas, $n = 364$). Los dos linajes mitocondriales divergentes en *D. cyanea* no resultaron grupos hermanos entre sí, sugiriendo una posible parafilia con respecto a *D. caerulescens*, que debe ser puesta a prueba con datos genómicos nucleares. No encontramos variación genética, diferencias de tamaño o estructura del canto entre las poblaciones del grupo del sur (desde la DNP hasta Bolivia), como tampoco entre las poblaciones del norte muestreadas (desde la DNP hasta Venezuela). Estas diferencias vocales parecen tener consecuencias en la discriminación de cantos y el reconocimiento entre especies de acuerdo con un estudio basado en experimentos de playback previamente publicado. Proponemos que la subespecie del sur sea elevada al rango de especie como *D. melanopsis*, una especie monotípica. A su vez, redefinimos a *D. cyanea*, que ahora se restringe a la mitad norte de los Andes tropicales como una especie polítípica con tres subespecies (*tovarensis*, *obscura* y *cyanea*). De acuerdo con los resultados, la subespecie *dispar* debe tratarse como un sinónimo de *cyanea*. Nuestro estudio demuestra la necesidad de seguir acumulando conjuntos complementarios de datos a partir de observaciones de campo, experimentos y evaluaciones basadas en colecciones para caracterizar mejor la historia evolutiva, la biogeografía, la bioacústica y la taxonomía de las aves montañas neotropicales.

Palabras clave: Andes, biogeografía, delimitación de especies, *Diglossopsis*, especiación, longitud del ala, vocalizaciones

INTRODUCTION

The degree of phenotypic divergence does not always parallel that of genetic divergence between separate populations. This decoupling between phenotypic trait differentiation and population genetic divergence may impede the characterization of biodiversity (Bickford *et al.* 2007, Freeman and Pennell 2021). Allopatric divergence driven by landscape changes or dispersal events is the most pervasive speciation mechanism underlying avian diversity (Graves 1985, Coyne and Orr 2004, Price 2008, Fjeldså *et al.* 2012). However, the extent of morphological, behavioral, and genetic divergence among daughter species may not be equally evident in all cases. Given sufficient time, the differentiation of geographically isolated populations accrues (Mayr 1942, Smith *et al.* 2014) at a pace that can be influenced by population size and natural selection (Nosil 2008, Winger and Bates 2015).

The speciation process in birds depends strongly on the interactions between topographic complexity, ecology, and dispersal ability, and the time required for divergence to act on morphological, behavioral, and genetic traits (Price 2008, Smith *et al.* 2014, Benham *et al.* 2015). More specifically, the humid montane habitats of Andean birds have generated distributions that are fragmented by barriers of various sizes that could prevent gene flow, setting the stage for population divergence and, ultimately, speciation (Remsen 1984, Graves 1988, 1991, Weir 2009, Fjeldså *et al.* 2012). Studies on several widespread avian species complexes of the Andes that incorporate vocal data have demonstrated that species-level diversity is highly underestimated (Krabbe and Schulenberg 1997, Cadena and Cuervo 2010, Isler *et al.* 2020, Musher *et al.* 2023). This is because the tempo and magnitude of divergence in characters associated with reduction of gene flow are often decoupled in the speciation process (Mayr 1963, Winger and Bates 2015). For instance, marked phenotypic differences exist between recently diverged, almost genetically indistinguishable populations of *Coeligena* hummingbirds (Palacios *et al.* 2019). On the other hand, phenotypically indistinguishable populations with deep genetic divergences are found in several Andean bird complexes, including ducks (Gutiérrez-Pinto *et al.* 2019), hummingbirds (Chaves *et al.* 2011, Benham *et al.* 2015), suboscines (Valderrama *et al.* 2014, Cadena *et al.* 2020, Chesser *et al.* 2020), and oscines (Gutiérrez-Pinto *et al.* 2012, Prieto-Torres *et al.* 2018, Cadena *et al.* 2019). That is, true diversity may be frequently overlooked because characterizations based on genetic or phenotypic traits alone are often insufficient.

Tanagers in the flowerpiercer clade *Diglossa* (Thraupidae) are ecologically and phenotypically specialized members of Neotropical montane bird communities (Moynihan 1968, Vuilleumier 1969), with a peak of diversity in the Andes. With 18 recognized species (Bock 1985, Isler and Isler 1999, Dickinson and Christidis 2014), *Diglossa* is one of the genera encompassing Andean birds that has revealed some of the most extraordinary and intriguing leapfrog patterns of geographic variation in birds (Moynihan 1979, Graves 1982, Vuilleumier 1984, Mauck and Burns 2009). Molecular data have shown a rapid diversification in the core *Diglossa* group (Hackett 1995, Mauck and Burns 2009), with a much more convoluted history than previously thought (see Gutiérrez-Zuluaga *et al.* 2021). The most morphologically similar taxa may not be sister lineages, implying a complex genetic basis of divergence yet to be revealed (Hiller *et al.* 2021). In contrast to the core *Diglossa* group, the 3 species in the *Diglossopsis* clade (*D. caerulescens*, *D. cyanea*, and *D. glauca*; Mauck and Burns 2009) show deeper divergences but only modest phenotypic differences. This group consists of similar-looking flowerpiercers with bluish plumages, facial masks, bright yellow to red irides, and reduced bill hooks; they are largely sympatric along the tropical Andes and are found across major topographic discontinuities such as the Marañón valley.

The Northern Peruvian Low (NPL) is the preeminent Andean dispersal barrier, largely defined by low summits and deep dry canyons (Vuilleumier 1968, 1984), especially the Porculla Pass, the Huancabamba Depression, and the Marañón river valley in northern Peru (Duellman 1979, Parker *et al.* 1985, Cuervo 2013). The NPL is well known to be a barrier that shapes geographic range limits (Vuilleumier 1968, Cracraft 1985) and gene flow of Andean birds (Cuervo 2013, Winger 2017), especially for those adapted to humid and forested ecosystems, as in most *Diglossa*. However, the effect of this geographic break on dispersal is not distributed equally across avian taxa (Vuilleumier 1984, Parker *et al.* 1985, Weir 2009, Cuervo 2013, Winger and Bates 2015, Winger 2017), including *Diglossa*.

The Masked Flowerpiercer is abundant and widespread in the tropical Andes where it occurs in montane and stunted forests, semi-open areas with isolated trees and scrubs, and forest borders. Although spanning a wide latitudinal range from northern Venezuela to central Bolivia (~4,500 km), its elevational range is restricted to 1,800–3,600 m.a.s.l. (Fjeldså and Krabbe 1990, Parker *et al.* 1996), which often amounts to only a few kilometers in linear distance.

Five subspecies are currently recognized (Dickinson and Christidis 2014), from south to north: *melanopis*, *dispar*, *cyanea*, *obscura*, and *tovarensis*. These taxa were defined on the basis of subtle differences in size, but especially the tone of the blue coloration in plumage (Hellmayr 1935, Zimmer 1942, Meyer de Schauensee 1951, Zimmer and Phelps 1952, Isler and Isler 1999). Range boundaries between subspecies are clearly delimited by geographic features, except for *D. c. cyanea* and *D. c. dispar*, whose distribution limits are unknown but are thought to replace each other in extreme southern Ecuador (FjeldsÅ and Krabbe 1990, Freile and Restall 2018) (Figure 1A). Although only qualitative characterization exists of the intraspecific phenotypic differences for northern *D. cyanea* populations, implying that they may not be phenotypically diagnosable units, the small differences between northern *D. cyanea* subspecies that have been described include subtleties in coloration and facial masks (Zimmer and Phelps 1952), and vocal peculiarities in *D. c. tovarensis*, albeit with small sample

sizes (FjeldsÅ and Krabbe 1990, Hilty 2003). The subspecies *D. c. dispar* is described to be similar in size and color to *D. c. cyanea* and *D. tovarensis*, but with a slight greenish tinge (Zimmer 1942, Zimmer and Phelps 1952). These differences between *D. cyanea* subspecies are slight and were described on the basis of a handful of specimens for comparisons (Vuilleumier 1969), hence, whether these recognized taxa are diagnosable units need to be addressed (Patten 2010, Remsen 2010). In contrast, the subspecies *D. c. melanopis* from south of the NPL in Peru south to Bolivia (Schulenberg *et al.* 2010, Herzog *et al.* 2017) is more clearly distinct. This southern subspecies is larger and duller, with a paler forecrown, less prominent white tips on the undertail coverts and less marked blue margins on tertials compared to the other 4 subspecies from north of the NPL (Hellmayr 1935, Zimmer 1942, Isler and Isler 1999).

Here, we conducted a geographically comprehensive analysis of genetic variation integrated with vocal and morphological data to assess (1) how the NPL bisects populations of

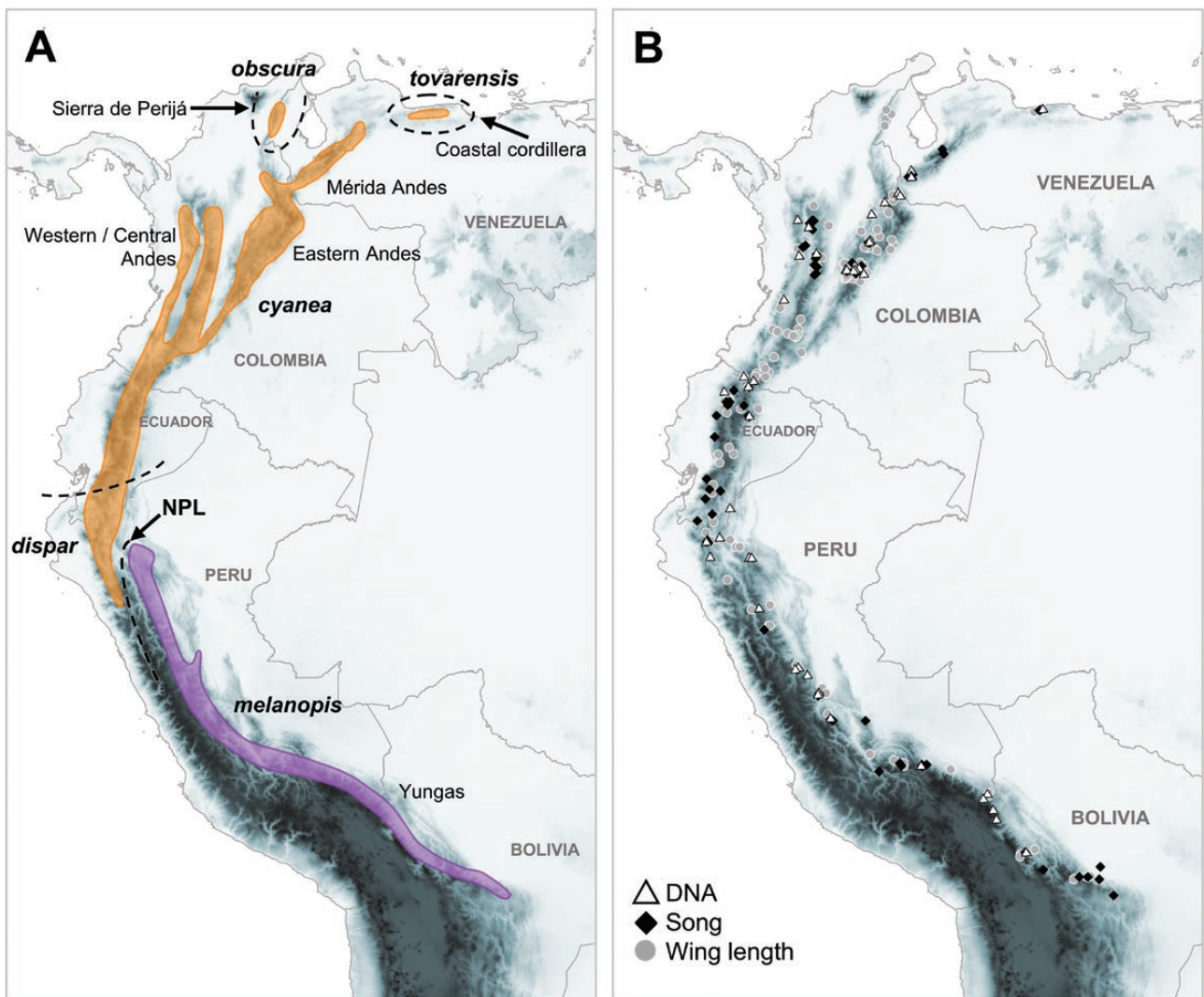


Figure 1. (A) Geographic distribution of the Masked Flowerpiercer (*Diglossa cyanea*). Dashed black lines indicate approximate range boundaries between the 5 subspecies currently recognized. Note that except for the northern limit of *D. c. dispar*, with uncertain precise location, subspecies boundaries coincide with low elevation gaps. The 2 colors denote the northern (orange) and southern (purple) groups separated by the NPL. (B) Symbols represent sample localities for vouchered specimens of *D. cyanea* used in the phylogeographic analysis (white triangles), wing length from study skins (gray dots), and song data (black diamonds). We were unable to obtain vocal and genetic samples for *D. c. obscura*.

D. cyanea and (2) whether genetic structure, vocal structure, and body size correspond to the current subspecies taxonomy. First, we explored the phylogeographic patterns of *D. cyanea* to evaluate the extent of its genetic structure in a geographic context. We discovered that 1 of the 2 major clades of *D. cyanea* seems closer to *D. caerulescens*; thus, we expanded the geographic sampling of molecular data in that species. Second, we compared song structure between the 2 *D. cyanea* groups revealed by the genetic analysis. Third, we quantified differences in wing length, as an index of body size, between the *D. cyanea* groups.

METHODS

Phylogeographic Structure

Mauck and Burns (2009) found that *D. cyanea* (Masked Flowerpiercer) and *D. caerulescens* (Bluish Flowerpiercer) are sister species; this would be one of the few cases in Andean birds of sister pairs that overlap for most of their geographic distributions. However, their analyses included only a single sample per species. Therefore, we expanded the geographic sampling to encompass the entire range of *D. cyanea* and, to a lesser extent, of *D. caerulescens*. To do this, we sequenced the mitochondrial gene NADH dehydrogenase subunit 2 (ND2, 1,041 base pairs [bp]) for 122 individuals of *D. cyanea* and 33 of *D. caerulescens*. We included the samples used by Mauck and Burns (2009) for both species. We included 3 outgroups: *D. glauca*, the other species in the *Diglossopsis* clade, and *D. albilatera* and *D. indigotica*, 2 species of the core *Diglossa* clade (see Supplementary Material Table S1 for details).

DNA extraction, amplification, and sequencing protocols followed Cuervo *et al.* (2014). Raw sequence data were inspected for ambiguities and stop codons using Sequencher 4.7 (GeneCodes Corp., Ann Arbor, MI) and were aligned using Geneious 9.1.8. We estimated ND2 gene trees using RAxML 8.2.12 (Stamatakis 2014) and MrBayes 3.2.7a (Ronquist *et al.* 2012) via the CIPRES Science Gateway 3.3 portal (Miller *et al.* 2010). For RAxML, we implemented the GTRCAT approximation for rate heterogeneity with 25 distinct categories, and automatic rapid bootstrapping search to assess nodal support after 650 replicates with the *autoMRE* option. For MrBayes, we implemented a partition scheme with a model of substitution for each codon position (first: HKY + Γ , second: HKY + I, third: GTR + I + Γ) as suggested by the Akaike information criterion with correction in PartitionFinder 2 (Lanfear *et al.* 2017). We ran 4 Markov chain Monte Carlo (MCMC) chains for 20 million generations, sampling every 1,000th, discarding the initial 50% as burn-in. We also used BEAST 2.6.3 (Bouckaert *et al.* 2019) to estimate divergence times while simultaneously estimating a tree topology (Heled and Drummond 2010). We applied a lognormal relaxed clock and used the default Yule Process as the tree prior. We used the average ND2 substitution rate (2.5% per million years) estimated for other tropical passerine birds (Smith and Klicka 2010). The alignment contained 158 taxa, 1,041 sites, and 256 variable sites. We ran 2 independent MCMC runs starting from random trees for 100 million generations, sampling every 5,000th and discarding the first 50% as burn-in in each run. Both posterior parameter values and tree files were combined with resampling in LogCombiner to obtain 10,002 post burn-in parameter estimates and trees, respectively. We used TreeAnnotator 2.6.3 (Bouckaert *et al.* 2019) to

calculate a maximum clade credibility tree with mean heights. We inspected convergence in the post burn-in MCMC parameter estimates for both Bayesian analyses in Tracer 1.7.1 (Rambaut *et al.* 2018). To further examine the genetic divergence between subspecies and major groups, we estimated genetic distances in MEGA (Kumar *et al.* 2018, Stecher *et al.* 2020) and built median-joining networks with $\epsilon = 0$ (Bandelt *et al.* 1999) for each major clade in PopART 1.7 (Leigh and Bryant 2015).

Vocal Variation

We examined audio recordings of *D. cyanea* archived in the Macaulay Library (ML; Cornell Lab of Ornithology, Ithaca, NY) and xeno-canto (XC; www.xeno-canto.org) (Figure 1, Supplementary Material Table S2). We retrieved a total of 88 recordings of *D. cyanea*, distributed by phylogeographic groups as 55 north of the NPL and 35 south of the NPL, and distributed by subspecies as follows: 35 of *D. c. melanopis*, 4 of *D. c. dispar*, 47 of *D. c. cyanea*, and 2 of *D. c. towarensis*. Recordings of *D. c. obscura* were unavailable. We excluded short or fragmented songs, or recordings with low signal-to-noise levels. Our sampling unit for the vocal analysis (see below) was at the individual level. We assumed that each audio recording from the same locality and date belonged to the same individual. We analyzed up to 10 song bouts (mean_[song bouts] = 4.32, SD = 2.81) of each recording to capture the intraindividual variation. We defined song bouts in recordings as clusters of vocal elements exceeding a duration of 0.5 s, and separation from other clusters was distinguished by silence intervals exceeding 1 s.

Songs of *D. cyanea* consist of a high-pitched, melodic warble of complex, accelerating tweet notes. To fully explore vocal variation, we analyzed the full song and up to 3 divided sections, as follows (also see Figure 2): (1) the full song is equivalent to the total length of the song (hereafter F); (2) we separately analyzed the first section of the song (hereafter S1), which corresponds to the introductory phrase, consisting of 2–5 short (<0.1 s) “tzi” notes, delivered at 0.1 to 0.4 s intervals; (3) the second section (hereafter S2) corresponds to the main phrase of the song: a complex, fast chatter of rich elements delivered at <0.08 s intervals; and (4) a third section of the song (hereafter S3), when present, is a series of 3–4 clear but strident whistling notes.

We measured 5 spectral and temporal traits in the full song (F) and each composing section (S1, S2, S3) by placing landmark boxes on spectrograms using Raven Pro 1.6 (Cornell Lab of Ornithology, Ithaca, NY), with custom visual settings (Hann type spectrograms; window size = 512 samples). These traits were as follows: duration, peak frequency, maximum frequency, minimum frequency, and bandwidth. Upon examination of all available vocal samples, we identified 2 clear song types segregated geographically, and a much less frequent alternative song. The latter consists of a single, main unit similar in structure to S2 of the typical song (Supplementary Material Figure S1).

To assess quantitative differences in songs between the southern and northern groups, and between the 4 sampled subspecies (*D. c. melanopis*, *D. c. dispar*, *D. c. cyanea*, and *D. c. towarensis*), we fitted multiple Bayesian linear mixed models (BLMMs) using MCMC techniques with the *MCMCglmm* package (Hadfield 2010) implemented in R 4.0.1 (R Core Team 2021). We used the Gaussian error distribution and

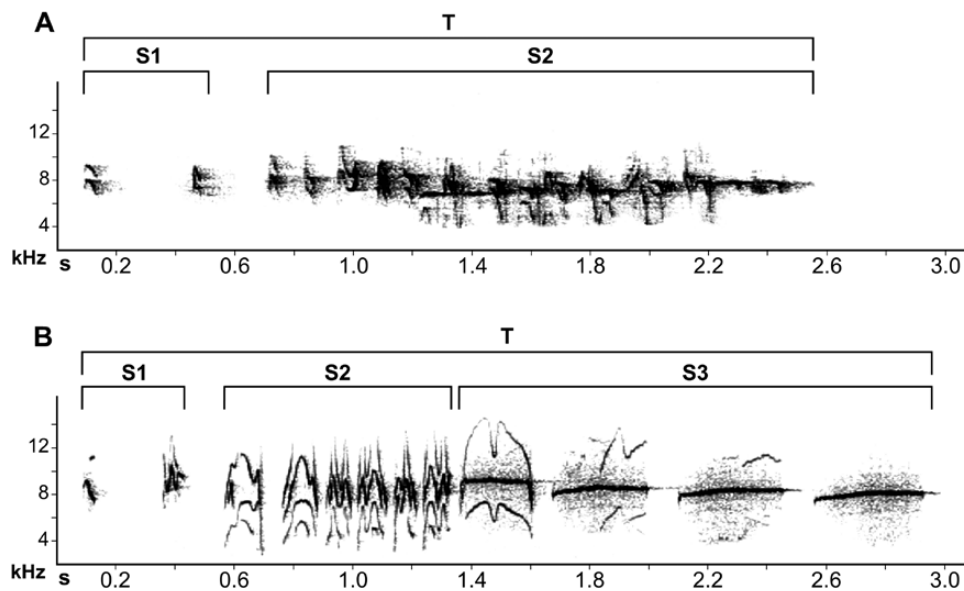


Figure 2. Spectrograms of representative samples of the typical song in the Masked Flowerpiercer (*Diglossa cyanea*). (A) An example from the northern group (*D. cyanea cyanea* from Caldas, Colombia, XC-373183), (B) an example from the southern group (*D. c. melanopis* from Cochabamba, Bolivia, ML-87666). Phrase sections (S1, S2, S3), and the full song (T) are indicated by brackets. The striking difference in the typical song between the northern (A) and southern (B) groups of *D. cyanea* is driven by the shorter warbling chatter (S2) and presence of the terminal phrase of strident whistle notes (S3) in the southern group songs.

used *MCMCglmm* default settings and priors. We report standardized estimates of regression coefficients as the mean 1,000 posterior distributions with 95% credible intervals (CIs) and considered effects to be statistically significant if CIs did not overlap with zero. Specifically, to examine vocal differences between the southern and northern groups, we fit 15 BLMMs as follows (also see Figure 1 and Supplementary Material Table S3): we first used the full song (F) and fitted, separately, 5 models, one per each vocal trait (duration, peak frequency, maximum frequency, minimum frequency, and bandwidth), which were used as response variables, and the geographical group as a binary fixed effect. We then fit 5 models for the phrase section S1 and 5 models for the section S2, using the same structure, to total 15 models. To incorporate intra-sample variation (Bolker *et al.* 2009), we included the recording ID as a random effect in all models. Importantly, the response variables were centered and standardized, using the *scale* function in R, to have a mean = 0, and standard deviation = 1, which allowed us to compare effect size across models (Nakagawa and Cuthill 2007, Schielzeth 2010). Following the same settings and model structure as described above, we fit another 15 models (see Supplementary Material Table S3); but instead of using group as a binary fixed effect, we used subspecies as a categorical fixed effect. We used the *relevel* function in R to obtain the statistical differences between pairs of subspecies in each model.

Body Size Variation

We examined body size variation of *D. cyanea* along its latitudinal distribution (Figure 1) using wing length (WL) as a surrogate for body size (Zink and Remsen 1986). Wing length may have problems in indexing body size for migratory birds where wing length varies in relation to the migratory activity, but for *Diglossa*, which are nonmigratory, that is not the case (J.V. Remsen, unpublished data). We measured WL (unflattened wing chord) using an end-stopped metallic ruler

(± 1.0 mm) on 364 round skins: 125 *D. c. melanopis*, 19 *D. c. dispar*, 207 *D. c. cyanea*, 4 *D. c. tovarensis*, and 9 *D. c. obscura* (Supplementary Material Table S4). Also, we reviewed specimen records from museums, ornithological datasets, and the literature (e.g., Paynter 1981, 1992, Stephens and Traylor 1983, Núñez-Zapata *et al.* 2016) to georeference localities of historical specimens. We only considered specimens categorized as adults without evidence of molt or worn primaries.

To examine variation in WL along the latitudinal distribution of the species (17.24°S to 10.41°N), we fit linear models (LM) in R (R Core Team 2021). On a first LM, we only included data from the southern group and used latitude and its interaction with sex as predictors (to obtain the slope for each sex) and WL as the response variable (Wing Length ~ Latitude*Sex). On a second LM, we used a similar model structure, but only included data from the northern group. Then, we fit 2 additional LMs to quantify statistical differences between the WL of males from the southern group vs. the northern group and between the WL of females from the southern group vs. the northern group (Wing Length ~ Geographical Group for males, and Wing Length ~ Geographical Group for females).

RESULTS

The NPL Divides *D. cyanea* Into Two Divergent Lineages

With strong statistical support, the samples of *D. cyanea* clearly fell into 1 of 2 clades that are sharply associated with geography (Figure 3). The uncorrected pairwise genetic distance between these 2 groups averaged 6.7% (Table 1). The northern group contained all *D. cyanea* samples to the west and north of the NPL from Cajamarca, Peru, through the Northern Andes up to Aragua, in the Coastal Cordillera in northern Venezuela. The low genetic variation within this group (average pairwise base differences = 0.0028) implies

Table 1. Pairwise genetic divergence between the *Diglossa cyanea* named taxa and *D. caerulescens* (last 6 taxa; marked with asterisk). The southern *D. cyanea* group (S) is solely represented by *D. c. melanopsis*, whereas the northern group (N) is represented by 3 of 4 named taxa (*D. cyanea obscura* was not sampled). Upper right cells contain the average number of base differences per site between groups (uncorrected *p*-distance), with the pairwise deletion option. Lower left cells show the net average differences per site between groups. Diagonal cells contain the within-group average number of base differences per site.

Subspecies	<i>Diglossa cyanea</i>				<i>Diglossa caerulescens</i> *					
	<i>tovarensis</i> (N)	<i>cyanea</i> (N)	<i>dispar</i> (N)	<i>melanopsis</i> (S)	<i>caerulescens</i>	<i>ginesi</i>	<i>saturata</i>	<i>ssp.</i>	<i>media</i>	<i>mentalis</i>
<i>tovarensis</i> (N)	—	0.0071	0.0077	0.0706	0.0740	0.0754	0.0740	0.0780	0.0730	0.0786
<i>cyanea</i> (N)	0.0058	0.0025	0.0029	0.0667	0.0740	0.0754	0.0740	0.0780	0.0729	0.0783
<i>dispar</i> (N)	0.0067	0.0007	0.0020	0.0673	0.0744	0.0755	0.0744	0.0784	0.0734	0.0788
<i>melanopsis</i> (S)	0.0670	0.0618	0.0626	0.0073	0.0799	0.0813	0.0804	0.0822	0.0788	0.0837
<i>caerulescens</i> *	0.0740	0.0727	0.0734	0.0762	—	0.0034	0.0043	0.0156	0.0106	0.0138
<i>ginesi</i> *	0.0749	0.0737	0.0740	0.0772	0.0029	0.0010	0.0044	0.0170	0.0120	0.0152
<i>saturata</i> *	0.0716	0.0703	0.0710	0.0744	0.0019	0.0015	0.0048	0.0167	0.0117	0.0149
<i>ssp.</i> *	0.0778	0.0766	0.0772	0.0783	0.0154	0.0163	0.0141	0.0004	0.0108	0.0159
<i>media</i> *	0.0730	0.0716	0.0724	0.0752	0.0106	0.0115	0.0093	0.0106	—	0.0070
<i>mentalis</i> *	0.0779	0.0763	0.0771	0.0794	0.0131	0.0140	0.0118	0.0150	0.0063	0.0015

Two Song Types in *D. cyanea* Reflect the Genetic Structure

We found 2 general song types that separate *D. cyanea* into a northern and a southern group across the NPL, reflecting the phylogeographic results. Songs clearly differ in note structure, duration, and spectral metrics between both sides of the NPL (Figure 4). Specifically, songs of the southern group (*D. c. melanopsis*) are characterized by the addition of a terminal series of strident whistling notes (S3) that are completely absent from our sample of recordings of the northern group (*D. c. tovarensis*, *D. c. cyanea*, and *D. c. dispar*); to the best of our knowledge, this terminal series has never been reported in field observations of *D. cyanea* north of the NPL. Additionally, the southern songs have a much shorter ($\beta_{\text{southern}} = 0.7$ s, 95% CIs: 0.5–0.8 s) warbling chatter (S2) than songs of the northern group ($\beta_{\text{northern}} = 1.9$ s, 95% CIs: 1.8–1.9 s, contrast $\beta_{\text{northern}} - \beta_{\text{southern}} = 1.2$ s, 95% CIs: 1.0–1.3 s; see details in Supplementary Material Tables S5 and S6). Although lacking the terminal whistling phrase (S3), songs of the northern group have longer phrases (S1, S2), yielding no statistical difference in full song duration (F) between groups. Moreover, songs of the southern group (*D. c. melanopsis*) are higher pitched (i.e., greater peak frequency values across comparable song sections; contrast $\beta_{\text{southern}} - \beta_{\text{northern}} = 1004$ Hz, 95% CIs: 860–1,156 Hz), and tend to occupy narrower bandwidths due to their higher minimum frequencies than the northern songs (bandwidth contrast $\beta_{\text{northern}} - \beta_{\text{southern}} = 1352$ Hz, 95% CIs: 902–1,785 Hz; see greater detail in Supplementary Material Tables S5 and S6).

To further explore vocal differences among the currently defined subspecies of *D. cyanea*, we compared the 5 vocal variables in the full song and the 2 shared sections (F, S1, S2) by considering each of the northern subspecies (*D. c. tovarensis*, *D. c. cyanea*, *D. c. dispar*) separately, except for *D. c. obscura* for which no vocal data exist. The southern subspecies (*D. c. melanopsis*) differed vocally from the other *D. cyanea* subspecies of the northern group, particularly for the higher pitch of its song elements, revealed by higher values of peak frequency and minimum frequency; see Figure 5, details in Supplementary Material Table S7). Lastly, the northern

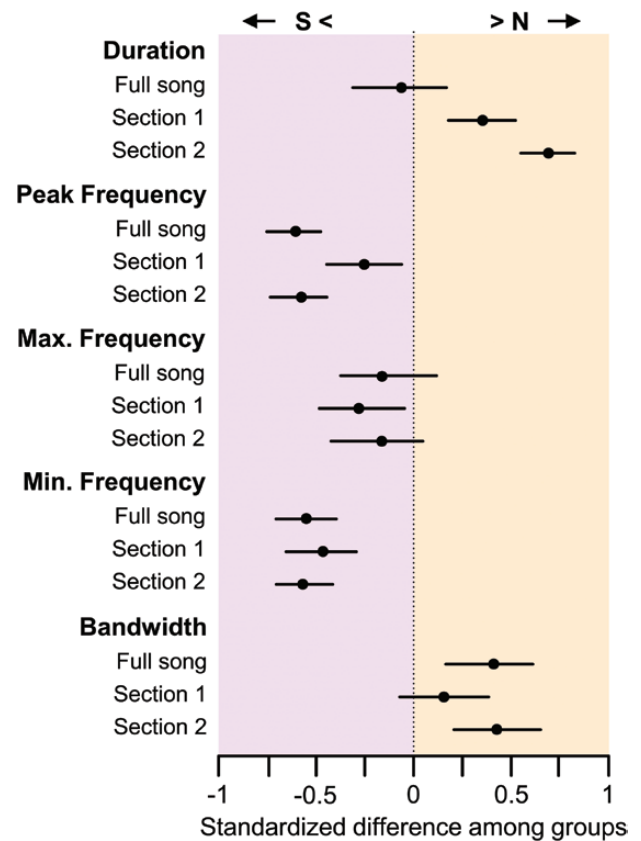


Figure 4. Acoustic differences and their confidence intervals (horizontal lines) in the songs of the 2 *Diglossa cyanea* groups, separated by the NPL. Differences were calculated as the mean values of the northern vocal samples minus those of the southern samples, where a standardized difference of 0 indicates no difference between the means of the 2 groups, positive standardized differences indicate larger values in the northern group (north and west of the NPL to Venezuela), and negative standardized differences indicate larger values in the southern group (*D. c. melanopsis* from the east and south of the NPL). Although full song duration was similar between the 2 groups, songs of southern groups were higher pitched with narrower bandwidths.

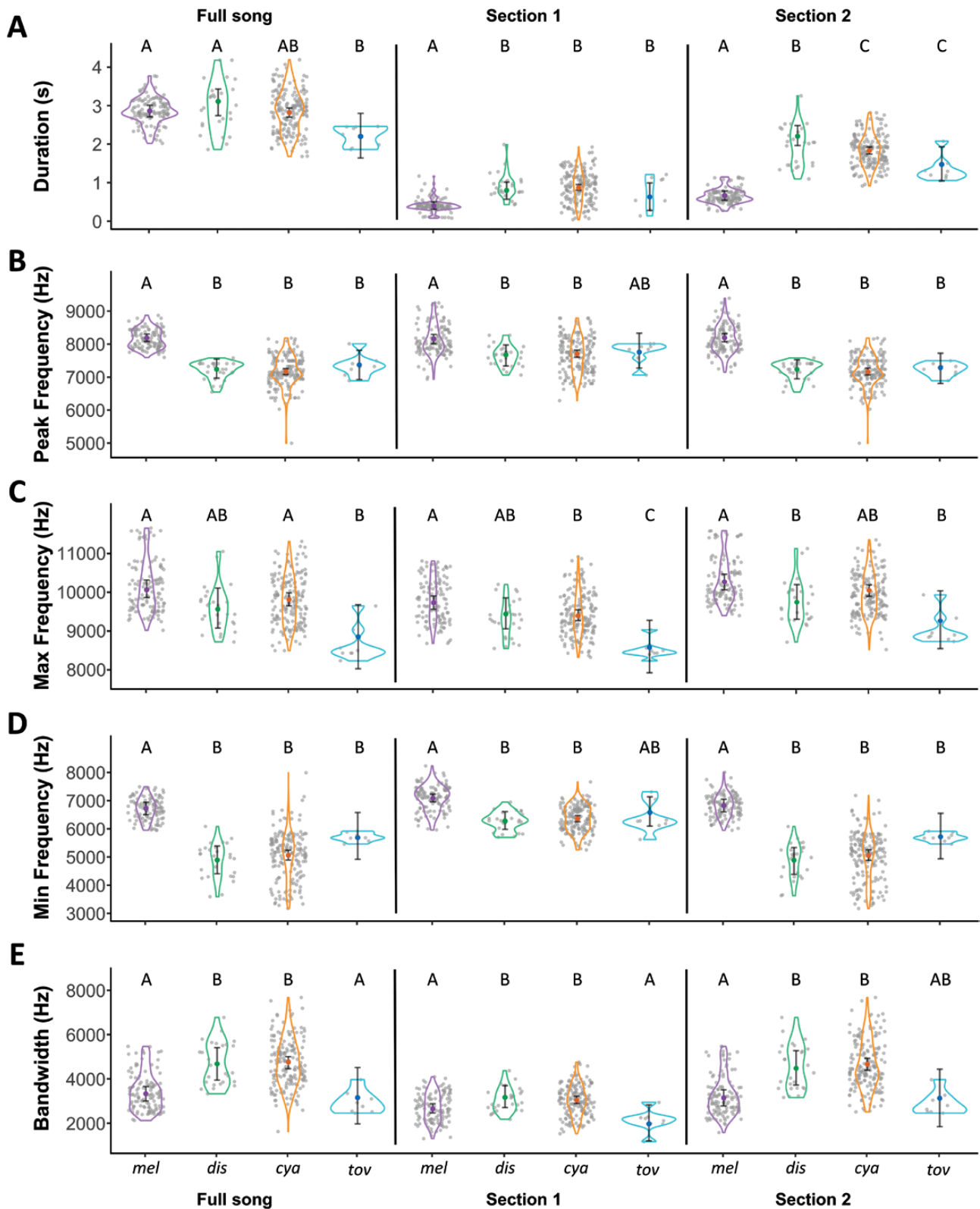


Figure 5. Quantitative acoustic differences among 4 subspecies of *Diglossa cyanea* (all except the unsampled *obscura*); *mel*: *melanopsis* (southern group); *dis*: *dispar*, *cya*: *cyanea*, *tov*: *tovarensis* (northern group). Acoustic variables analyzed included (A) duration, (B) peak frequency, (C) maximum frequency, (D) minimum frequency, and (E) frequency bandwidth. The full song and phrase sections 1 and 2 are compared among taxa. In most cases, the southern subspecies *D. c. melanopsis* showed the most dissimilar acoustic traits, being most different to songs of the northern subspecies *D. c. cyanea*.

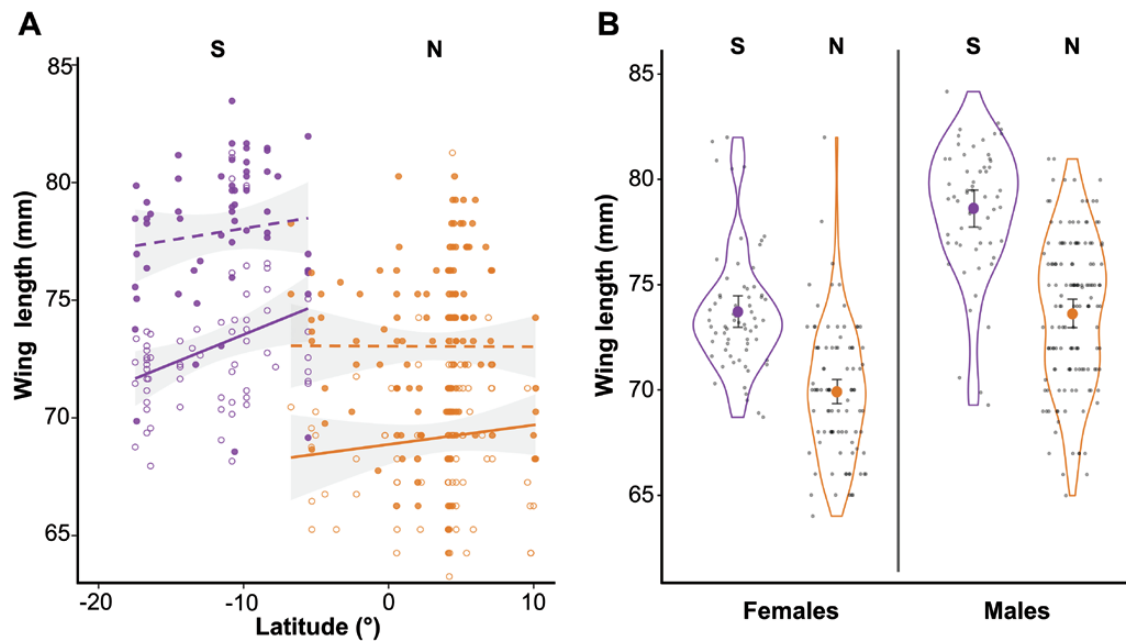


Figure 6. Geographic variation in wing length in *Diglossa cyanea* along its latitudinal range along the Andes, depicting the southern group (*D. c. melanopis*) in purple, and the northern group in orange. **(A)** Wing length does not show a range-wide latitudinal pattern of variation, although the southern group tended to have shorter wings towards its southern range limits. Females (open circles, solid fit line) and males (filled circles, dashed fit line) are indicated. **(B)** Both females and males of *D. c. melanopis*, south of the NPL (in purple) tended to be larger than individuals of their respective sex in the northern group (in orange). Despite the high variance in wing length and partial overlap between groups, linear models distinguish both the southern and northern groups based on wing-length data.

subspecies (*D. c. towarensis*) has a shorter full song (contrast $\beta_{dispar} - \beta_{tovarensis} = 0.9$ s, 95% CIs: 0.2–1.5 s), accompanied by a much lower maximum frequency in S1 and S2 overall, and outstanding visual characteristics of the notes in comparison to other northern *D. cyanea* subspecies, although sample sizes are limited ($n = 2$) (Supplementary Material Table S7 and Supplementary Material Figure S4).

In addition to the song differences between the 2 *D. cyanea* groups from opposite sides of the NPL, we found that in about half of the available recordings of the southern group (*D. c. melanopis*), an alternative or secondary song was present (see Supplementary Material Figure S1). The alternative song consists of a single, continuous chatter phrase, largely different in structure from the typical song on either side of the NPL, characterized by a slightly shorter duration ($\beta_{alternative} = 2.5$ s, 95% CIs: 2.2–2.7 s; $\beta_{southern} = 3.0$ s, 95% CIs: 2.8–3.1 s) and lower frequencies (both peak and minimum) than the typical southern song (Supplementary Material Table S8).

WL Differs Between the Southern and Northern Groups

We only found a statistically significant (and positive) association between latitude and WL for females in the southern group ($\beta_{females\ southern} = 0.25$, $P = 0.01$, Figure 6A, Supplementary Material Table S9). Wings of *D. c. melanopis* females tended to be longer toward its northern range limit. In sharp contrast, neither WL of males nor females of the northern group showed any statistically significant variation along latitude (i.e., confidence intervals of slopes included 0, $\beta_{males\ southern} = 0.099$, $P = 0.34$, $\beta_{females\ northern} = 0.082$, $P = 0.37$, $\beta_{males\ northern} = -0.003$, $P = 0.98$). More importantly, we found that WL differed statistically between the 2 groups

across the NPL for both females ($\beta_{females\ southern} - \beta_{females\ northern} = 3.8$ mm, $P < 0.001$) and males ($\beta_{males\ southern} - \beta_{males\ northern} = 4.8$ mm, $P < 0.001$, Figure 6B, Supplementary Material Table S10). Simply put, southern females have longer wings than do northern females, and southern males have longer wings than do northern males (Figure 6B, Supplementary Material Table S10).

DISCUSSION

By integrating genetic, vocal, and morphological data, we documented the existence of 2 divergent lineages within the Masked Flowerpiercer (*D. cyanea*). Despite the extremely subtle plumage differences between these 2 lineages, they exhibit deep genetic structure on par with the amount of genetic divergence between other blue-plumaged flowerpiercer species in the *Diglossopsis* clade. In addition, the 2 lineages have distinct songs in terms of structure and spectral traits, and dissimilar wing lengths in both males and females. Our results suggest 2 biological species in *D. cyanea* separated by the NPL, as follows: *D. melanopis* from Peru and Bolivia, and *D. cyanea* sensu stricto from extreme northern Peru, Ecuador, Colombia, and Venezuela, including the subspecies *cyanea*, *obscura*, and *tovarensis*. Further evidence is needed to determine the degree of genetic, vocal, and phenotypic differentiation of *D. c. obscura* of the Sierra de Perijá and *D. c. towarensis* of the Coastal Cordillera of Venezuela.

The diversity within *Diglossa* has likely been shaped by time and geographical isolation, accompanied by genetic divergence, and possibly adaptive divergence in relation to environmental or social and behavioral factors (Moynihan 1979) that strengthen the effect of physical barriers (Vuilleumier 1984, Smith *et al.* 2014, Gutiérrez-Zuluaga *et al.* 2021). We

have shown that in *D. cyanea*, the abrupt topographic and environmental turnover of the humid montane forest belt at the NPL maintains 2 diverging populations isolated since at least the Pliocene. Furthermore, the lack of gene flow indicated by the sharp genetic break between the northern and southern populations of *D. cyanea* suggests the evolution of differences in traits, such as song, that could maintain separate lineages even when environmental changes permitted increased connectivity between populations, for example, by shifting forest belts during glacial periods (Hooghiemstra and Van der Hammen 2004, Ramírez-Barahona and Eguiarte 2013, Flantua and Hooghiemstra 2018).

Genetic divergence between the southern *D. melanopis* was stronger with respect to northern *D. cyanea* (sensu stricto) than to *D. caerulescens*, and the ND2 gene tree showed a non-monophyletic *D. cyanea* as currently recognized. Although our genetic sampling encompassed most of the relevant geographic regions along the *D. cyanea* range, a few caveats in this phylogenetic inference should be noted. First, this gene tree may not reflect the true species history between these 3 taxa (Funk and Omland 2003, McKay and Zink 2010), so that a larger genomic dataset of nuclear loci would be necessary to assess whether this phylogenetic hypothesis holds, especially given the extreme similarity in phenotype between the 2 *cyanea* groups. A plausible hypothesis to be tested is that the northern *D. cyanea* and the southern *D. melanopis* are indeed sister species that originated from vicariance or from dispersal over the NPL with subsequent differentiation, and this speciation event occurred shortly after the stem separation from the lineage leading to *D. caerulescens*. An approximation to address this first point would consist of vocal analyses that include *D. caerulescens* songs in the pairwise comparisons, which was beyond the scope of this study. A qualitative inspection of audio recordings of *D. caerulescens* songs indicates geographic variation across its range. Songs of *D. caerulescens* north of the NPL tend to be more similar to songs of *D. cyanea*, whereas south of the NPL *D. melanopis* and *D. caerulescens* seem much less similar. Differences in the bandwidth and duration of song sections are worthy of further research.

Another issue to be addressed is whether the absence of phenotypic or genetic samples of *D. c. obscura*, of which few specimens exist (Zimmer and Phelps 1952), affects the analysis. Endemic to the Sierra de Perijá, this taxon is rare there (Hilty 2003, López-O *et al.* 2014), in contrast to the abundance of other subspecies of *D. cyanea* within their ranges. This hints at ecological differences that might represent an additional cause of speciation. Finally, based on our limited genetic and vocal samples, we found that *D. c. tovarensis* is indeed a distinct population from the nominate *D. cyanea* of the Venezuelan and Colombian Andes; however, without additional data, we refrain from making additional taxonomic recommendations. It would be insightful to test experimentally via song playback how *tovarensis* reacts to *D. c. cyanea* songs.

In the oscine passerines, in addition to cultural evolution, plasticity in songs is common due to variation in learning abilities among individuals (Slater 1989, Price 2008), however, the genetically programmed, conservative template that predisposes learning of “own” species songs is informative of its phylogenetic history (Remsen 2005, Cadena and Cuervo 2010). In our study, the most significant differences in songs were found between the populations separated by the NPL.

Most remarkably, the southern *D. c. melanopis* revealed 2 types of song, whereas the northern populations have only 1. Additionally, the final whistling notes (S3), which are completely absent in the northern populations, may represent an innovation in song that is fixed in the southern *D. c. melanopis*, which in turn became its most distinctive vocal characteristic. Mirroring the genetic data, no vocal variation was observed within the large distribution of *D. c. melanopis*, or within that of the northern *D. cyanea* subspecies, except for the apparently distinct features of *D. c. tovarensis*. Freeman *et al.* (2022) found that *D. c. melanopis* discriminates between “own” songs and those of northern *D. c. cyanea*, and this reinforces the hypothesis that this level of song divergence might work as a reproductive barrier in case of secondary contact between these 2 lineages, provided cross learning of songs is insignificant.

Our morphological analysis showed that wing length also shifts across the NPL. Although the only association with latitude was a slight decrease in WL toward the southern range limits, in Bolivia, of *D. c. melanopis*, the extent of sexual dimorphism in this trait is well conserved within *D. c. cyanea* and *D. c. melanopis* on both sides of the NPL (Figure 6). Remarkably, WL differs on average, between the northern and southern populations, by more than 5 mm for males and 4 mm for females, representing a ~6% length difference between sexes based on the total WL.

In this study, we found evidence for an independent evolutionary history of the 2 *D. cyanea* lineages separated by the NPL that is expressed in unique vocal and phenotypic traits. Despite the conspicuous genetic, vocal, and size differences, minimal variation in plumage coloration and patterns has masked this diversity, as has been the case for multiple tropical birds (e.g., D’Horta *et al.* 2013, Smith *et al.* 2018, Berv *et al.* 2021). The evolutionary assembly of the Neotropical montane avifauna still has much to understand considering the complexity and diversity of evolutionary histories and the wide range of phenotypic divergences between populations (Weir 2009, Fjeldså *et al.* 2012, Cuervo 2013, Winger and Bates 2015, Cadena *et al.* 2020). In flowerpiercers, patterns of speciation and phenotypic evolution within the core *Diglossa* clade (Mauck and Burns 2009, Gutiérrez-Zuluaga *et al.* 2021), in which marked plumage diversity is evident but vocal variation is slight (personal observation), contrasts with our findings in the *Diglossopsis* clade. Our results demonstrate that analyzing multiple characters reveals that the ecological and evolutionary patterns in *Diglossa* flowerpiercers are far more complex than previously recognized.

Taxonomic Implications

Based on our analysis of vocal divergence combined with insights on their behavioral isolation consequences from playback experiments (see Freeman *et al.* 2022), we recommend that the 2 primary lineages be treated as separate species under the Biological Species Concept (Mayr 1942), specifically *D. melanopis* and *D. cyanea* (sensu stricto). The depth of genetic divergence in ND2 (6.7%) is even higher than differences between most other currently recognized species in *Diglossa* (Mauck and Burns 2009). These 2 species have been evolving in isolation for at least 2.7 million years and exhibit song differences that indicate behavioral isolation (Uy *et al.* 2018). The subtle but consistent differences in the described

plumage coloration and size, as indicated by the wing length data, are consistent with either species or subspecies rank under the BSC but are evidence for species rank under the diagnosability-based Phylogenetic Species Concept (Cracraft 1983). More broadly, these 2 taxa can be recognized as independent species-level lineages that are phenotypically and genetically distinct (de Queiroz 1998, Barraclough 2019).

Diglossa cyanea sensu stricto (Lafresnaye, 1840)

Warbling Masked-Flowerpiercer

Types

Two syntypes: Museum of Comparative Zoology, Harvard University MCZ 76723 and 76724, from Bogotá (formerly, Santa Fe de Bogotá), Colombia.

Protonym

Uncirostrum cyaneum Lafresnaye 1840.

Diagnosis

Extremely similar to larger *D. melanopsis* but with brighter blue coloration overall. The whitish markings of the undertail coverts, and the blue outer margins of flight feathers and tertials are much more distinctly marked. Its song consists of variable warbles, lacking the high-pitched, pure terminal whistles of *D. melanopsis* songs (Figure 2).

Distribution

North and west of the Marañón river valley, from Cajamarca, Peru through the Northern Andes, including the Sierra de Perijá, to the Coastal Cordillera of Venezuela (Figure 1).

English name

Because *D. cyanea* is here redefined, keeping the original name for the more restricted taxon could create confusion. The proposed common name refers to the effusive warbles that make up its song, lacking whistle components.

Subspecies

Polytypic, with 3 subspecies. First, the widespread nominate subspecies of the Northern Andes of Venezuela, Colombia, Ecuador, and extreme northern Peru. Second, *D. c. towarensis* Zimmer and Phelps 1952, restricted to the Coastal Cordillera of northern Venezuela. Third, the much rarer *D. cyanea obscura* Phelps and Phelps 1952 of the Sierra de Perijá, and until now only known from a handful of specimens collected on the eastern (Venezuelan) slope of the range. The limited data and small number of specimens of *obscura* impede assessing its diagnosability, but it might be better subsumed with *cyanea*. We consider *D. cyanea dispar* Zimmer 1942 to be a junior synonym of the nominate subspecies, given its lack of genetic and vocal differentiation and lack of consistent diagnostic features (see Discussion).

Diglossa melanopsis Tschudi 1844

Whistling Masked-Flowerpiercer

Holotype

Muséum d'Histoire Naturelle de Neuchâtel MHNN 92.8934, from Junín, Peru (Hellmayr 1935).

Protonym

Diglossa melanopsis Tschudi 1844.

Diagnosis

On average, *D. melanopsis* is larger than *D. cyanea*. The bluish tones are duller, but its crown lighter blue. The whitish edges of the vent patch, and the bluish outer margins in the wing feather and tertials, are more diffused and less distinct. Songs of *D. melanopsis* end with a unique phrase of 3-4 pure whistling notes and are significantly higher in pitch and narrower in bandwidth than that of *D. cyanea* (Figure 2). Both *D. cyanea* and *D. melanopsis* are distinguishable from the sympatric *D. caerulescens* by their larger size, longer and deeply hooked bills, brighter violaceous blue coloration, complete black facial mask including auriculars, forefront and chin, and bright red irides.

Distribution

South and east of the Marañón river valley, from Amazonas, Peru south to Santa Cruz, Bolivia, along the Amazonian versant of the Andes (Figure 1).

English name

The proposed English name refers to the distinctive song of *D. melanopsis*, which starts with warbles but ends with pure-note whistles.

Subspecies

Monotypic. No phenotypic variation has ever been recognized from northern Peru to Bolivia, and we found no indication otherwise.

Supplementary material

Supplementary material is available at *Ornithology* online.

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Ethics statement

We complied with ethical standards and legal rules and regulations of scientific research.

Nomenclatural statement

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Author contributions

S.C.M. and A.M.C. conceived the idea and designed the study and together with J.V.R. examined specimens in collections and recollected wing length data. A.M.C. and R.T.B. collected and analyzed genetic data. S.C.M. and C.E.L. conducted the morphometric and vocal statistical analyses. The manuscript was largely written by S.C.M. and A.M.C., with significant contributions of all other authors. All were involved in interpretation of the results, implemented reviewers' suggestions, and contributed substantial materials, resources, and funding.

Data availability

Sequences generated in the present study were deposited in GenBank under access numbers OR032417–OR032569. Analyses reported in this article can be reproduced using the data and scripts provided by [Martínez-Gómez et al. \(2023\)](#).

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