Genetic and phenotypic differentiation in a Neotropical passerine with a disjunct distribution in the Andean and Atlantic forests (Thamnophilus ruficapillus)

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Abstract

The Andean and Atlantic forests are separated by the open vegetation corridor, which acts as a geographic barrier. However, these forests experimented cycles of connection and isolation in the past which shaped the phylogeographic patterns of their biotas. We analyzed the evolutionary history of the Rufous-capped Antshrike (Thamnophilus ruficapillus), a species with a disjunct distribution in the Atlantic and Andean forests and therefore an appropriate model to study the effect of the open vegetation corridor and the Andes on the diversification of the Neotropical avifauna. We performed a phylogenetic/phylogeographic analysis including the five subspecies, using mitochondrial and nuclear genomic DNA, and also studied their differences in vocalizations and plumage coloration. Both the mitochondrial and nuclear DNA evidenced a marked phylogeographic structure with three differentiated lineages that diverged without gene flow in the Pleistocene (1.0-1.7 million years ago): one in the Atlantic Forest and two in the Andean forest. However, the two Andean lineages do not coincide with the two disjunct areas of distribution of the species in the Andes. Vocalizations were significantly different between most subspecies, but their pattern of differentiation was discordant with that of the nuclear and mitochondrial DNA. In fact, there is no song differentiation between the subspecies of the Atlantic Forest and that of the northwestern Bolivian Andes, even though they differ genetically and belong to different lineages. Consistently, no differences were found in plumage coloration between the subspecies of the Atlantic Forest and that of the southern Andes. Our results suggest a complex evolutionary history in this species, which differentiated both due to dispersion across the open vegetation corridor, likely during a period of connection between the Andean and Atlantic forests, and as a consequence of a geographic barrier in northern Bolivia. In both cases Pleistocene climatic oscillations appear to have influenced the species diversification.

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Keywords: Andean forest, Atlantic Forest, mitochondrial and genomic DNA, vocalizations, plumage coloration, dispersion, Pleistocene climatic oscillations.

Introduction

The main factors that have promoted the diversification of the Neotropical avifauna include the Andes Mountains (Weir 2006, Brumfield and Edwards 2007, Sedano and Burns 2010, Weir and Price 2011), wide rivers (Ribas et al. 2012, Naka and Brumfield 2018, Kopuchian et al. 2020, Thom et al. 2020) and the closure of the Isthmus of Panama (Weir et al. 2009, Smith and Klicka 2010). Although with a more prominent role in the Northern Hemisphere and the southern extreme of South America, the Pleistocene glacial cycles also played a relevant role as avian diversification drivers throughout the Neotropics (Lessa et al. 2003, Weir and Schluter 2004, Lovette 2005, Campagna et al. 2012, Kopuchian et al. 2016, Acosta et al. 2020, Bukowski et al. 2023). This is clearly the case for highland Andean taxa (Weir 2006, Jetz et al. 2012), but also in the lowlands Pleistocene climatic oscillations have driven speciation by promoting cycles of contraction, fragmentation and expansion of forest ranges, which generated vicariance, divergence and in some cases secondary contact of both forest and dry habitat species (Rull et al. 2011, Trujillo-Arias et al. 2017, 2020, Silva et al. 2019, Bolívar-Leguizamón et al. 2020, Thom et al. 2020).

Another relevant driver of diversification in the Neotropical region, which until recently has been less studied than those mentioned above, is the open vegetation corridor. This dry strip formed by the Caatinga, Cerrado and Chaco biomes isolates the Amazon and Andean forests from the Atlantic Forest, thus affecting the connectivity of three of the most biodiverse rainforests in the world (Orme et al. 2005). Either by vicariance due to the establishment of the open vegetation corridor in the Neogene in a previously continuous forest or as a consequence of dispersion through these dry habitats after its formation, multiple species of birds are disjunctly codistributed in these forests, with varying degrees of differentiation between forests (Lavinia et al. 2015, 2019, Trujillo-Arias et al. 2017, 2018, 2020, Cabanne et al. 2019, Bocalini et al. 2023).

Even though the Andean and Atlantic forests are currently isolated, they have experienced cycles of connection and isolation associated with the geotectonic processes and climatic fluctuations of the Neogene and the Quaternary (Nores 1992, Silva 1994), adding more complexity to this system and its effect on the avifauna of the region (Trujillo-Arias et al. 2017, 2018, 2020, Cabanne et al. 2019, Lavinia et al. 2019). The cyclic connection between these rainforests could have occurred during glacial maxima through the expansions of forests into the Cerrado (Silva 1994), a possibility supported by palynological studies (Ledru 1991, 1993, Oliveira-Filho and Ratter 1995) and avian data (Carnaval and Moritz 2008, Cabanne et al. 2016, 2019, Trujillo-Arias et al. 2017). The contact could have also occurred during interglacial periods through gallery forests in the Chaco region (Olrog 1963, Nores 1992), a hypothesis based mainly on forest bird distribution patterns but without clear evidence (Zurita et al. 2014, Trujillo-Arias et al. 2017). Irrespective of their differences in timing and location, these proposed connections could have acted in combination (Trujillo-Arias et al. 2017, 2018, 2020).

As shown with other geographic barriers or habitat mosaics in the Neotropics (Smith et al. 2014, Naka and Brumfield 2018, van Els et al. 2021), the effect of this dynamic rainforest history also depends on species-

specific ecological characteristics and dispersal abilities (Trujillo-Arias et al. 2018, 2020, Lavinia et al. 2019). Several studies have focused on the evolutionary history of species that inhabit exclusively the Amazon forest or the Atlantic Forest, providing insights into the historical diversification processes operating within these regions (e.g. Ribas et al. 2012, Maldonado-Coelho et al. 2013, Cabanne et al. 2016, Silva et al. 2019). Fewer comprehensive evolutionary studies have been performed on species that inhabit both the Atlantic and the Andean or Amazonian forests (Lavinia et al. 2015, 2019, Trujillo-Arias et al. 2017, 2018, 2020, Cabanne et al. 2019, Bolívar-Leguizamón et al. 2020).

The Rufous-capped Antshrike (*Thamnophilus ruficapillus*) has three disjunct areas of distribution, one of them in the Atlantic Forest and the other two in the Andean forest (Figure 1a). In the latter, it inhabits humid and semi-humid forests (with different degrees of human alteration) and patches of dense shrubs bordering watercourses in predominantly open areas east of the Andes (Brumfield and Edwards 2007, del Hoyo et al. 2020). In the Atlantic Forest it occurs in low elevation areas of northern Argentina and southern Brazil (Brumfield and Edwards 2007, del Hoyo et al. 2020). *T. ruficapillus* belongs to the group of suboscine passerines and it is an insectivorous, monogamous and sexually dichromatic species (del Hoyo et al. 2020). Currently, five subspecies are recognized (del Hoyo et al. 2020, Clements et al. 2022): *T. r. jaczewskii* (in the Andean forest of northern Peru), *T. r. marcapatae* (in southeastern Peru, particularly Puno and Cusco departments), *T. r. subfasciatus* (in the Yungas of northwestern Bolivia), *T. r. cochabambae* (in southern Bolivia to northwestern Argentina) and *T. r. ruficapillus* (in the Atlantic Forest of southeastern Brazil and northeastern Argentina) (see Figure 1a). Consistent with these designations, previous analyses of mitochondrial DNA have shown intraspecific divergences of around 4% in the cytochrome c oxidase subunit I (COI) gene between *T. r. cochabambae* in the Andean forest and *T. r. ruficapillus* in the Atlantic Forest (Kerr et al. 2009).

Here we leverage the distribution of *T. ruficapillus* to use this species as a model for the study of the effect of the open vegetation corridor and the Andes on the diversification of the Neotropical avifauna. For doing this, we analyzed the evolutionary history of this species using a comprehensive approach that includes both genetic and genomic analyses (mitochondrial and nuclear genomic DNA) as well as phenotypic analyses (study of vocalizations and plumage coloration) to better understand its phylogeographic patterns and the role played by the aforementioned Neotropical diversification drivers.

Materials and methods

Taxon sampling for genetic and genomic analyses

We analyzed 30 specimens of *T. ruficapillus* from 18 collection sites covering the main distribution range of the species and including representatives of the five subspecies that have been described to date (Figure 1a, see also Table S1 in the Supporting information Appendix). We also included one specimen of *T. doliatus* and one specimen of *T. caerulescens* to be used as outgroups (we could not include *T. torquatus* , which is the sister species of *T. ruficapillus* ; Brumfield and Edwards 2007). Subspecies were determined according to the distribution range described by del Hoyo et al. (2020) and Clements et al. (2022).

Laboratory protocols for mitochondrial markers

We extracted DNA from fresh tissue (pectoral muscle or blood) following the silica-based protocol described by Ivanova et al. (2006), using individual spin columns (Lijtmaer et al. 2012). We amplified two mitochondrial genes: cytochrome c oxidase I (COI) and cytochrome *b* (cyt *b*). Primers used for the amplification of the 695 base pairs (bp) of the COI were BirdF1 (Hebert et al. 2004) and COIbirdR2 (Kerr et al. 2009) and to amplify 1007 bp of the cyt *b* we used L14841 (Kocher et al. 1989) and H16065 (Lougheed et al. 2000).

PCR amplification reaction cocktails and the thermocycling profile for COI from fresh tissue followed Lijtmaer et al. (2012). To amplify the cyt *b* we followed the protocol in Arrieta et al. (2013) and the PCR cocktail was prepared in 20 µl volume with the following reagents: 3 µl of genomic DNA, 1x PCR buffer, 0.2 mM dNTPs, 3 mM Cl_2Mg/SO_4Mg, 0.5 µM primer forward, 0.5 µM primer reverse and 1U Taq DNA polymerase (Invitrogen). The PCR thermocycling profile was as follows: 3 min at 94 ^{o}C; 40 cycles of 45 sec

at 94 ºC, 30 sec at 53 ºC and 1 min at 72 ºC; and finally 10 min at 72 ºC.

Sequencing of both mitochondrial markers was conducted at Macrogen Korea (Seoul, Korea) and performed bidirectionally with the same primers used for amplification. GenBank accession numbers for all sequences generated in this study are included in Table S1.

Laboratory protocols for nuclear genomic markers

We extracted genomic DNA from fresh tissue samples using DNeasy tissue extraction kit (Qiagen, CA, USA). To generate the genomic data we used double-digest restriction site-associated DNA sequencing (ddRADseq) following the protocol of Peterson et al. (2012) with modifications described in Thrasher et al. (2018). Briefly, for each sample we isolated ˜500 ng of DNA, at a standardized concentration of 20 ng/µl, and digested it with the restriction enzymes SbfI High Fidelity (8 base bp recognition site; 5'-CCTGCAGG-3') and MspI (4 base bp recognition site; 5'-CCGG-3') (New England BioLabs, MA, USA). The digested DNA was ligated to P1 and P2 adapters on both ends (sequences are available in Peterson et al. 2012). We size-selected groups of 20 uniquely barcoded samples (index groups), retaining fragments between 400 and 700 bp using Blue Pippin (Sage Science, MA, USA). To incorporate the full Illumina TruSeq primer sequences and unique indexing primers into each library, we performed low cycle number PCR with Phusion High-Fidelity DNA Polymerase (New England BioLabs), with the following thermocycling profile: 98°C for 30 sec followed by 11 cycles at 98°C for 5 sec, 60°C for 25 sec, and 72°C for 10 sec with a final extension at 72°C for 5 min. We visualized the product of this amplification on a 1% agarose gel and performed a final 0.73 AMPure cleanup to eliminate DNA fragments smaller than 200 bp. Finally, sequencing was performed on an Illumina HiSeq 2500 lane at the Cornell University Biotechnology Resource Center as part of a larger sequencing batch, obtaining single-end 150-bp sequences, with an average mean sequencing depth of 151 reads per locus per individual (range: 104-220).

Genetic diversity and population structure based on mitochondrial markers

To perform the genetic analyses, we edited and aligned COI and cyt*b* sequences using CodonCode Aligner 4.0.4 (CodonCode Corporation, Dedham, MA) and carefully checked the chromatograms for ambiguities and sequences to detect the presence of any stop codons, as well as alignment gaps.

Because mitochondrial sequences are linked within the same genome, COI and cyt *b* sequences were concatenated for the analyses. First, to analyze the genetic variation within the species and understand the relationship among haplotypes, we calculated the average p distances using MEGA 5.2 (Tamura et al. 2011) and generated haplotype networks using the median joining algorithm implemented in PopART 1.0 (< http://popart.otago.ac.nz >), respectively. We conducted an analysis of molecular variance (AMOVA) in Arlequin 3.5 (Excoffier and Lischer 2010) to explore the distribution of genetic variation within *T. ruficapillus* and specifically test whether there are differences in the frequency of mitochondrial haplotypes among subspecies. The Φ_{ST} values between pairs of subspecies were computed using uncorrected genetic distance matrices between haplotypes and significance was tested through 2,000 random permutations.

Phylogenetic analyses and divergence time estimations based on mitochondrial DNA

We inferred gene trees with Bayesian and maximum parsimony (MP) methodologies using MrBayes 3.2.2 (Ronquist et al. 2012) and TNT 1.1 (Goloboff et al. 2003), respectively. The best-fit model of nucleotide substitution for each locus for the Bayesian analysis was selected using the Bayesian information criterion (BIC) implemented in jModelTest 2.1.1 (Darriba et al. 2012). HKY+I was chosen for COI and KHY for cyt *b*(Hasegawa et al. 1985). Both loci were placed in unlinked partitions allowing parameters to vary and to be estimated independently (except for topology and branch lengths). We conducted two independent runs of 10 million generations sampling trees every 100 generations under default priors for all parameters. We discarded the first 25% of the sampled trees as burn-in and the remaining 75,000 trees of each run were combined to generate a majority rule consensus tree. The standard deviation of split frequencies (SDSF) between runs was always < 0.01 indicating convergence. Using Tracer 1.6 (Rambaut et al. 2014), we assured that both runs reached the stationary phase and that we had a good sample of the posterior probability

distributions. For the MP analysis we ran heuristic searches based on 1,000 random addition sequences (RAS) coupled with the tree bisection reconnection (TBR) branch-swapping algorithm, saving 10 trees per replication. A strict consensus tree was estimated from all the most parsimonious trees. To estimate node support we conducted a bootstrap analysis (Felsenstein 1985) that consisted of 1,000 pseudoreplicates of 100 RAS + TBR saving 10 trees per replicate.

The age of the most recent common ancestor between *T. ruficapillus* and *T. doliatus* and the time of separation of the mitochondrial lineages within *T. ruficapillus* were estimated by generating a time-calibrated ultrametric tree with the Bayesian approach implemented in BEAUTi/BEAST 1.8 (Drummond et al. 2012). Both mitochondrial markers were placed in separate partitions with unlinked substitution and clock models selected with jModelTest: HKY+I for COI and HKY for cyt *b* . The tree models were linked since the mitochondria is a single unit of inheritance. We specified a Yule speciation tree prior, assuming a constant population size and a relaxed uncorrelated lognormal clock. We used a calibration of 2.1% per million years (My) for cyt *b* (1.05×10^{-2}substitutions/site/lineage/My; Weir and Schluter 2008) and 1.17×10^{-2} substitutions/site/lineage/My for COI (Lavinia et al. 2016). The analysis was run for 100 million generations sampling every 1,000 generations. We checked for stationarity in the estimation of the parameters and adequate ESS values using Tracer. We discarded the first 10% of the sampled trees (burn-in = 10,000) and then estimated the 95% highest posterior density (HPD) intervals of divergence dates using TreeAnnotator 1.8 (Drummond et al. 2012).

Population structure and phylogenetic analysis based on genomic DNA

We demultiplexed raw reads and applied standard quality filters in Ipyrad 0.7.28 (Eaton and Overcast 2020). We discarded sequences when a single base had a Phred quality score below 10 or more than 5% of bases had a Phred quality score below 20. Additional filtering was applied to only retain reads that did not have enzyme cleavage sites, adaptor sequences or index sequences and included up to 3 ambiguous sites. Finally, we only kept loci that were present in at least 80% of the samples. The ddRAD data is available in Dryad (doi: xxx).

We performed a principal component analysis (PCA), using the gdsfmt and SNPRelate packages (Zheng et al. 2012) in R 3.5.1 (R Core Team, 2018), to visualize possible clusters in the data. We used all the SNPs of the dataset (including multiple SNPs per locus), but because the PCA is sensitive to missing data, SNPs missing from at least one individual were removed. We also assigned individuals to genetic clusters (K) using Structure 2.3.4 (Pritchard et al. 2000). For this analysis we used a single random SNP from each RAD locus. We implemented the admixture ancestry model with correlated allele frequencies and an allele frequency prior of $\lambda = 1$. We conducted 10 runs for each value of K = 1-4, and each run consisted in 500,000 generations following a burn-in of 100,000. The most likely value of K was determined following the ΔK method described by Evanno et al. (2005) and implemented in Structure Harvester 0.6.94 (Earl and vonHoldt 2012). We averaged results across the 10 runs using the greedy algorithm in the program CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) and visualized results using the conStruct package (Bradburd et al. 2018) in R.

We used RAxML version 8.2.4 (Stamatakis 2014) to infer a maximum likelihood phylogenetic tree. We implemented the ASC_GTRGAMMA model and the Lewis correction for ascertainment bias and we conducted 200 bootstrap replicates to assess node support. We included one specimen of *T. caerulescens* as outgroup (we obtained ddRADseq data for this specimen from a previous study; Kopuchian et al. 2020; see Table S1).

Demographic history modeling

We used the Generalized Phylogenetic Coalescent Sampler (G-PhoCS) version 1.2.3 (Gronau et al. 2011) to estimate divergence times, effective population sizes and gene flow. Because of the computationally intensive nature of this analysis, we restricted our data set to a maximum of 10 individuals per subspecies, selecting one sample per location. We ran G-PhoCS using the standard MCMC settings described in Gronau et al. (2011) and default parameters with 75,000 burn-in generations and 750,000 additional sampling generations. We assessed adequate mixing and convergence using Tracer. Divergence times and effective population sizes

were converted from mutation scale to generations (T) and individuals (Ne) respectively, by assuming an average mutation rate of 10^{-9} mutations per bp per generation (Kumar and Subramanian 2002). The model implemented in G-PhoCS is conditioned upon a given phylogenetic topology. Thus, we ran G-PhoCS using the topology of the nuclear genomic tree (which is concordant with the Bayesian and MP mitochondrial trees, see the Results section below).

Song analyses

We analyzed 50 recordings belonging to the five subspecies of *T. ruficapillus* and covering the entire geographic distribution range of the species (see Table S2). Recordings were in 'wav' format and were obtained from the Macaulay Library of Natural Sounds (Cornell Lab of Ornithology) and Xeno-Canto.

We generated and analyzed the spectrograms using Raven Pro 1.5 (http://www.birds.cornell.edu/raven). The conditions for the analyses were a 512 fast Fourier transform length with a 50% overlap, a Hamming window type, and a grayscale color scheme. We analyzed one song per individual choosing the one with the best signal-to-noise ratio. Songs in this species consist of a repetition of similar notes. The first note is the longest, followed by successively shorter notes, until the last note, which is longer and similar to the first one. We measured five variables: mean duration of the note, mean duration of the interval between notes, fundamental frequency (calculated as the difference in frequency between the harmonics and measured in the center of each harmonic), bandwidth of the note (difference between the maximum and minimum frequencies of the note) and the number of notes in each song (Figure S1). Variables were measured on the first note, then additionally averaged between the second, fourth and sixth note to obtain a measurement of the intermediate notes, and finally again on the last note.

To assess vocal variation we performed a PCA and analyzed the data with a one-way analysis of variance (ANOVA) followed by Bonferroni's contrasts to assess differences among subspecies in their PC scores (all the principal component scores met the assumptions of homoscedasticity and normality). All variables were standardized previous to the analysis and we considered that variables were significantly correlated with a PC only when the module of their factor loading value was greater than 0.7 (Tabachnick and Fidell 2001).

Finally, we also performed an ANOVA using the original song variables, followed by Bonferroni's contrasts, because this can provide a more detailed analysis of specific differences among subspecies.

Plumage coloration analyses

To objectively describe plumage colour variation within *T. ruficapillus* , we obtained reflectance spectra measurements from museum skins deposited at the MACN (Table S3). We measured five males and four females of *T. r. cochabambae* and 30 males and 18 females of*T. r. ruficapillus* , which are the only two subspecies present in Argentina and available in the MACN collection. We used only adult specimens that were in excellent preservation condition for reflectance measurements.

Male plumage in this species is mostly brown (*T. r. cochabambae*and *T. r. ruficapillus*) or dark gray (*T. r. jaczewskii* ,*T. r. marcapatae* and *T. r. subfasciatus*), with rufous wings, a rufous or chestnut cap and barred underparts and tail. The species is sexually dichromatic, with brown females in all subspecies (i.e., no gray plumage) and either lack or very faint barring in their underparts and no white marking in their tales (Figure S2). Taking into account this colour patterning, we measured reflectance in six plumage patches on each specimen: throat, breast, belly, crown, nape and back. All reflectance measurements were performed with an Ocean Optics USB 2000 spectrometer (Ocean Optics Inc., Dunedin, Florida) with a PX-2 pulsed xenon light source (effective range of emission from 220 to 750 nm), calibrated against a WS-1 diffuse reflectance white standard (Ocean Optics, Inc., Dunedin, Florida, USA). Plumage was illuminated and reflectance data were collected with a bifurcated probe housed in a prismatic holder that was held against the chosen region on the study skin. The probe was held with an angle of 90^{o} to the surface of the plumage patch. The diameter of the circular measured area was approximately 6 mm, and the distance between the probe and the plumage was 23 mm.

We performed analyses using the pavo package in R (Maia et al. 2013). Because this species is sexually

dichromatic, males and females were analyzed separately. We evaluated colour differentiation in the chromatic component between subspecies for each plumage patch by estimating a perceptual distance ([?]S) using the Vorobyev and Osorio (1998) colour discrimination model. Colour perceptual distances are expressed in terms of just noticeable differences (jnd) and a value of 1.0 jnd represents the theoretical threshold for discrimination of two colours, meaning that it is the distance in the perceptual colour space at which two colours would be visually discernible (Barreira et al. 2021). Thus, [?]S > 1.0 jnd indicates significant colour discrimination by the birds in all light conditions (Vorobyev et al. 1998, Siddiqi et al. 2004). In addition, we determined the statistical significance of the estimated [?]S values for each plumage patch comparison between subspecies (Maia and White 2018). For this, we estimated the geometric average of [?]S values for all comparisons performed between subspecies and calculated the 95% confidence interval (CI) through a bootstrap analysis with the bootcoldist function of pavo in R. We also tested the significance of these comparisons through a PERMANOVA analysis with the adonis function of the vegan package in R (Oksanen et al. 2008).

Results

Genetic diversity and population structure based on mitochondrial markers

We analyzed the concatenated mitochondrial DNA sequences (COI + cyt b = 1,130 bp) of 24 individuals of *T. ruficapillus* belonging to the five subspecies (Table S1). The specimens were collected in 16 localities from Peru, Bolivia, Argentina and Uruguay, covering a wide range of the species distribution (Figure 1a). Our results showed considerable variation within *T. ruficapillus* and, in particular, the subspecies that inhabits the Atlantic Forest, *T. r. ruficapillus* , clearly differed from the four Andean subspecies, with a mean genetic differentiation between 2.6% and 3.6% (Table 1). There was also considerable variation among the Andean subspecies, with *T. r. cochabambae* showing the highest genetic divergence both with respect to *T. r. ruficapillus* and with the remaining Andean subspecies (Table 1). Lower levels of differentiation were observed between *T. r. subfasciatus* , *T. r. marcapatae* and *T. r. jaczewskii* and in fact we found almost no differentiation (0.2%) between *T. r. subfasciatus* and *T. r. marcapatae* .

Both the haplotype network (Figure 1b) and the AMOVA (Table 1) show a marked phylogeographic structure within *T. ruficapillus* , with three clearly differentiated lineages: one formed by the subspecies present in the Atlantic Forest (*T. r. ruficapillus*), another one by the subspecies distributed in the Andes of southern Bolivia and northwestern Argentina (*T. r. cochabambae*), and the last one containing the remaining Andean subspecies from northern Bolivia to Peru (see Figure 1b). Consistently, Φ_{ST} values from the AMOVA were high and significant between the subspecies of these three different lineages (Φ_{ST} 0.7-0.83), but were not significant between the three subspecies of the northern lineage (*T. r. subfasciatus* , T. *r. marcapatae* and *T. r. jaczewskii* ; see Table 1). The haplotype network confirmed a lack of differentiation and shared haplotypes between *T. r. subfasciatus*and *T. r. marcapatae* , and low genetic differentiation between these two subspecies and *T. r. jaczewskii* , despite its geographically isolated and disjunct distribution.

Phylogenetic analyses and diversification dating based on mitochondrial DNA

Bayesian and maximum parsimony (MP) reconstructions recovered *T. ruficapillus* as monophyletic in relation to *T. doliatus* with maximum statistical support (Figure S3). The time-calibrated ultrametric phylogeny (Figure 1c) shows that the split between them occurred 1.65 million years ago (Mya) (95% HDP: 0.82-2.27). These reconstructions show three clades that correspond to the lineages described above: one includes the subspecies from the Atlantic Forest (*T. r. ruficapillus*), another one is formed by *T. r. cochabambae* and the third one by *T. r. subfasciatus* , *T. r. jaczewskii* and*T. r. marcapatae* (see Figure 1c and Figure S3). In the case of the Bayesian and MP reconstructions the oldest split was between the subspecies from the Atlantic Forest and those from the Andean forest (Figure S3), with low values of both posterior probability and bootstrap support between the two Andean clades (0.63 and 63%, respectively). However, the time-calibrated ultrametric phylogeny indicated that the first split within the species, which occurred in the Pleistocene approximately 1.29 Mya (95% HPD: 0.71-2.10 Mya), was between the southernmost Andean subspecies (*T. r. cochabambae*) and the rest of the subspecies (Figure 1c). This analysis suggested a more recent divergence between the subspecies from the Atlantic Forest (*T. r. ruficapillus*) and the clade that

includes the rest of the Andean subspecies (*T. r. subfasciatus* , *T. r. jaczewskii* and *T. r. marcapatae*), approximately 0.97 Mya (95% HPD: 0.52-1.57 Mya). This split, however, also had low support (posterior probability: 0.58; Figure 1c).

Population structure, phylogenetic tree and demographic history modelling based on ddRAD markers

We obtained reduced-representation genomic data for 18 specimens of *T. ruficapillus* collected in 11 localities from Bolivia, Argentina and Uruguay that belong to the subspecies *T. r. ruficapillus* , *T. r. cochabambae* and *T. r. subfasciatus*(Figure 1a, Table S1) and represent the three clades obtained with mitochondrial DNA. The *de novo* assembly produced a total of 6,165 RADseq loci (13,485 SNPs) present in at least 80% of the individuals. This data set was used to perform the analyses described below.

Nuclear genomic variation was congruent with the results described for mitochondrial DNA, showing a clear differentiation between the three lineages. First, individuals clustered in the PCA in three clearly differentiated groups which corresponded to each of the subspecies (Figure 2a). The Structure analysis also supported the three main groups, as K = 3 was the model with the highest likelihood (Figure S4) and the three genomic clusters correspond to the mitochondrial lineages (Figure 2b). Only one individual of *T. r. ruficapillus* from Uruguay presented mixed genomic content, but the proportion of its genome belonging to the *subfasciatus* cluster was very low (less than 5%). The rest of the individuals did not show admixture in their genome, suggesting a lack of gene flow among these three subspecies.

The maximum likelihood reconstruction recovered a topology with the same three clades obtained in the Structure and PCA analyses, which also coincide with the mitochondrial lineages (Figure 2c). All nodes had maximum bootstrap support. The first split within the species occurred between *T. r. ruficapillus* (from the Atlantic Forest) and the subspecies from the Andean forest (*T. r. cochabambae* and *T. r. subfasciatus*), with a subsequent separation between these Andean subspecies. This topology is congruent with those obtained in the MP and Bayesian analysis with mitochondrial data (but differ from the topology of the calibrated ultrametric tree; see Figure 1c and Figure S3).

Our demographic analysis in G-PhoCS was based on the topology of the nuclear phylogeny, which indicated that the initial split occurred between the eastern T. r. ruficapillus and the Andean subspecies (*T. r. cochabambae* and *T. r. subfasciatus*), with a subsequent separation between the latter. The result, however, indicated that the three lineages split almost at the same time (Figure 3): *T. r. ruficapillus* diverged from the other two subspecies almost 1,7 million generations ago (Mga) (95% confidence interval: 1,5-2,0 Mga) and the two Andean subspecies diverged from each other only 440 generations after this first split. The effective population size of each of the subspecies is between one third and half of the ancestral effective population size estimated for the species (˜1.5M individuals). However, the most noticeable result in this regard is the very small estimated ancestral effective population size of the lineage leading to *T. r. subfasciatus* and *T. r. cochabambae* , which was estimated to be only 313 individuals. This result suggests that this population could have experienced a bottleneck after (or during) its split from *T. r. ruficapillus* , expanding afterwards to reach the current effective population size of the Andean subspecies (˜0.4M and ˜0.55M individuals; Figure 3). This is consistent with the fact that Andean populations could have been isolated in refugia during the glacial periods after their split from *T. r. ruficapillus* (Chaves et al. 2011). Finally, this analysis suggests very low gene flow between subspecies (Figure 3), a result consistent with the PCA and Structure analyses.

Vocalization analyses

The PCA evidenced differences in vocalizations among subspecies. The first three PCs explained 65% of song variation. PC1 correlated with the fundamental frequency of the first and the last note, PC2 correlated with the mean duration of the first and intermediate notes and PC3 did not strongly correlate with any song variable (Table S4). Subspecies statistically differed in PC1 (F = 11.32, p ¡ 0.01) and PC2 (F = 18.43, p ¡ 0.01), whereas no significant differences were found for PC3 (F = 2.87, p = 0.09). In particular, pairwise comparisons showed that *T. r. jaczewskii* significantly differed from *T. r. ruficapillus* and *T. r. cochabambae* in PC1 and from *T. r. marcapatae* in PC2 (Table S5). In turn, *T. r. cochabambae*differed from all subspecies in PC2 (Table S5). These differences can be observed in the space delimited by the first two PCs (Figure

4). No differences were found in the rest of the pairwise comparisons between subspecies for PC1, PC2 or PC3 (Table S5).

The results of the individual song variables provided more detail about song variation and showed substantial differentiation among subspecies. In fact, the ANOVA indicated significant differences for most song variables (Tables S6 and S7). Moreover, the Bonferroni contrasts showed significant differences in at least one variable for almost all subspecific comparisons, with the only exceptions of *T. r. subfasciatus* vs. *T. r. ruficapillus* and *T. r. subfasciatus* vs. *T. r. marcapatae* (Table S8). Therefore, and consistently with the PCA result, both *T. r. jaczewskii* and *T. r. cochabambae* significantly differed in their songs from all other subspecies, being the subspecies with the most differentiated vocalizations. In addition. *T. r. jaczewskii* was the subspecies that differed in more song variables from the rest (and particularly so from *T. r. cochabambae* and *T. r. ruficapillus*) (Table S8).

Coloration analyses

The quantification of plumage chromatic distances between females of *T. r. ruficapillus* (from the Atlantic Forest) and *T. r. cochabambae* (from the Andean forest) using the Vorobyev and Osorio (1998) model indicated that five plumage patches had differences in colour above the discrimination threshold ([?]S > 1.00 jnd): throat, breast, belly, crown and nape (Table 2). In turn, the comparison between males of these same two subspecies showed differences higher than 1.00 jnd for three plumage patches: throat, belly and nape (Table 2). The PERMANOVA, however, was not significant for any of the comparisons (Table 2), suggesting that the variation between subspecies was not significantly higher than the variation within each of them neither for females nor for males. This relatively subtle, non-significant colour differentiation between subspecies can also be observed in the reflectance spectra of the patches (Figure S5).

Discussion

The combination of mitochondrial and nuclear DNA, complemented with data on vocalizations and plumage coloration, allowed us to study the evolutionary history of *T. ruficapillus* . Our findings provide a good starting point towards understanding the history of diversification of this species, the relative patterns of genotypic and phenotypic variation, and the role played by the open vegetation corridor and the Andes Mountains in this process.

First, the mitochondrial DNA results suggest that the split between *T. doliatus* and the lineage that includes *T. ruficapillus*and *T. torquatus* (which are sister species; Brumfield and Edwards 2007) occurred 1.65 Mya, within the Pleistocene. This indicates that the origin of *T. ruficapillus* took place even more recently within the Pleistocene. The subsequent diversification within the species generated a phylogeographic structure with three main genetic lineages: one in the Atlantic Forest (*T. r. ruficapillus*), another one including the southernmost Andean subspecies (*T. r. cochabambae*), and finally one lineage formed by the three remaining Andean subspecies (*T. r. subfasciatus* , *T. r. marcapatae* and *T. r. jaczewskii*). The various analyses based on both the mitochondrial DNA and the nuclear genome recovered these three genetic clusters (although we note that for genomic analyses we were only able to include *T. r. subfasciatus* as a representative of the northern Andean lineage).

The relationship between these three main lineages is not clear and it can actually be considered a polytomy. The Bayesian and MP mitochondrial reconstructions, as well as the nuclear genomic phylogeny, suggested that the first split within the species took place between the lineage from the Atlantic Forest and an Andean lineage, with a subsequent separation within the latter. However, the time-calibrated ultrametric phylogeny inferred that the first split occurred between the two Andean lineages. Surprisingly, the topology of this ultrametric phylogeny indicates that shortly after the Andean split, the colonization of the lowland areas of the Atlantic Forest occurred from the northern Andean lineage and not from the lineage that inhabits northwestern Argentina and southern Bolivia, which is much geographically closer to the Atlantic Forest. This discordance in the relationship among linages could be due to the temporal proximity of both splits, a result common to all analyses. In particular, the time-calibrated ultrametric phylogeny based on mitochondrial DNA suggested that the first split within *T. ruficapillus* (in this reconstruction the split between

the two Andean lineages) occurred approximately 1.3 Mya, and the colonization of the Atlantic Forest approximately 1 Mya. The G-PhoCS analysis, which was based on nuclear DNA, suggested that the two splits between lineages took place approximately 1.7 million generations ago. If we consider that the generation time for passerines could be estimated to be around 1 year on average (Wang 2004, Cabanne et al. 2008), these two results (from the analyses of mitochondrial and nuclear genomic DNA) are relatively concordant. Consistent with the temporal proximity of these two deepest splits and their different sequence of occurrence depending on the reconstruction methodology, one should note that the support of the nodes that define the relationship of these three lineages was very low in all mitochondrial reconstructions (63% bootstrap support in the MP analysis, 0.63 posterior probability in the Bayesian phylogeny and 0.58 posterior probability in the time-calibrated phylogeny; see Figure 1c and Figure S3). Regardless of the subtle difference in the timing of the splits and the uncertainty in their order, our results are congruent in suggesting that the main events of diversification of *T. ruficapillus* that generated its current patterns of population diversity emerged in a short time period during the Pleistocene, shortly after the origin of the species.

Taking together our genetic and genomic results, including the lack of gene flow between the Atlantic Forest and Andean forest populations, we can confirm the role of the open vegetation corridor as a geographic barrier for *T. ruficapillus* . Nevertheless, the origin of the allopatric populations of this species should be explained as a result of dispersal through the open vegetation corridor and not as a vicariance event due to its establishment. This is because the Atlantic Forest and the Andean forest became isolated by this barrier before the Pleistocene (Costa 2003, Trujillo-Arias et al. 2017, Cabanne et al. 2019, Lavinia et al. 2019) and therefore before the origin of *T. ruficapillus* and the split between its Atlantic and Andean lineages (which is dated to approximately 1-1.7 Mya by our analyses). This suggests a relevant role of the Pleistocene climatic cycles on the diversification of *T. ruficapillus* , since the dispersion through the open vegetation corridor could have occurred during one of the periods of connection between the Atlantic and Andean forests. If the first split within the species occurred between the lineage from the Atlantic Forest and the ancestral lineage from the Andean forest (as suggested by most of our analyses, including the phylogeny based on genomic DNA), it is difficult to establish the direction of the dispersion and colonization. On the other hand, if the first split occurred within the Andes (as suggested by the time-calibrated Bayesian analysis), it could be considered that the species originated in the mountain range and after its first split between the Andean lineages the dispersion occurred to the east, allowing the colonization of the Atlantic Forest. In this second scenario, the Bayesian phylogeny indicates that the colonization occurred from the northern lineage of the Andean forest, suggesting that it could have taken place across the Cerrado as proposed by Silva (1994), and not across the Chaco as hypothesized by Nores (1992). We note that this is only a possibility since our lack of sampling on the northern portion of the species distribution in the Atlantic Forest prevents us from drawing robust conclusions about the route/s of connection. Moreover, the fact that *T. doliatus* (the sister species of *T. ruficapillus*) is widely distributed to the north of *T. ruficapillus* and also has populations in the east and west of the Neotropics, precludes us from assuming which is the area in which these two species may have split, which could have helped distinguishing among hypothesized colonization scenarios. Further studies with a more geographically comprehensive sampling of *T. ruficapillus* and the inclusion of *T. doliatus* are needed to fully elucidate this matter.

The Andes Mountains have also played a relevant role in the diversification of *T. ruficapillus* , as indicated by the existence of two distinct Andean lineages without evidence of gene flow. Surprisingly, these two lineages do not coincide with the two disjunct areas of distribution of this species in the Andes (at least from a mitochondrial perspective; see Fig. 1): one lineage is formed by the populations of *T. r. cochabambae* (from northwestern Argentina and southern Bolivia) and the other one is constituted by the three remaining Andean subspecies (*T. r. subfasciatus* , *T. r. marcapatae* and *T. r. jaczewskii* from northern Bolivia and Peru, which span both areas of disjunct distribution in the mountain range and only exhibit a shallow differentiation in the case of *T. r. jaczewskii* , the northern subspecies that is geographically isolated). This pattern suggests that there is a geographic barrier in northern Bolivia, which in fact coincides with phylogeographic breaks found in the same area in various other montane bird species (Cadena et al. 2007, Weir 2009, Chaves et al. 2011, Valderrama et al. 2014, Gutiérrez-Pinto et al. 2019). Given that our analyses indicated that the

divergence between these two Andean lineages occurred around 1.3-1.7 Mya, during the Pleistocene, one could hypothesize that Pleistocene climatic oscillations have also played a role in the diversification of this species in the Andes (Weir 2006, 2009, Chaves et al. 2011, Valderrama et al. 2014).

The vocal analyses showed significant differences in one or various song variables between most of the subspecies, which at first glance seems to be consistent with the presence of a marked phylogeographic structure. In fact, only two of the subspecies comparisons did not show vocal differentiation. One of them was between *T. r. subfasciatus* and *T. r. marcapatae* , which were also the pair of subspecies with the lowest genetic divergence and shared haplotypes. The second pair of subspecies without song differences was *T. r. ruficapillus* and *T. r. subfasciatus* , an unexpected result given that they belong to different mitochondrial lineages and their areas of distribution are disjunct, with the former present in the Atlantic Forest and the latter in the Andean forest of northwestern Bolivia. On the other extreme of our results, *T. r. jaczewskii* was one of the most vocally differentiated subspecies, significantly differing in its song from all other subspecies. This result could be expected given that *T. r. jaczewskii* is geographically isolated from the other populations of the species, but contrasts with the relatively shallow mitochondrial DNA differentiation in this subspecies (nuclear DNA could not be analysed), particularly when compared with *T. r. subfasciatus* and *T. r. marcapatae* , which in fact are part of its same lineage but significantly differed in their songs.

The finding of these discordances between the genetic/genomic patterns of divergence and vocal differentiation in *T. ruficapillus* is surprising given that this species is a suboscine, and therefore its song is likely developed without learning (Kroodsma and Konishi 1991, Isler et al. 1998, Touchton et al. 2014), being free from the influence of cultural evolution and possessing a more direct connection with genetic divergence than in oscines (Seddon 2005). In fact, previous intraspecific studies of suboscine song variation have shown more concordant patterns of vocal and genetic structuring (Isler et al. 2005, García et al. 2018, Acero-Murcia et al. 2021, Bukowski et al. 2024), although with some cases of divergent lineages lacking song differentiation in suboscines (García et al. 2018) and other birds with innate songs (Nwanko et al. 2018), as was the case for *T. r. ruficapillus* and *T. r. subfasciatus* in this study. Future vocal studies of this species should analyse the putative effect of natural and sexual selection, which could cause different patterns of song variation compared to neutral genetic markers even in species with innate songs (García et al. 2018, 2023). On the other hand, the vocal pattern of variation in this species can also be affected by the adaptation to habitat characteristics and their effect on sound transmission, which could delay or accelerate song differentiation in relation to genetic phylogeographic patterns, depending on the similarity or disparity of the various forest types along the species distribution (Morton 1975, Wiley 1991, Seddon 2005, Tubaro and Lijtmaer 2006).

Finally, and in spite of their allopatry and marked mitochondrial and nuclear DNA differentiation, no significant differences were found between the Atlantic Forest subspecies *T. r. ruficapillus* and the Andean subspecies *T. r. cochabambae* in plumage coloration (neither for males nor for females). The lack of correlation between colour differentiation and genetic divergence among intraspecific lineages has been found in previous studies of Neotropical birds (e.g. Trujillo-Arias et al. 2020, Paulo et al. 2023), but analyses including the remaining three subspecies, as well as a larger geographic sampling, are needed to better assess the pattern of colour variation in this species.

Final remarks

Our analyses of the evolutionary history of *T. ruficapillus* shows that this species originated in the Late Pleistocene, within the last 1.7. million years, and diversified into three clearly differentiated lineages: an Atlantic Forest lineage and two different lineages in the Andes, with a split in western Bolivia. This pattern of genetic variation highlights the relevance of the open vegetation corridor and the Andes Mountains as diversification drivers for this species, likely in combination with the effect of the Pleistocene climatic cycles. Levels of phenotypic variation, however, are partially discordant with this genetic structuring. Songs are significantly differentiated among subspecies, but their geographic pattern of variation does not coincide with that shown by mitochondrial or nuclear DNA. We found clearly differentiated songs between subspecies that are genetically similar and, on the contrary, lack of detectable song differentiation between the Atlantic Forest subspecies and that of northwestern Bolivia in the Andes, which present a substantial genetic differentiation

and belong to different lineages. Similarly, plumage colouration between the Atlantic Forest subspecies and that of the southern Andes did not differ significantly. Future studies should address these discordances with an increased sampling and the analysis of the effect of evolutionary and ecological pressures on song and colour. Finally, our results are in accordance with those of other birds with disjunct populations in the Atlantic and Andean forests for which the open vegetation corridor has promoted diversification (e.g. Lavinia et al. 2015, 2019, Trujillo Arias et al. 2018, 2020, Cabanne et al. 2019). However, these studies show that the level of differentiation between the populations of these forests is notoriously heterogeneous, suggesting an idiosyncratic effect of this barrier and the cycles of connection and isolation of these forests and highlighting the importance of comparative analyses to better understand the role of the main drivers of avian Neotropical diversification and speciation.

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Table 1. Genetic pairwise comparisons among the five subspecies of*Thamnophilus ruficapillus* based on mitochondrial DNA (COI + cyt$b$). Above the diagonal: pairwise Φ_{ST} values; significant values ($p < 0.05$) in bold. Diagonal: mean uncorrected genetic distances within each subspecies. Below the diagonal: mean uncorrected genetic distances between pairs of subspecies. Genetic distances are expressed in percentage values.

Subspecies	*T. r. jaczewskii*	*T. r. marcapatae*	*T. r. subfasciatus*	*T. r. cochabambae*	*T. r. ruficapillus*
T. r. jaczewskii	0.1	0.92	0.59	**0.83**	**0.73**
T. r. marcapatae	0.6	0	0.15	**0.82**	**0.70**
T. r. subfasciatus	0.8	0.2	0.4	**0.83**	**0.73**
T. r. cochabambae	3.5	3.3	3.4	0.7	**0.74**
T. r. ruficapillus	3	2.6	2.7	3.6	0.3

Table 2. Colour differentiation ([?]S) among females of *T. r. ruficapillus* and *T. r. cochabambae* , and among males of *T. r. ruficapillus* and *T. r. cochabambae* . [?]S represents relative chromatic perceptual distance between females of the two subspecies analyzed and between males of the two subspecies analyzed for each plumage patch. Values are geometric means (and 95% confidence intervals) for all comparisons. The unit of [?]S is jnd (just noticeable differences). Values above 1.0 jnd, the theoretical threshold for colour discrimination, are underlined. The PERMANOVA results were statistically not significant in all comparisons.

Plumage patch	Females	Females	Females	Males	Males	Males
	N *T. r. ruficapillus*	N *T. r. cochabambae*	[?]S	N *T. r. ruficapillus*	N *T. r. cochabambae*	[?]S
Throat	18	4	1.39 (0.22-3.24)	30	5	1.12 (0.39-2.32)
Breast	18	4	1.63 (0.48-2.77)	30	5	0.91 (0.24-1.89)
Belly	18	4	1.53 (0.54-2.59)	30	5	1.61 (0.67-2.57)
Crown	18	4	2.31 (0.59-4.45)	30	5	0.71 (0.41-2.61)

Plumage patch	Females	Females	Females	Males	Males	Males
Nape	18	4	2.34 (0.89-3.75)	30	5	1.20 (0.42-6.95)
Back	18	4	0.99 (0.24-2.72)	30	5	0.46 (0.24-1.99)

Figure legends

Figure 1. Distribution of *T. ruficapillus* , sampling information and analyses of mitochondrial DNA. (a) Distribution map (shaded in violet) showing sampling localities, subspecies identity and the type of sequence data obtained for each sample. The distribution map is based on BirdLife International and NatureServe (2014). (b) Median-joining haplotype network of concatenated mDNA (COI + cyt *b* ; 1,130 bp). Circles represent haplotypes and their size is proportional to haplotype frequency. The length of the branches connecting haplotypes is proportional to the number of nucleotide differences between them, which are indicated by the number of line marks on each branch. Colors represent the subspecies according to the scheme incorporated in the figure. Black circles represent unsampled hypothetical haplotypes. Bird illustrations are from del Hoyo (2020). (c) Bayesian phylogenetic tree with divergence times obtained from the analysis of the concatenated mitochondrial dataset (1,130 bp). The numbers near the nodes indicate the mean divergence time estimates and the numbers between square brackets correspond to the 95% highest posterior density intervals of those estimates. Divergence times and the numbers between square brackets are in millions of years ago. Posterior probability values are shown below the divergence times. The colors to the right of the tree indicate subspecies information.

Figure 2. Genomic analyses of *T. ruficapillus* . (a) Principal Component Analysis (PCA) based on 2,306 SNPs (SNPs present in all individuals). (b) Structure plot for K=3 based on 5,073 SNPs (one random SNP per RAD locus). (c) Phylogeny inferred with maximum likelihood; the colours to the right of each individual indicate the subspecies. The tree was rooted with *Thamnophilus caerulescens* . Bootstrap support values are indicated in the nodes.

Figure 3. Demographic reconstruction of *T. ruficapillus* based on 6,165 RADseq loci (13,485 SNPs). Estimates of divergence times (T; in millions of generations), migration rates (mg; in migrants per generation) and current and ancestral effective population sizes (Ne; in millions of individuals) are reported with their 95% confidence intervals.

Figure 4. Analysis of the vocalizations of *T. ruficapillus* . Song differentiation among subspecies is shown based on a PCA performed with the song variables (mean duration of the note, mean duration of the interval between notes, fundamental frequency, bandwidth of the note and the number of notes in each song; the first four variables were measured for the first note).