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Non-monophyly and deep genetic differentiation across low-elevation barriers in a Neotropical montane bird (*Basileuterus tristriatus*; Aves: Parulidae)

Natalia Gutiérrez-Pinto [a,*,1], Andrés M. Cuervo [b,1], Jhonathan Miranda [c], Jorge L. Pérez-Emán [d,e], Robb T. Brumfield [b], Carlos Daniel Cadena [a]

[a] Laboratorio de Biología Evolutiva de Vertebrados, Departamento de Ciencias Biológicas, Universidad de Los Andes, Bogotá, Colombia
[b] Department of Biological Sciences and Museum of Natural Science, Louisiana State University, Baton Rouge, LA 70803, United States
[c] Postgrado de Ecología, Facultad de Ciencias, Universidad Central de Venezuela, Av. Los Ilustres, Los Chaguaramos, Apartado Postal 47058, Caracas 1041-A, Venezuela
[d] Instituto de Zoología y Ecología Tropical, Facultad de Ciencias, Universidad Central de Venezuela, Av. Los Ilustres, Los Chaguaramos, Apartado Postal 47058, Caracas 1041-A, Venezuela
[e] Colección Ornitológica Phelps, Apartado 2009, Caracas 1010-A, Venezuela

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ABSTRACT

Most widespread birds of Neotropical cloud forests exhibit phenotypic variation that is partitioned geographically suggesting allopatric divergence, but little is known about the extent to which such phenotypic differentiation is consistent with genetic variation. We studied geographic patterns of genetic differentiation in the Three-striped Warbler (*Basileuterus tristriatus*), a polytypic and widespread understory bird of the foothills and mid-elevation zone of the tropical Andes and adjacent mountains of Central and South America. We sequenced mitochondrial DNA for 196 samples covering the entire range of *B. tristriatus*, as well as 22 samples of its putative closest relatives: the Three-banded (*B. trifasciatus*) and Santa Marta (*B. basilicus*) warblers. We found deep genetic structure across the range of *B. tristriatus*, which consisted of ten major clades including *B. trifasciatus*, a species that was nested within *B. tristriatus*. In contrast, *B. basilicus* was not closely related to *B. tristriatus* but part of a clade of *Myiothlypis* warblers. Geographic boundaries among clades were clearly related to lowland gaps separating subspecies groups. The subspecies *melanotis* of the mountains of Central America was sister to a large clade including *B. t. tacarcunae*, and the rest of South American clades, including *B. trifasciatus*. Five clades are found in the northern Andes, where no signs of gene flow were found across barriers such as the Táchira Depression or the Magdalena valley. Our study highlights the importance of valleys in promoting and maintaining divergence in a lower montane forest bird. The substantial genetic and phenotypic differentiation, and the paraphyly uncovered in *B. tristriatus*, may call for revising its species boundaries.

1. Introduction

Understanding the processes underlying spatial patterns of biological diversity is one of the central goals of evolutionary biology. One outstanding biogeographic pattern is the high diversity of avian species in humid forests of the tropical Andes (Fjeldså and Irestedt, 2009; Hawkins et al., 2007; Stotz et al., 1996). The high avian diversity in Neotropical mountains resulted from a number of processes involved in its evolutionary assembly, including population divergence of isolated populations after vicariance or range shifts (Brumfield and Edwards, 2007; Fjeldså and Irestedt, 2009; Weir, 2009). The topographically and ecologically complex landscapes of the Neotropical mountains offer multiple opportunities for diversification and speciation (Kattan et al., 2004; Ruggiero and Hawkins, 2008; Rull, 2011; Vuilleumier, 1970). However, permeability of barriers is dynamic, and coupled with environmental fluctuations, may elicit demographic changes in diverging populations (d'Horta et al., 2011; Excoffier et al., 2009; Smith et al., 2011). Although broadly distributed along the Andean spine and adjacent mountain systems, humid montane forests are not continuous; rather, they are abruptly interrupted by low-elevation gaps (e.g., dry valleys) and by high elevation ridgelines. Restricted dispersal between isolated montane populations may lead to population differentiation. An indication of this in the Neotropical montane avifauna is the pervasive geographic variation in plumage associated with geographic barriers (Graves, 1985; Remsen, 1984; Vuilleumier, 1970). Accordingly, geographically variable

* Corresponding author.
E-mail addresses: n.gutierrez126@uniandes.edu.co (N. Gutiérrez-Pinto), acuerv1@tigers.lsu.edu (A.M. Cuervo), biojhonathan@gmail.com (J. Miranda), jorge.perez@ciens.ucv.ve (J.L. Pérez-Emán), brumfld@lsu.edu (R.T. Brumfield), ccadena@uniandes.edu.co (C.D. Cadena).
[1] These authors contributed equally to this work.

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species with wide but discontinuous geographic ranges in tropical mountains allow testing the role of low-elevation gaps on population divergence and speciation.

The Three-striped Warbler (*Basileuterus tristriatus*, Parulidae) is an understory nine-primaried oscine bird that inhabits mature and secondary forests in humid slopes of tropical mountains from Costa Rica and Panama in Central America, and along the Andes and associated mountain ranges from northeastern Venezuela to Bolivia (Curson, 2010; Stotz et al., 1996). *Basileuterus tristriatus* exhibits high phenotypic diversity (Chapman, 1924; Zimmer, 1949), with 12 currently recognized subspecies (Curson, 2010; Howard and Dickinson, 2003) mostly separated by geographic barriers (Fig. 1).

The geographic pattern of phenotypic variation in *B. tristriatus* suggests that its populations have differentiated in isolation and may indicate ongoing speciation associated with geographic barriers. In addition, the degree of phenotypic differentiation among some populations of *B. tristriatus* arguably exceeds the divergence in plumage pattern and coloration existing among several species of *Basileuterus*, suggesting *B. tristriatus* might comprise more than one species. Moreover, some authors have suggested that two *Basileuterus* species with restricted and allopatric distributions with respect to *B. tristriatus* could be close relatives based on superficial plumage resemblance: the Three-banded Warbler (*B. trifasciatus*) of the arid foothills of Tumbes and the dry Huancabamba valley of southwestern Ecuador and northwestern Peru (Hellmayr, 1935; Todd, 1929), and the Santa Marta Warbler (*B. basilicus*) of the Sierra Nevada de Santa Marta in northern Colombia (Hellmayr, 1935; Todd, 1929). Although *B. trifasciatus* was also hypothesized to be conspecific with *B. culicivorus* (Curson, 2010; Zimmer, 1949), it has been found to be sister to *B. tristriatus* (Lovette et al., 2010); however, with only one individual sampled per species it was not possible to discern whether *B. trifasciatus* and *B. tristriatus* constitute reciprocally monophyletic groups. The evolutionary affinities of *B. basilicus* remain uncertain because it has not been included in any phylogenetic study.

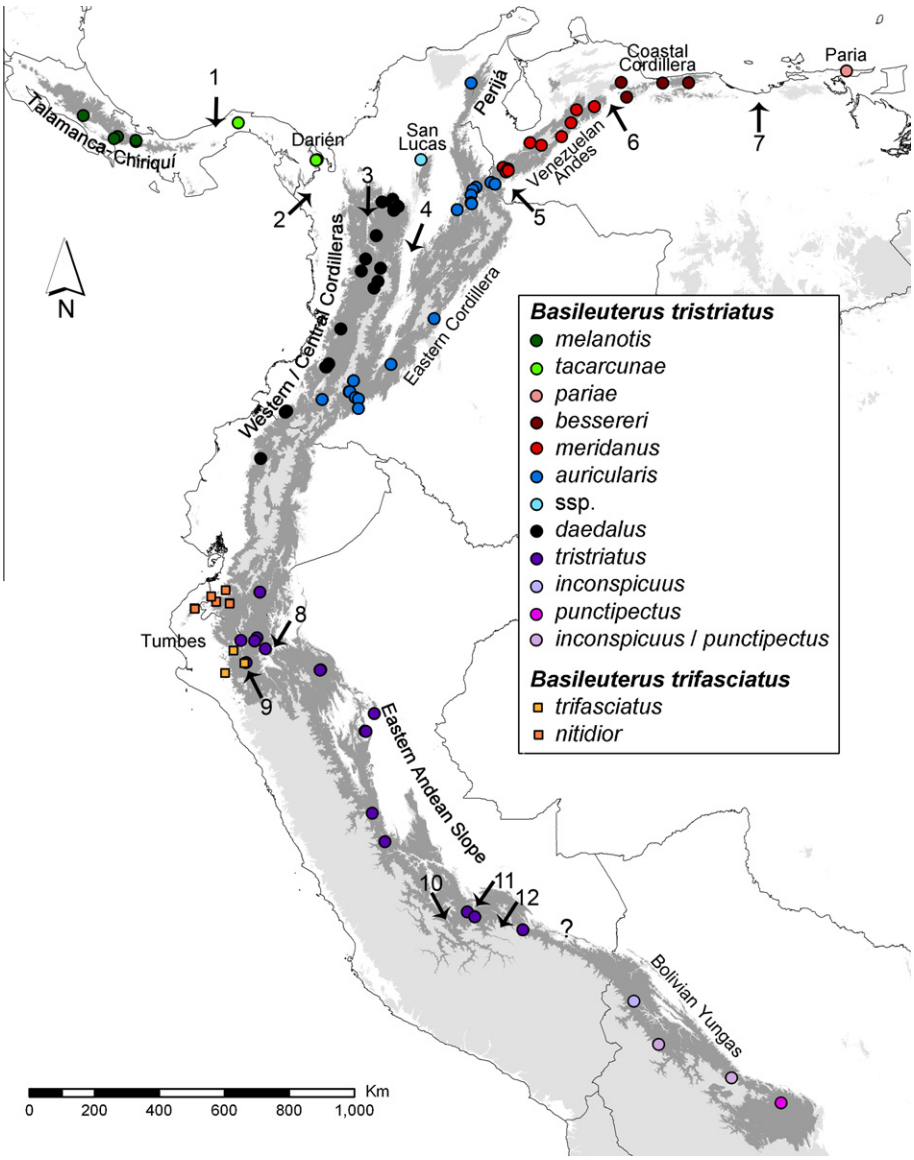

Fig. 1. Map of montane regions in Central and South America (light gray) with the approximate geographic distributions (in dark gray), and sampling localities of the Three-striped (*Basileuterus tristriatus*, colored dots) and Three-banded Warbler (*B. trifasciatus*, colored squares). Each color corresponds to the assumed sampled subspecies. Numbers correspond to potential barriers or geographic locations mentioned in the text: (1) central Panama lowlands; (2) Urabá lowlands; (3) Cauca valley; (4) Magdalena valley; (5) Táchira Depression; (6) Turbio-Yaracuy Depression; (7) Unare Depression; (8) North Peruvian Low (NPL); (9) dry Huancabamba valley; (10) Apurímac valley; (11) Urubamba valley; (12) Vilcanota cordillera. The question mark denotes the uncertain location where *B. t. tristriatus* and *B. t. inconspicuus* replace each other in Puno, Peru. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Here we use a large sample of mitochondrial DNA (mtDNA) sequences obtained from specimens covering the distribution of *B. tristriatus* to investigate the impact of historical processes (divergence in isolation, gene flow, demographic processes) on genetic differentiation with a focus on patterns across geographic barriers. We first investigated the phylogenetic position of *B. basilicus* and *B. trifasciatus* with respect to different populations of *B. tristriatus*. Second, we examined the geographic correspondence between genetically defined populations and phenotypically defined units in the group. Third, we inferred levels of gene flow across putative barriers and assessed historical changes in population size.

2. Materials and methods

2.1. Sampling

Vouchered tissue samples were collected in the field or obtained from natural history collections either as frozen tissues or toe pads of study skins. We aimed to cover all major geographic regions across the range of *B. tristriatus* and to include samples in the vicinity of geographic breaks. A small number of unvouchered samples (18) were included to augment sample size of certain subspecies (Appendix Table A1). Samples were unavailable from the remote coastal range of Serranía de la Macuira in northern Colombia (Marinkelle, 1970). Individual samples were ascribed to subspecies based on a comparative examination of specimens relative to original descriptions and their geographic provenance, but in some cases it was difficult to assign individuals to subspecies due to phenotypic intergradation at intermediate locations (e.g. *inconspicuus* vs. *punctipectus* and *bessereri* vs. *meridanus*), in which case we used only geographic provenance to assign them to a population (see Todd, 1929; Zimmer, 1949). We generated sequence data for 196 individuals referable to 10 of the 12 subspecies of *B. tristriatus*, for 20 individuals of the two subspecies of *B. trifasciatus*, and for two individuals of *B. basilicus*. We sampled representatives of all named forms with the exception of *B. t. baezae* from the eastern slope of the Ecuadorian Andes and *B. t. canens* from Santa Cruz, Bolivia (probably part of a *inconspicuus*/*punctipectus* cline, see below). We sequenced or obtained data from GenBank for 12 outgroup taxa selected based on previous phylogenetic studies (Lovette and Bermingham, 2002; Lovette et al., 2010).

2.2. DNA isolation, amplification and sequencing

Genomic DNA was extracted from ~20 mg of tissue using either the DNeasy extraction kit (Qiagen, Valencia, CA), following the manufacturer's protocol, or using the phenol-chloroform (PC) method. The PC method was applied as follows. First, we added 300 μl of pre-heated (60 °C) CTAB and 2 μl of Proteinase-K to a crushed piece of tissue for cell lysis, incubating for 24 h at 65 °C. We then followed the phenol DNA extraction protocol as described by Sambrook and Russell (2001), with two ethanol precipitations (98% and 70%, respectively) and elution of the DNA pellet in 40 μl of TE buffer. In the case of toe pad tissue samples, lysis time was extended up to 5 days and the elution volume was 30 μl.

We amplified the entire second subunit of the NADH dehydrogenase mitochondrial gene (ND2; 1041 bp) using primers L5215 (Hackett, 1996) and H6313 (Johnson and Sorenson, 1998), and internal primers L5758 and H5766 for sequencing (Brumfield et al., 2007). PCR conditions and sequencing protocols used are described elsewhere (Brumfield et al., 2007). Raw sequence data from light and heavy strands were inspected, edited, and aligned using Sequencher 4.7 (GeneCodes Corp., Ann Arbor, MI) or Geneious v5.4 (Drummond et al., 2011). Sequences generated in this study were deposited in GenBank (accession numbers JQ727209-JQ727429).

2.3. Phylogenetic delineation of the Basileuterus tristriatus complex

We first sought to evaluate whether *B. trifasciatus* and *B. basilicus* are members of the *B. tristriatus* complex as hypothesized by earlier workers. To investigate the relationships among these taxa, we conducted phylogenetic analyses including two samples of *B. basilicus*, two of *B. trifasciatus*, four from disjunct populations of *B. tristriatus*, and nine representatives of major clades of Parulidae, including the type species of the genera *Myiothlypis* (*M. nigrocristata*) and *Basileuterus* (*B. culicivorus*) (Lovette et al., 2010). Two outgroup taxa were chosen, the distantly related oscine passerines *Zeledonia coronata* and *Spindalis zena* (Lovette and Bermingham, 2002).

We first estimated the ND2 gene tree topology and divergence times jointly in the Bayesian program BEAST 1.6.1 (Rambaut and Drummond, 2007). We ran the analyses assuming a relaxed uncorrelated lognormal molecular clock model, a Yule speciation tree prior, a UPGMA starting tree, and a GTR + Γ substitution model. The clock model was estimated from a lognormal distribution with a mean of 0.0125 substitutions/site/lineage/myr (SD = 0.1), a prior distribution sufficiently wide to include the 2.1% divergence per myr rate estimated for cytochrome *b* (Lovette, 2004; Weir and Schluter, 2008) and the faster rates (~2.5%) of the avian ND2 gene (Smith and Klicka, 2010). The analysis ran for 50 million steps sampled every 1000 steps. The output was examined using Tracer 1.5 (Rambaut and Drummond, 2007) to confirm convergence among MCMC parameter estimates. All parameters effective sample size values (ESS) were at least 1000 and generally >2000. The first 10 million steps were discarded as burn-in. In addition, we also estimated the ND2 genealogy using a maximum-likelihood (ML) analysis in RAxML 7.0.4 (Stamatakis, 2006) and another Bayesian inference (BI) analysis in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), via the Bioportal at the University of Oslo, Norway. The ML inference in RAxML implemented the general-time reversible model of nucleotide substitution with gamma-distributed rate-heterogeneity among sites (GTR + Γ) and 25 rate categories for Γ (instead of the typical 4) to account for invariant sites (I) as recommended by the RAxML author (Stamatakis, 2006). All free model-parameters were estimated by RAxML, and nodal support was assessed with 1000 through-bootstrap replicates. The BI analysis consisted of four independent runs of one cold and three heated Metropolis-coupled Markov chains (MCMC) with incremental heating temperature of 0.125 and ran for 20 million generations, sampling parameters and trees every 1000 generations. MrModelTest 2.3 (Nylander, 2004) was used in conjunction with PAUP* 4.0b10 (Swofford, 2002) to find the best-fit substitution model under the Akaike information criterion (AIC). The best-fit model of nucleotide substitutions for the data set employed was GTR + I + Γ. The graphical output of Tracer 1.5 (Rambaut and Drummond, 2007) and convergence diagnostics from MrBayes were used to confirm convergence among independent MCMC runs. Posterior probabilities of clades were estimated discarding the first 10,000 trees as burn-in. We used the package SumTrees 3.1 to calculate nodal support and summarize trees (Sukumaran and Holder, 2010).

2.4. Phylogeography and historical demography

We estimated an ND2 gene tree using RAxML (ML) and MrBayes (BI), including 196 samples of *B. tristriatus*, 20 samples of *B. trifasciatus*, and a subset of six outgroup taxa (*Seiurus aurocapilla*, *Setophaga ruticilla*, *Myioborus miniatus*, *Basileuterus culicivorus*, *B. lachrymosus*, and *B. basilicus*). A partition-by-codon-position

scheme was selected via AIC as the best of three partition alternatives analyzed in RAxML. The partitioned ML analysis implemented the GTR + Γ substitution model for each codon position with individual per partition branch length optimization and 20,000 bootstrap replicates. The best-fit models of nucleotide substitutions selected by MrModelTest for each codon partition and implemented in the BI analysis were GTR + I + Γ for the first and third codon positions, and HKY + I for the second codon position. All estimated parameters were unlinked across partitions. The BI analysis consisted of six MCMC chains ran for 20 million generations, sampling every 1000 generations. Posterior probabilities of clades and the consensus tree were estimated after discarding the first 10,000 trees (50%) as burn-in. To visualize genetic diversity and possible geographic associations among haplotypes, we constructed median joining networks for each major clade identified in phylogenetic analysis using the program Network v.4.6 (Bandelt et al., 1999). We characterized genetic diversity for the complex for each major clade calculating the following summary statistics in Arlequin 3.5 (Excoffier and Lischer, 2010): number of polymorphic sites (*var*), haplotype number (*h*), gene diversity (*Hd*) and nucleotide diversity (π), as well as Tajima's *D* and Fu's *F* to assess deviations from neutrality. We calculated pairwise genetic distances within and between clades using MEGA5 (Tamura et al., 2011). Genetic differentiation among populations was evaluated using Φ_{ST} statistics calculated from a pairwise distance matrix among haplotypes, and their significance was estimated via 20,000 permutations of haplotypes among populations using Arlequin 3.5 (Excoffier and Lischer, 2010).

To assess to what extent prominent geographical features and their associated ecological discontinuities serve as barriers to gene flow among populations of *B. tristriatus*, we estimated gene flow across six potential barriers: (1) the Turbio-Yaracuy Depression, (2) the Táchira Depression, (3) the Magdalena River valley, (4) the Cauca River valley, and (5) the North Peruvian Low (Fig. 1). These geographic features were chosen because they had enough sampling at both sides of the barrier, and coincide with variable levels of phenotypic (Graves, 1982; Vuilleumier, 1969) and genetic differentiation (Bonaccorso, 2009; Cadena et al., 2007; Chaves and Smith, 2011; Miller et al., 2007) of other Andean species, or correspond to subspecies boundaries in *B. tristriatus*. We estimated the migration rate between all available samples of the populations pairs separated by these low-elevation gaps using the MDiv program (Nielsen and Wakeley, 2001). Each run consisted of five million generations using the finite sites model, discarding the first 1.25 million as burn-in and setting the maximum values for the scaled migration rate to 10 and for the scaled divergence time to 5.

Finally, we looked for evidence of historical signatures of fluctuations in population size for each major clade by examining Bayesian skyride plots (Minin et al., 2008) in BEAST 1.6.1 (Rambaut and Drummond, 2007), which depicts a coalescent-based estimation of population size change over time. We used a strict molecular clock with a rate of 2.5% Ma^{-1}, a starting UPGMA tree, the best-fit substitution model for each population, and ran each analysis for 50 million generations sampling parameters every 1000 generations. We discarded the first 5 million (10%) generations as burn-in, and all ESS parameters were at least 1000 and generally >2000.

3. Results

3.1. Phylogeny of the Basileuterus tristriatus complex

Phylogenetic reconstructions by ML and BI analyses recovered the same pattern and topology as the time-calibrated gene tree estimated using BEAST; thus, we present only the latter. We found that *B. tristriatus* is paraphyletic because *B. trifasciatus* is nested within it with high statistical support (Fig. 2). We refer to the clade formed by these two species as the *B. tristriatus* complex. The expanded geographic sampling used for phylogeographic analyses (Section 3.2 below) also supports the position of *B. trifasciatus* as a highly differentiated lineage within *B. tristriatus*. In contrast, contrary to expectations from superficial phenotypic resemblance, particularly of the immature plumage (Hellmayr, 1935), we found that the Santa Marta endemic *B. basilicus* is not part of the *B. tristriatus* complex. Rather, the ML and BI results indicate that *B. basilicus* is part of a clade of *Myiothlypis* warblers (Fig. 2). The molecular-clock analysis indicated that the crown age of the *B. tristriatus* complex dates from the late Pliocene to the early Pleistocene, between 3.5 and 1.8 Ma (mean 2.6 Ma). Based on the topology, the lineage leading to *B. trifasciatus* diverged soon after, between 2.3 and 0.9 Ma (mean 2.0 Ma), which is roughly coincident with the timing of differentiation of all the other major lineages of *B. tristriatus* in South America (Fig. 2, Appendix Fig. A1).

3.2. Phylogeography

3.2.1. Sequence variation

The ingroup alignment consisted of 216 sequences of the complete ND2 gene (including 20 of *B. trifasciatus*); 176 sites (16.9%) of the total 1041 bp were variable and 143 (13.7%) parsimony-informative. Among variable sites, 154 substitutions were synonymous and 19 non-synonymous.

3.2.2. Population structure

The ND2 gene tree revealed deep genetic structure across the range of the *B. tristriatus* complex. The group consists of at least 10 major lineages (Fig. 3; Table 1). A Central American lineage (clade A-subspecies *melanotis*) was consistently recovered, albeit with moderate support, as sister to the rest of the complex (Fig. 3). The lowland gap of central Panama separates clade A from populations of the San Blas and Darién mountains (clade B-*tacarcunae*). In agreement with the phylogenetic results (Section 3.1), *B.*

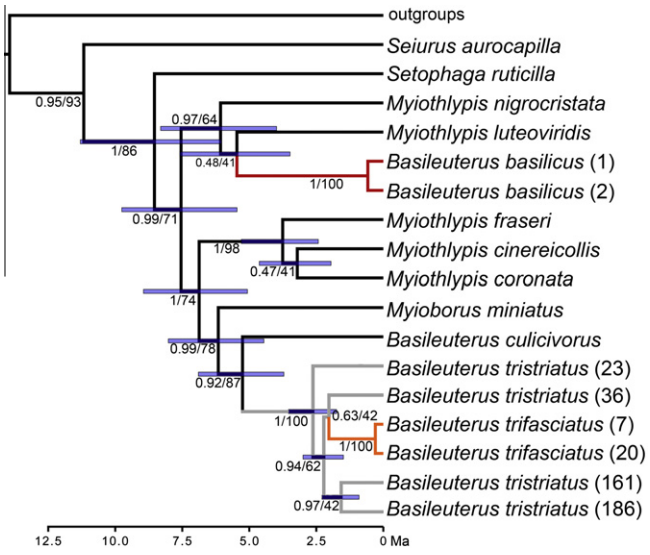

Fig. 2. Time-calibrated consensus ND2 tree from the BEAST analysis. The taxon set included representatives from the Parulidae to evaluate the phylogenetic position of the Three-striped Warbler (*Basileuterus tristriatus*, gray), Santa Marta Warbler (*B. basilicus*, red), and the Three-banded Warbler (*B. trifasciatus*, orange). Branch numbers denote posterior probabilities (from MrBayes) and bootstrap values (from RAxML), respectively. Node bars represent the 95% high posterior density of mean divergence time in millions of years (Ma). Numbers at the end of some samples refer to the individuals used for this analysis, as they are identified in Table A1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

trifasciatus was found as divergent clade (clade C) with no clear affinities within the *B. tristriatus* complex. No structure was found within *B. trifasciatus* associated with subspecies (*trifasciatus* and *nitidior*) nor geography.

Five of seven South American clades of *B. tristriatus* are found in mountain ranges in the northern part of the continent. One occurs in the Central and Western cordilleras of Colombia and Ecuador (clade F-*daedalus*) and is largely separated by the Magdalena valley from its closest relative: the population of the Eastern cordillera and the Serranía de Perijá (clade G-*auricularis*). A differentiated population is found in the isolated Serranía de San Lucas in central Colombia (Fig. 1); this population (clade D) has no clear affinities within the complex (Fig. 3) and was significantly divergent from the adjacent populations of the Central ($\Phi_{ST} = 0.801$, $p < 0.005$; mean uncorrected distance = 0.046) and Eastern cordillera ($\Phi_{ST} = 0.816$, $p < 0.005$; mean uncorrected distance = 0.039). The last two clades from northern South America correspond to the distinctive population of the Venezuelan Andes and Coastal cordillera (clade I-*meridanus*/*bessereri*), which formed a group sister to the lineage of the highlands of the Paria Peninsula in eastern Venezuela (clade J-*pariae*).

The remaining two clades of *B. tristriatus* occur along the eastern slope of the Andes. One is widespread, ranging from southeastern Ecuador to southern Peru (clade H-*tristriatus*) and showing genetic subdivision associated with two barriers, the North Peruvian Low and the Apurímac valley (Fig. 3). Genetic divergence among these three subclades is comparable to the divergence between other clades in the complex, but we treat them as a single group (clade H) because they are geographically cohesive and have no known phenotypic structure. The other clade is the southernmost group, consisting of samples from the cloud forests of Puno in southern Peru and the Bolivian Yungas (clade E-*punctipectus*/*inconspicuus*). The geographic boundaries of major clades are clearly related to low-elevation gaps dissecting montane forests and separating subspecies or subspecies groups (Figs. 1 and 3). The levels of genetic differentiation based on the Φ_{ST} values found in this study are similar or higher than the divergence found among differentiated populations in other Neotropical bird species

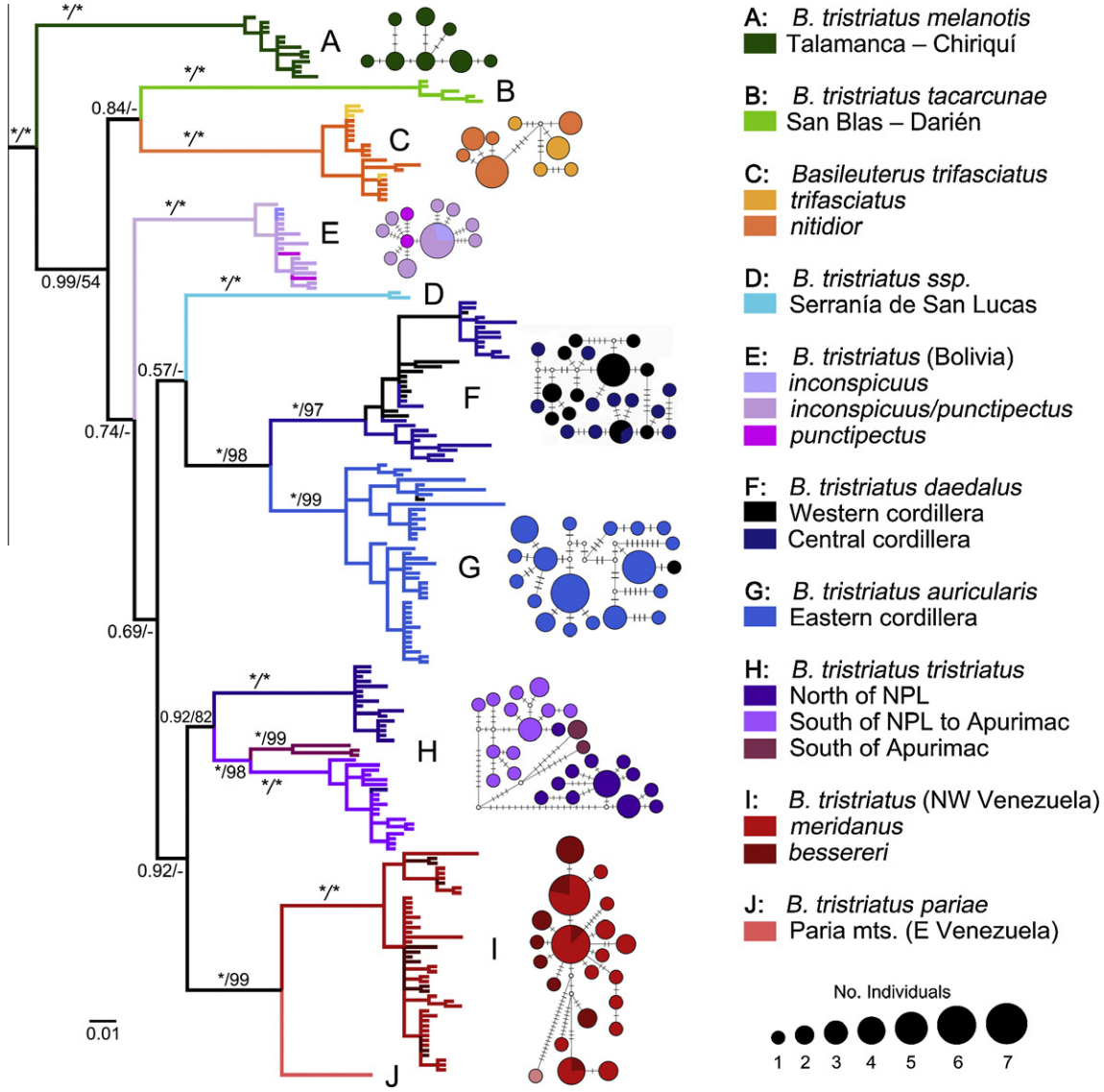

Fig. 3. On the left, 50% majority-rule consensus tree from the BI analysis showing the genetic divergence and phylogenetic relationships among members of the *Basileuterus tristriatus* complex based on sequences of the ND2 gene. On branches, nodal support is indicated by asterisks for maximum support (1.0 BI posterior probability/100 ML bootstrap) or stated numerically if greater than 0.5/50. In front of major clades (A–J), median-joining haplotype networks are presented for populations with sufficient sample sizes. Haplotype networks show the genetic diversity and relationships among haplotypes within divergent populations. Each color denotes the geographic provenance of samples (see also Fig. 1). An interconnecting line represents a mutational step.

Table 1
Pairwise comparisons among each major clade recovered in the phylogeographic analysis of the Three-striped Warbler *(Basileuterus tristriatus)*. Letters in parentheses as in the clades depicted in Fig. 3. Above diagonal: pairwise Φ_{ST} values; significant differentiation in bold ($P < 0.005$, or if marked with an asterisk $P \leqslant 0.05$). Diagonal: average uncorrected pairwise genetic distances among individuals within clades. Below diagonal: pairwise average uncorrected genetic distances.

Population	1 (**C**)	2 (**A**)	3 (**B**)	4 (**I**)	5 (**J**)	6 (**G**)	7 (**F**)	8 (**D**)	9 (**H**)	10 (**E**)
1 – Tumbes (*B. trifasciatus*; **C**)	0.004	**0.933**	**0.922**	**0.904**	0.902	**0.871**	**0.849**	**0.913**	**0.703**	**0.914**
2 – Talamanca–Chiriquí (*melanotis*; **A**)	0.055	0.002	**0.954**	**0.919**	0.945	**0.894**	**0.871**	**0.954***	**0.727**	**0.942**
3 – San Blas-Darién (*tacarcunae*; **B**)	0.054	0.063	0.003	**0.918**	0.930	**0.886**	**0.853**	**0.944***	**0.709**	**0.944**
4 – Venezuela (*meridanus*/*bessereri*; **I**)	0.046	0.051	0.056	0.004	0.783	**0.873**	**0.857**	**0.886**	**0.693**	**0.899**
5 – Paria (*pariae*; **J**)	0.044	0.046	0.054	0.023	–	0.845	0.809	0.969	0.467	0.930
6 – Eastern cordillera (*auricularis*; **G**)	0.046	0.056	0.057	0.046	0.046	0.007	**0.722**	**0.816**	**0.675**	**0.861**
7 – Central/Western cordilleras (*daedalus*; **F**)	0.046	0.056	0.056	0.046	0.047	0.027	0.009	**0.801**	**0.676**	**0.837**
8 – Serranía de San Lucas (unnamed ssp.; **D**)	0.052	0.056	0.062	0.042	0.041	0.039	0.046	0.001	**0.535**	**0.928**
9 – SE Ecuador and Peru (*tristriatus*; **H**)	0.042	0.052	0.055	0.038	0.035	0.040	0.042	0.038	0.019	**0.656**
10 – Bolivia (*inconspicuus*/*punctipectus*; **E**)	0.039	0.046	0.050	0.039	0.039	0.039	0.039	0.039	0.035	0.003

complexes (Cabanne et al., 2011; Vilaça and Santos, 2010), which highlight the marked genetic structure in *B. tristriatus* populations.

3.2.3. Genetic diversity, gene flow, and historical demography

In addition to the marked phylogeographic structure described above, all populations exhibited relatively high levels of haplotype and nucleotide diversity (Table 2). In the 216 sampled individuals of *B. tristriatus* and *B. trifasciatus*, we found 135 haplotypes, none of which was abundant (Fig. 3). Nucleotide diversity was similar among populations (Table 2), but slightly higher in the genetically subdivided lineage *B. t. tristriatus* (clade H). This lineage is deeply structured across two of the most pervasive barriers of the eastern Andean slope, yet it was the only case where no known phenotypic traits are associated with marked genetic subdivisions. Overall, no haplotypes were shared among major clades. The only two exceptions corresponded to a sample from the Western cordillera (Clade F) that nested within the Eastern cordillera clade (clade G), and an individual from northern Peru exhibiting a haplotype nested within the subclade from the opposite side of the North Peruvian Low (clade H). Coalescent analyses suggest no gene flow across two putative barriers, the Táchira Depression and the Magdalena valley (Fig. 4b and 4c, respectively; Fig. 1). In contrast, a signal of gene flow was recovered between population pairs separated by the Turbio-Yaracuy Depression (Fig. 4a) and the Cauca River valley (Fig. 4d), and less so, by the North Peruvian Low (Fig. 4e).

The high genetic diversity within populations and the generally short internal branches relative to the long branches subtending clades are indicative of population divergence occurring over a short period of time, and a subsequent, relatively long and stable history of isolation of populations. Overall, we found weak support for changes in effective population size; a slight signature of expansion was found in *daedalus* (clade F) (Table 2; Appendix Fig. A2).

4. Discussion

4.1. The Basileuterus tristriatus complex and possible taxonomic implications

Our analyses suggest that *B. trifasciatus* is a highly differentiated lineage within *B. tristriatus*. Specifically, we found that within the *B. tristriatus* clade, *B. trifasciatus* is a phenotypically distinct population from the dry Huancabamba valley and the Tumbesian region, which split off within the time frame of rapid differentiation of most other lineages of *B. tristriatus* (Fig. 2). The paraphyly of *B. tristriatus* with respect to *B. trifasciatus* calls for an evaluation of the species rank of *B. trifasciatus* or of species boundaries within *B. tristriatus*, considering the phenotypic diagnosability of major linages as well. According to different criteria under a number of species concepts (see de Queiroz (2007) for a review), four different species could be recognized minimally: *melanotis*, *tacarcunae*, *trifasciatus*, and *tristriatus*, which would include the rest of the Andean clades (and thus might allow further subdivision). However, there is not enough evidence to question the species rank of *B. trifasciatus* or to reform species limits within *B. tristriatus* according to the Biological Species Concept (Mayr, 1942), and despite the paraphyly of *B. tristriatus* in the ND2 gene tree. The asymmetry of range sizes of *B. tristriatus* and *B. trifasciatus* and the isolated distribution of the latter in the dry slopes of the Huancabamba and Tumbesian regions (Fig. 1) suggest that its origin resulted from the differentiation of a peripheral ancestral population, which may well have attained reproductive isolation

Table 2
Summary statistics describing the genetic diversity and demographic changes of each major clade recovered in the phylogeographic analysis of the *Basileuterus tristriatus* complex (see map in Fig. 1 for geographic details, letters as in clades of Fig. 3). The total number of individuals (*N*), number of haplotypes (*h*), number of variable sites (*var*), gene diversity (*Hd*), nucleotide diversity (π), Tajima's *D*, and Fu's *F*.

Population	*N*	*h*	*var*	*Hd* ± SD	π ± SD	Tajima's *D*	Fu's *F*
Tumbes (*B. trifasciatus*; **C**)	20	9	15	0.87 ± 0.05	0.0039 ± 0.0022	−0.31	−0.89
Talamanca–Chiriquí (*melanotis*; **A**)	13	8	10	0.92 ± 0.05	0.0023 ± 0.0015	−1.01	−2.96*
San Blas-Darién (*tacarcunae*; **B**)	5	4	6	0.90 ± 0.16	0.0031 ± 0.0022	0.76	−0.23
Venezuela (*meridanus*/*bessereri*; **I**)	45	20	36	0.92 ± 0.02	0.0037 ± 0.0020	−1.84*	−8.24**
Paria (*pariae*; **J**)	1	1	–	–	–	–	–
Eastern cordillera (*auricularis*; **G**)	40	25	39	0.94 ± 0.03	0.0068 ± 0.0036	−1.16	−11.13**
Central/Western cordilleras (*daedalus*; **F**)	34	29	58	0.98 ± 0.02	0.0086 ± 0.0045	−1.59*	−18.55**
Serranía de San Lucas (unnamed ssp.; **D**)	2	2	1	1.00 ± 0.50	0.0013 ± 0.0019	–	–
SE Ecuador and Peru (*tristriatus*; **H**)	38	26	72	0.97 ± 0.01	0.0178 ± 0.0090	0.34	−2.76
Bolivia (*inconspicuus*/*punctipectus*; **E**)	18	11	19	0.86 ± 0.08	0.0025 ± 0.0015	−2.05*	−5.21**
Mean	21.6	13.5	28.4	0.93	0.0056		

** *p*-Value <0.01.
* *p*-Value <0.05.

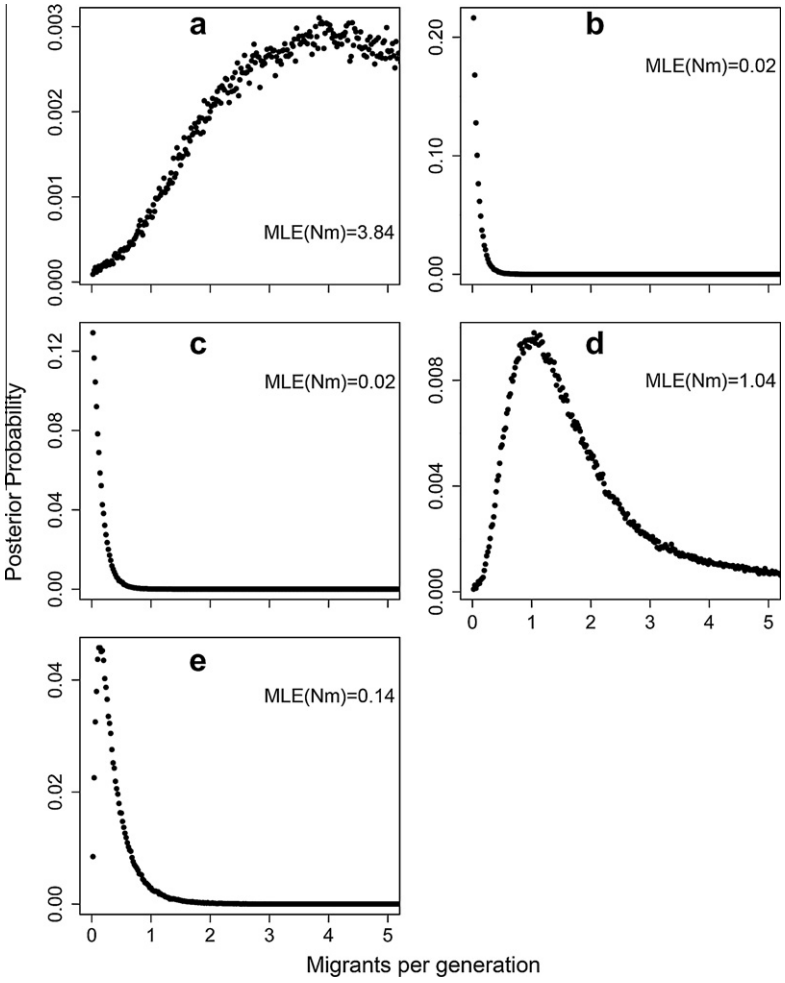

Fig. 4. Posterior probability distribution of the number of migrants per generation estimated using MDiv for population-pairs separated by low-elevation gaps. (a) Turbio-Yaracuy Depression between the Venezuelan Andes (*meridanus*) and Coastal cordillera (*bessereri*); (b) Táchira Depression between the Venezuelan Andes (*meridanus*) and Eastern cordillera (*auricularis*); (c) Magdalena valley between the Eastern (*auricularis*) and Central cordillera (*daedalus*); (d) Cauca valley between the Western and Central cordilleras (*daedalus*); (e) North Peruvian Low (NPL) between southeastern Ecuador-northern Peru and central Peruvian populations (*tristriatus*). Note the change of scale in posterior probabilities across comparisons. MLE(Nm) denotes the Maximum Likelihood estimate for the number of migrants per generation.

from the rest of the group (Barraclough and Nee, 2001; Funk and Omland, 2003). Although formal quantitative analyses have not been conducted, *B. trifasciatus* clearly possesses the most dissimilar plumage pattern and coloration in the *B. tristriatus* group (see Curson, 2010) and uses the most different habitat type (xerophytic scrub vs. humid forest of *tristriatus*), and it is also genetically differentiated. Thus, we suggest that, despite the paraphyly of *B. tristriatus*, *B. trifasciatus* should continue to be considered a separate species pending detailed additional analyses. We note, however, that *B. trifasciatus* and *B. tristriatus* do not overlap in range and exhibit markedly similar vocalizations (Schulenberg et al., 2007). Whether other lineages of *B. tristriatus* merit species rank could be a matter of taxonomic taste and the species definition adopted, but any decision in such respect would require an integrative study of species delimitation (see for example Cadena and Cuervo, 2010; Carstens and Dewey, 2010; Zapata and Jiménez, 2012), including an expanded genetic sampling, as well as behavioral, phenotypic, and ecological information. Thus, those taxonomic recommendations are beyond the scope of this study.

On the other hand, contrary to expectations from overall plumage pattern indicating a close resemblance between *B. tristriatus* and *B. basilicus* (Hellmayr, 1935; Todd, 1929), we found that the latter is not even a member of the recently redefined genus *Basileuterus* (Lovette et al., 2010). Rather, *basilicus* appears to be related to a clade comprising some of the South American warblers in the genus *Myiothlypis*. Our results also show that *Myiothlypis* is likely paraphyletic (Fig. 2), which is also reflected in some of the tree reconstructions included in the comprehensive phylogenetic analysis of the family (Fig. 3 in Lovette et al., 2010). We thus propose to transfer the species *B. basilicus* to the genus *Myiothlypis*, although the affinities of *basilicus* within *Myiothlypis* remain unknown.

4.2. Divergence across low-elevation barriers

Deep genetic structure in *B. tristriatus* reveals a long history of divergence resulting from genetic isolation across low-elevation gaps. Differentiation initiated in the Late Pliocene, but most divergence within South America occurred within a relatively short time interval during the early Pleistocene. Phylogeographic breaks exist in central Panama, the Urabá lowlands and Magdalena valley in Colombia, the Unare and Táchira depressions in Venezuela, the North Peruvian Low, and the Apurímac valley. In general, some of these breaks observed in the *B. tristriatus* complex coincide with breaks identified for other avian species with similar distributions (e.g., Bonaccorso, 2009; Pérez-Emán, 2005), suggesting that a common set of geographic barriers has promoted diversification in the Neotropical montane avifauna. In addition, high-elevation habitats could also restrict genetic exchange between slopes of the same

Andean range (i.e. *daedalus* vs. *tristriatus* in Ecuador). Downward elevational shifts of the montane forest during the Pleistocene in the northern Andes (Cárdenas et al., 2011; Hooghiemstra and Van der Hammen, 2004) likely created corridors of suitable habitat across narrow valleys allowing dispersal of mid-elevation and foothill birds. However, such shifts in vegetation may not have affected differentiation in some of the *B. tristriatus* lineages. For instance, the striking genetic and phenotypic divergences between the two lineages separated by the narrow Táchira Depression (*auricularis* vs. *meridanus*) were likely maintained during periods of habitat connectivity because their divergence predates the latest Pleistocene cycles. Another example of an association between a low-elevation gap and a phylogeographic break is at the Unare Depression, between the populations of the Venezuelan Coastal cordillera (*bessereri*) and the northeastern mountains (Turimiquire and Paria; *pariae*). The latter mountains are home to highly differentiated populations of a number of montane forest birds (Cadena and Cuervo, 2010; Pérez-Emán, 2005; Pérez-Emán et al., 2010).

The North Peruvian Low emerged as an important phylogeographic break within the subspecies *tristriatus* (clade H). However, we concur with previous studies that failed to detect any indication of phenotypic variation in series of specimens covering both sides of this barrier (Hellmayr, 1935; Zimmer, 1949). Genetic distance across the North Peruvian Low was slightly higher (0.029) than that observed between the phenotypically distinct populations *auricularis* and *daedalus* across the Magdalena valley, and *pariae* and *meridanus*/*bessereri* across the Unare Depression (Table 1). The Marañón valley (i.e., the North Peruvian Low in general) is recognized as one of the most intriguing biogeographic areas in the Andes (Bates and Zink, 1994; Bonaccorso, 2009; Miller et al., 2007; Parker et al., 1985; Vuilleumier, 1969), and our study emphasizes the importance of this barrier in promoting cryptic population genetic divergence. In contrast, a major phylogeographic break in *B. tristriatus* lies in the foothills of southern Peru, but this does not correspond to any major geographical or habitat discontinuity. The subspecies *B. t. tristriatus* extends south and east of the Urubamba valley and is replaced by *B. t. inconspicuus/punctipectus* somewhere in Puno, east of the Vilcanota cordillera. A similar phylogeographic pattern in that area has been documented in another oscine passerine (*Arremon torquatus*, Emberizidae; Cadena et al., 2007; Cadena and Cuervo, 2010) and a hummingbird (*Adelomyia melanogenys;* Chaves and Smith, 2011). Such phylogeographic pattern in the southern Andes where physical barriers are apparently not involved has been speculated to result from long-term ecological and climatic stability of that region in the form of a refugium, would have allowed divergence of southern Andean populations in the absence of a physical geographic barrier (Fjeldså et al., 1999).

Flanked by the lowlands of the Magdalena, Nechí, and Cauca river drainages, the isolated Serranía de San Lucas in central Colombia harbors a distinct population of *B. tristriatus* that seems to have been evolving independently from populations of the other Andean ranges for a substantial period of time. The San Lucas lineage averaged 0.046 uncorrected sequence divergence with respect to all other *B. tristriatus* clades (range: 0.023–0.063). This form is phenotypically closest to the nominate subspecies of the eastern slope of the Ecuadorian and Peruvian Andes and to birds of the northern Central cordillera (N.G.P. and A.M.C. unpubl. data). Although only two specimens of this population exist and confirmation with more samples is desirable, its genetic divergence is remarkable (Fig. 3) and comparable to the divergences observed among the other major clades of *B. tristriatus* including *B. trifasciatus* (Table 1). Considering the short geographic distance between the Serranía de San Lucas and the northern Central cordillera, and that the low pass in between is relatively high in elevation (600–700 m) and covered by humid lowland forests (Cuervo et al., 2008), it was unexpected that populations of this range had attained such a high degree of genetic differentiation. Because the San Lucas specimens are indeed phenotypically distinct (also see Curson, 2010; Salaman et al., 2002), we will present a description of this new taxon elsewhere.

We found evidence of gene flow between the Central and Western cordilleras across the Cauca valley (Colombia; Fig. 4d), and between the Venezuelan Andes and Coastal cordillera across the Turbio-Yaracuy Depression (Venezuela; Fig. 4a). Although these putative barriers could be separating the montane forest avifaunas in the present time by delimiting the geographic range of a number of bird species (Cracraft, 1985; Fitzpatrick, 1973), they seemingly have played no major role in the historical structuring of genetic variation in *B. tristriatus*. However, we note that incomplete lineage sorting between those populations could not be entirely discounted if these barriers are effectively interrupting gene flow but not enough time has elapsed for the populations to achieve reciprocal monophyly. Support for ongoing gene flow across these same barriers was also observed in *Arremon brunneinucha*, another widespread mid-elevation forest bird (Cadena et al., 2007).

4.3. Genetic and phenotypic delimitation of lineages and patterns of phenotypic variation in B. tristriatus

Our results revealed an apparent discordance between phenotypic variation and patterns of genetic differentiation in several cases in which two or more subspecies formed a single well-defined phylogroup within which there was no genetic structure associated with geographic variation in external phenotype. Specifically, the Venezuelan subspecies *meridanus* and *bessereri* (clade I), the southern subspecies *inconspicuus* and *punctipectus* (clade E), and the two subspecies of *B. trifasciatus* (Clade C; Fig. 3) were not genetically differentiated. We believe that the apparent discordance observed is actually artificial because the named subspecies involved likely do not correspond to diagnosable populations. First, *bessereri* and *meridanus* may not be fully diagnosable, given that their supposed diagnostic characteristics could indicate individual variation rather than fixed population differences (Todd, 1929). Other authors, although recognizing *bessereri*, remarked that it is only slightly paler than *meridanus* (Hellmayr, 1935) or ignored any difference between them (Chapman, 1924). Second, the three taxa from southern Peru to the Bolivian Yungas (*inconspicuus*, *punctipectus* and *canens*) may be better treated as a single entity; Zimmer (1949), surprisingly, described the subspecies *inconspicuus* and *canens* as distinct from *punctipectus*, acknowledging that their alleged diagnostic traits were only subtle, and that the assignment of specimens with intermediate plumage pattern to any form was ambiguous. In fact, an augmented modern series of Bolivian specimens demonstrates that this variation is not discrete but likely corresponds to a cline along the continuous range of *B. tristriatus* from Puno to Samaipata (S. Herzog, pers. comm.). When this gradual variation is considered and subspecies designations restricted to fully diagnosable taxa, the deep genetic differentiation across low-elevation gaps uncovered in the *B. tristriatus* complex is indeed remarkably congruent with phenotypically delimited populations. The only exception is the nominate subspecies *tristriatus*, for which we failed to detect in series of specimens any indication of phenotypic differentiation associated with the deep genetic structure uncovered along its range in the eastern Andean slope, corresponding to the location of the North Peruvian Low and the Apurímac river valley (clade H, Fig. 3). Phylogeographic studies of Neotropical passerines have provided mixed results for the correspondence between phenotypic (subspecies or plumage traits) and genetic groups (e.g. Brumfield, 2005; Cabanne et al., 2011; Cadena et al., 2007; Vilaça and Santos, 2010; Weir et al., 2008). This discordance could be the result of the disconnection between sampled markers

and loci involved in plumage coloration and pattern, as well as random genetic drift, selection to different habitat types, introgression, clinal variation, or undocumented cryptic phenotypic variation.

4.4. The Central to South American connection

The short internodes and moderate support for the nodes connecting *B. t. tacarcunae*, *B. trifasciatus*, and the rest of the Andean lineages may be the result of rapid colonization of the Andean montane forests followed by simultaneous isolation that ultimately resulted in differentiation. The population from the Chiriquí–Talamanca highlands of Costa Rica and western Panama (*melanotis*) was the most differentiated lineage, diverging in the Late Pliocene (95% HPD 2.5–4.9 Ma; Appendix Fig. A1). Although a number of bird lineages of North American origin, mostly from the lowlands, colonized South America congruently with the rise of the isthmus in the Late Pliocene (Smith and Klicka, 2010), studies on montane birds are scarce, and one with *Chlorospingus*, showed complicated patterns of back colonization from the Andes to Central America (Weir et al., 2008). We cannot test competing biogeographic scenarios (e.g. north-to-south spatial mode) with the information content of the current data. Further studies that include an expanded molecular sampling (e.g. genomic data, slow-evolving genes) would be informative to resolve basal short internodes, although other phylogeographic studies involving Andean species also report basal polytomies that may be the product of an early rapid differentiation (Cadena et al., 2007; Chaves and Smith, 2011; Pérez-Emán, 2005; Weir et al., 2008).

5. Conclusion

The present study highlights the important role of geographical discontinuities such as low-elevation gaps in promoting and maintaining population differentiation in an avian complex of the mid-elevation zone of the Neotropical montane biome. Our comprehensive study with thorough sampling over much of the Neotropical montane region allowed us to: (1) reject the hypothesis that the Santa Marta endemic *B. basilicus* is part of the *Basileuterus tristriatus* complex and to demonstrate that it is instead a member of the *Myiothlypis* group; (2) discover the non-monophyly of *B. tristriatus* because *B. trifasciatus* emerged as a differentiated lineage within this avian complex; (3) uncover deep genetic structure that largely mirrors phenotypic variation in *B. tristriatus* and correspond to interrupted gene flow across low-elevation gaps; and (4) reveal cryptic diversity along the eastern Andean slope and a differentiated disjunct population in the Serranía de San Lucas in Colombia. The accumulation of phylogeographic studies that encompass the geographic distribution of widespread species are critical for characterizing cryptic biological diversity in the tropics, and form the basis of comparative tests on the evolutionary assembly of species-rich biotas.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.03.011.

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