

Ecological speciation along an elevational gradient in a tropical passerine bird?

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Abstract

Local adaptation of populations along elevational gradients is well known, but conclusive evidence that such divergence has resulted in the origin of distinct species in parapatry remains lacking. We integrated morphological, vocal, genetic and behavioural data to test predictions pertaining to the hypothesis of parapatric ecological speciation associated with elevation in populations of a tropical montane songbird, the Grey-breasted Wood-wren (*Henicorhina leucophrys*: Troglodytidae), from the Sierra Nevada de Santa Marta, Colombia. We confirmed that two distinct populations exist along the elevational gradient. Phylogenetic analyses tentatively indicate that the two populations are not sister taxa, suggesting they did not differentiate from a single ancestor along the gradient, but rather resulted from separate colonization events. The populations showed marked divergence in morphometrics, vocalizations and genetic variation in mitochondrial and nuclear loci, and little to no evidence of hybridization. Individuals of both populations responded more strongly to their own local songs than to songs from another elevation. Although the two forms do not appear to have differentiated locally in parapatry, morphological and vocal divergence along the elevational gradient is consistent with adaptation, suggesting a possible link between adaptive evolution in morphology and songs and the origin of reproductive isolation via a behavioural barrier to gene flow. The adaptive value of phenotypic differences between populations requires additional study.

Introduction

Ecological speciation is the process whereby reproductive isolation between lineages arises as a consequence of natural selection acting on ecologically relevant traits (Schluter, 2001, 2009; Coyne & Orr, 2004; Rundle & Nosil, 2005; Nosil, 2012). Natural selection can lead to reproductive isolation if such traits are directly involved in mate choice or if the process results in divergence in other traits involved in reproductive isolation via pleiotropic effects (Grant & Grant, 2007; Jiggins, 2008;

Servedio *et al.*, 2011). For example, properties of avian songs, which can play a crucial role in reproductive isolation in birds (Slabbekoorn & Smith, 2002a; Price, 2008), can evolve as a result of direct selection imposed by ambient noise or habitat-dependent sound propagation (Morton, 1975; Wiley & Richards, 1982; Slabbekoorn & Smith, 2002a, b), or as a by-product of adaptive evolution in morphological traits such as body and beak size (Slabbekoorn & Smith, 2000; Podos, 2001; Seddon, 2005). If song divergence is accompanied by divergence in signal perception leading to assortative mating, then this process of adaptive evolution can result in the origin of new species (Slabbekoorn & Smith, 2002a,b; Grant & Grant, 2007; van Doorn *et al.*, 2009).

Although ecological speciation can occur in any geographic context provided that the origin of barriers to

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reproduction is driven by natural selection (Templeton, 1989; Fitzpatrick *et al.*, 2008), considerable theoretical and empirical work on ecological speciation has focused on populations continuously distributed in parapatry along environmental gradients. This is because the formation of new species in the absence of geographic isolation is most likely to occur when natural selection is sufficiently strong to counteract the effects of gene flow, a probable scenario along environmental gradients. Empirical evidence of parapatric ecological speciation in nature remains scarce (Coyne & Orr, 2004; Price, 2008), although numerous examples exist of adaptive divergence of populations along environmental gradients in the face of gene flow (Smith *et al.*, 2005b; McCracken *et al.*, 2009; Ribeiro *et al.*, 2011). Moreover, models indicate that parapatric speciation driven by divergent selection in the presence of gene flow is theoretically plausible (Gavrilets, 2004). Therefore, speciation along environmental gradients with no physical restriction to gene exchange might be more common than traditionally thought (Nosil, 2008; Pinho & Hey, 2010).

A major challenge for studies testing for ecological speciation in parapatry is to distinguish whether the distribution of distinct populations along environmental gradients reflects primary *in situ* differentiation, or rather secondary contact between formerly allopatric populations (Endler, 1982). To distinguish between these alternatives and to better understand the mechanisms promoting speciation, we need studies that combine phylogenetic approaches with analyses of gene flow, as well as knowledge of the ecological factors promoting divergence (Coyne & Orr, 2004; Sobel *et al.*, 2010). More generally, although there is ample evidence of the importance of natural selection for the formation of new species (Schluter, 2001, 2009), we require more integrative research connecting the study of adaptive variation, including traits involved in reproductive isolation, with assessment of the phylogenetic affinities of populations and patterns of gene flow.

Population differentiation in response to varying selective pressures along elevational gradients has been an important focus for researchers interested in adaptive evolution and ecological speciation (Patton & Smith, 1992; Angert & Schemske, 2005; McCormack & Smith, 2008; Cheviron & Brumfield, 2009; McCracken *et al.*, 2009; Milá *et al.*, 2009; Storz *et al.*, 2012). Avian song is expected to diverge as a result of varying selection in different elevational zones because changes in vegetation structure affect the ability of sounds to propagate and changes in animal assemblages might lead to varying levels of acoustic interference (Slabbekoorn *et al.*, 2002; Dingle *et al.*, 2008; Kirschel *et al.*, 2009). Moreover, because songs are correlated with morphological traits (i.e. bill and body size) and because those traits can show adaptive evolution associated with elevation (Price, 1991; McCormack & Smith, 2008; Milá

et al., 2009), song divergence may arise as a result of pleiotropic effects.

Although some phylogenetic studies indicate that species replacing each other along elevational gradients are close relatives, suggesting their divergence might have occurred in parapatry, convincing empirical evidence for this mode of speciation in mountain systems is largely lacking (Cadena, 2007; Cadena *et al.*, 2012). Specifically, we are unaware of studies showing evidence of speciation resulting from adaptation to different elevational zones involving these elements: phylogenetic evidence that species replacing each other along elevational gradients are sister taxa, divergence in phenotypic traits and/or signalling traits (e.g. songs) associated with elevation, patterns of genetic variation suggesting reproductive isolation between forms replacing each other at different elevations, and evidence that divergence in signals is associated with assortative mating.

Here, we integrate morphological, vocal, genetic and behavioural data to test predictions of the hypothesis of ecological speciation associated with elevation in a tropical songbird, the Grey-breasted Wood-wren (*Henicorhina leucophrys*: Troglodytidae). Populations of this bird occurring in the Sierra Nevada de Santa Marta (SNSM) in northern Colombia are an ideal study system for addressing this issue because two forms replace each other along an elevational gradient with no apparent physical barriers to dispersal and gene flow (Todd & Carriker, 1922; Hilty & Brown, 1986). We conducted (1) phylogenetic analyses using DNA sequence data to determine whether these forms diversified *in situ* from a single ancestor that colonized the SNSM (i.e. parapatric divergence along the elevational gradient) or whether their divergence involved an allopatric phase, with current elevational parapatry reflecting secondary contact. We then (2) characterized the patterns of morphometric and vocal variation along the elevational gradient to determine whether differences associated with elevation exist and (3) tested whether divergence in such traits correlated with patterns of genetic variation at multiple molecular markers. Finally, we (4) conducted behavioural experiments to determine whether divergence in songs associated with elevation was a likely mechanism promoting assortative mating.

Materials and methods

Study area

The SNSM is an isolated mountain massif located in northern Colombia. Reaching elevations of nearly 5800 m and being only *c.* 40 km from the Caribbean coast, the SNSM is the highest coastal mountain range in the world. Owing to its isolation, the SNSM has high levels of endemism across multiple groups (Cleef *et al.*, 1984).

Our study was conducted on the San Lorenzo slope, located in the north-western SNSM (Fig. 1). Mean annual precipitation in San Lorenzo is 2840 mm at 600 m and 2520 mm at 2250 m elevation (Cleef *et al.*, 1984). We focused on lower- and upper-montane forests, which extend from *c.* 600 to 2500–2700 m and from *c.* 2500 to 2800 m, respectively. Lower-montane forests are characterized by trees with dense foliage and often with buttressed roots, and a 25–35 m-tall canopy; arborescent ferns and palms are common in the understory, and vascular epiphytes are abundant (Cleef *et al.*, 1984). Upper-montane forests are characterized by a high incidence of cloud cover. Trees are smaller (8–20 m), unbuttressed, and there are high densities of epiphytes (Bromeliaceae, Orchidaceae, pteridophytes, bryophytes and lichens). Owing to selective timber extraction and deforestation, some areas at upper elevations have been transformed into early secondary habitats, with

Chusquea bamboo dominating the understory (Cleef *et al.*, 1984).

Study system

The Grey-breasted Wood-wren is a highly vocal and territorial bird of the forest understory broadly distributed in montane areas of Central and South America (Kroodsmas & Brewer, 2005). Recent studies focused on Ecuadorian populations (Dingle *et al.*, 2008, 2010) have demonstrated song divergence presumably driven by adaptation to different acoustic environments along an elevational gradient. Birds from lower elevations sang within more-restricted bandwidths and lower-frequency ranges than birds at higher elevations, possibly a result of cicadas calling at high frequencies having a dominant presence only at lower elevations. Vocal divergence also correlated well with genetic differentiation.

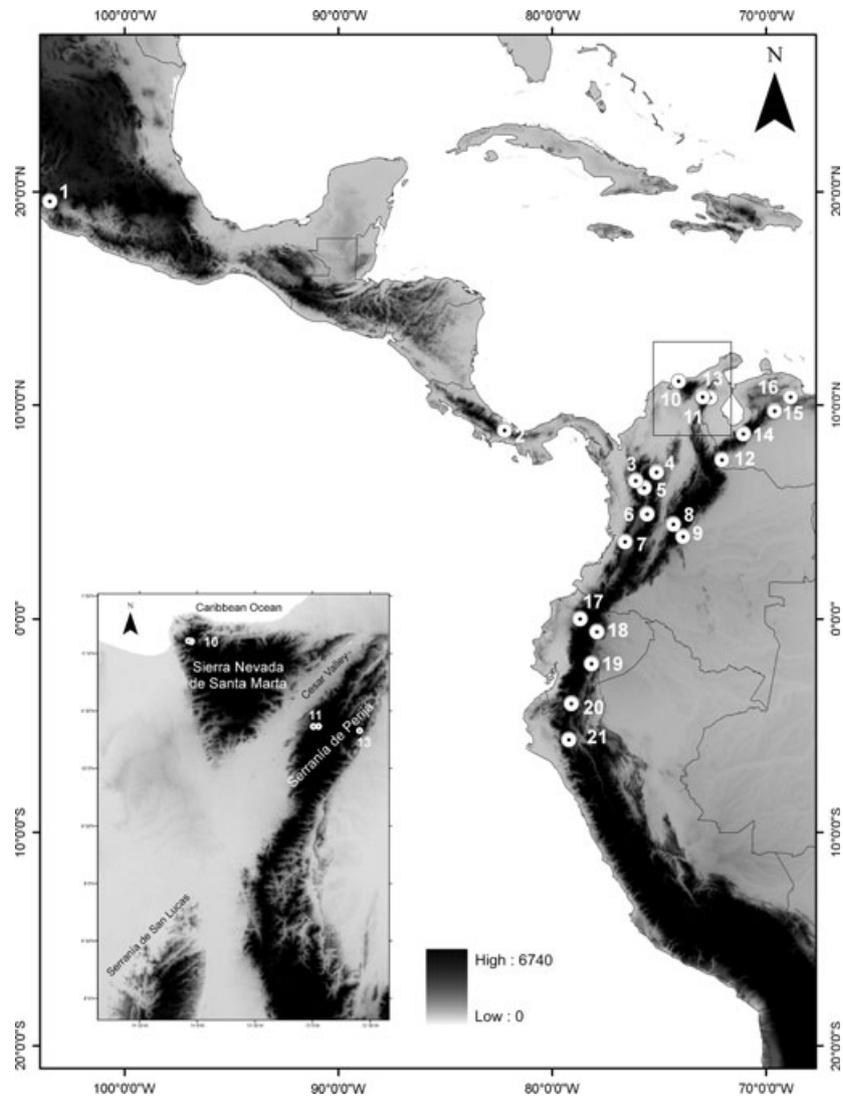


Figure 1 Geographic distribution of samples of *Henicorhina leucophrys* used in phylogenetic analyses (large map) and map of northern Colombia showing the location of sampling sites on the San Lorenzo slope, located in the north-western sector of the SNSM, and in the Serranía de Perijá (inset). Numbers correspond to the ID column in Table 1; note that all sampling sites in San Lorenzo, SNSM, are depicted with a single locality ID number (10).

Two different forms of Grey-breasted Wood-wren referred to as *H. l. bangsi* and *H. l. anachoreta* replace each other along the elevational gradient in the SNSM (Bangs, 1899; Ridgway, 1903). Although Todd & Carriker (1922) indicated that these two forms occupy distinct elevations separated by a distributional gap, more recent data suggest they are mostly parapatrically distributed but with partial sympatry, with *H. l. bangsi* ranging from c. 600 to 2100 m, and *H. l. anachoreta* from c. 1800 to 3600 m (Hilty & Brown, 1986). Prior to this study, however, the distribution of these forms had been described based only on data from a few specimens and nonquantitative field observations. Therefore, the existence and extent of a zone of elevational overlap had been inferred only tentatively and the pattern of elevational replacement was not clear. Hence, we avoid referring to the different subspecies names owing to the uncertainty in their distributions and to the fact that, *a priori*, we were unable to rule out the possibility that variation along the elevational gradient was clinal. By studying patterns of morphological, vocal, genetic and behavioural variation along the elevational gradient in the SNSM, we can test predictions of the hypothesis of parapatric ecological speciation and also determine whether patterns of variation are consistent with those seen in similar studies in Ecuador (Dingle *et al.*, 2008, 2010), which would be suggestive of parallel adaptive differentiation in separate mountain systems.

Field sampling

To characterize patterns of genetic, phenotypic and vocal variation in San Lorenzo, we collected data along an elevational transect ranging from c. 1100 to 2810 m (the location of the San Lorenzo transect in the SNSM and the Neotropics is shown in Fig. 1). Sampling efforts were concentrated in three main areas: (1) around Finca La Victoria (1100–1360 m), (2) El Dorado Natural Bird Reserve (1780–2200 m) and (3) around the headquarters of the Sierra Nevada de Santa Marta National Park (2250–2810 m). In each of these areas, we collected samples for genetic analyses, took morphological measurements and recorded songs from birds in 20 territories. We also captured birds at other elevations to collect morphological data and samples for genetic analyses, seeking to cover the elevational gradient as thoroughly as possible.

Testing the geographic pattern of differentiation

To determine whether the two forms existing in the SNSM originated as a result of parapatric divergence from a single ancestor along the elevational gradient, or whether they colonized the area independently implying their divergence involved an allopatric phase

(i.e. secondary contact), we examined their phylogenetic relationships with respect to other populations of *H. leucophrys*. We based our phylogenetic analyses on sequences of the mitochondrial ATPase 6 and ATPase 8 genes (842 base pairs) owing to the existence of previous and ongoing extensive phylogeographic studies of the genus *Henicorhina* using these markers (Dingle *et al.*, 2006, 2008; J.L. Pérez-Emán *et al.*, unpubl. data).

Samples from 101 individuals captured using mist-nets were collected along the elevational gradient in the SNSM from June to July 2009. A sample of c. 0.05 mL of blood from the brachial vein was obtained with a heparinized capillary tube and stored in lysis buffer (White & Densmore, 1992). These samples were supplemented with five tissue samples associated with voucher specimens collected previously, for a total of 106 individuals. We extracted genomic DNA from blood or tissue using a DNeasy Kit (QIAGEN, Valencia, CA, USA), and we amplified and sequenced ATPase 6 and 8 using standard approaches (Cadena *et al.*, 2007). We were able to generate ATPase sequences for 100 of the 106 samples available.

Preliminary phylogenetic analyses considering our 100 sequences from the SNSM and sequences of more than 300 individuals from populations thoroughly covering the distribution range of *H. leucophrys* and of the other three species in the genus *Henicorhina* indicated that all individuals from the SNSM are assignable to one of two strongly supported monophyletic groups nested within a South American clade, and that these populations are most closely related to neighbouring populations from Northern South America. Thus, to test whether the forms occurring in the SNSM are sister taxa (as predicted by the hypothesis of parapatric divergence) or not (a scenario favouring the hypothesis of double colonization), we conducted phylogenetic analyses using a database including only the five individuals from the SNSM with associated voucher specimens plus sequences from populations selected to represent biogeographic areas relevant to address the question of interest (Fig. 1, Table 1). Such areas corresponded to localities in Venezuela (Serranía de Perijá, Mérida Andes, Cordillera de la Costa, $n = 5$ individuals), Colombia (Serranía de Perijá and all three Andean cordilleras, $n = 10$), Panama (Chiriquí, Bocas del Toro, $n = 2$) and Ecuador and Northern Peru (localities on both slopes of the Andes, $n = 5$). We also included in analyses one sequence of *H. leucophrys* from Mexico and considered two sequences of the White-breasted Wood-wren (*H. leucosticta*, from Belize and Ecuador), together with representatives of the genera *Cyphorhinus*, *Cantorchilus* and *Pheugopedius*, as outgroup taxa (Table 1).

Phylogenetic analyses were conducted using maximum-parsimony and maximum-likelihood methods. Maximum-parsimony analyses consisted of a heuristic search with 100 stepwise-addition replicates and 1000 bootstrap replicates in PAUP*4.0b10 (Swofford, 2002). For maximum-likelihood analysis, we implemented the

Table 1 Sources and geographic origin of samples of Grey-breasted Wood-wrens (*Henicorhina leucophrys*) and outgroups included in phylogenetic analyses. GenBank accession numbers are given together with references for previously published sequences. Acronyms: ANDES-BT, Museo de Historia Natural Universidad de Los Andes; COP, Colección Ornitológica Phelps; CTR, Center For Tropical Research, University of California Los Angeles; FMNH, The Field Museum; IAvH, Instituto Alexander von Humboldt; LSUMZ, Louisiana State University Museum of Natural Science; STRI, Smithsonian Tropical Research Institute. See Fig. 1 for geographic locations.

ID	Taxon	Catalogue No.	Accession	Reference	Locality
1	<i>Henicorhina leucophrys</i>	FMNH 393980	KC209528	This study	Mexico, Jalisco, Sierra de Manantlan
2	<i>H. leucophrys</i>	STRI JTW 133	AY304314	Dingle <i>et al.</i> , 2006	Panama, Bocas del Toro, Palo Seco
2	<i>H. leucophrys</i>	LSUMZ B-26442	KC209529	This study	Panama, Chiriqui, Fortuna
3	<i>H. leucophrys</i>	IAvH BT5224	KC209530	This study	Colombia, Antioquia, Páramo Frontino
4	<i>H. leucophrys</i>	IAvH 2151	KC209531	This study	Colombia, Antioquia, El Encanto
5	<i>H. leucophrys</i>	ANDES-BT AMC 739	KC209532	This study	Colombia, Antioquia, Angelópolis
6	<i>H. leucophrys</i>	IAvH- 4516	KC209533	This study	Colombia, Risaralda, La Linda
7	<i>H. leucophrys</i>	IAvH 12499	KC209534	This study	Colombia, Valle del Cauca, Chicoral
8	<i>H. leucophrys</i>	IAvH 13944	KC209535	This study	Colombia, Cundinamarca, Los Robles
9	<i>H. leucophrys</i>	ICN 32612	KC209536	This study	Colombia, Meta, Serranía Aguas Claras
10	<i>H. leucophrys</i>	ANDES-BT CDC 079	KC209537	This study	Colombia, Magdalena, SNSM, San Lorenzo, 2000 m
10	<i>H. leucophrys</i>	ANDES-BT AMR 016	KC209538	This study	Colombia, Magdalena, SNSM, San Lorenzo, 2000 m
10	<i>H. leucophrys</i>	ANDES-BT CDC 080	KC209539	This study	Colombia, Magdalena, SNSM, San Lorenzo, 2400 m
10	<i>H. leucophrys</i>	ANDES-BT CDC 081	KC209540	This study	Colombia, Magdalena, SNSM, San Lorenzo, 2400 m
10	<i>H. leucophrys</i>	ANDES-BT AMR 017	KC209541	This study	Colombia, Magdalena, SNSM, San Lorenzo, 2400 m
11	<i>H. leucophrys</i>	ANDES-BT AMC 1038	KC209542	This study	Colombia, Cesar, Perijá, Manaure, San Antonio
11	<i>H. leucophrys</i>	ANDES-BT NGP 036	KC209543	This study	Colombia, Cesar, Perijá, Manaure, El Cinco
12	<i>H. leucophrys</i>	ANDES-BT 0910	KC209544	This study	Colombia, Norte de Santander, Tamá
13	<i>H. leucophrys</i>	COP IC-807	KC209527	This study	Venezuela, Zulia, Serranía de Perijá, Las Lajas
13	<i>H. leucophrys</i>	COP IC-827	KC209526	This study	Venezuela, Zulia, Serranía de Perijá, Las Lajas
14	<i>H. leucophrys</i>	COP 07N0446	KC209525	This study	Venezuela, Mérida, La Mucuy
15	<i>H. leucophrys</i>	COP 07N0195	KC209524	This study	Venezuela, Lara, PN Yacambú, El Blanquito
16	<i>H. leucophrys</i>	COP JP408	KC209523	This study	Venezuela, Yaracuy, Sierra de Aroa
17	<i>H. leucophrys</i>	NA	EU022425	Dingle <i>et al.</i> , 2008	Ecuador, Pichincha, Bellavista
18	<i>H. leucophrys</i>	NA	EU022434	Dingle <i>et al.</i> , 2008	Ecuador, Napo, Yanayacu
19	<i>H. leucophrys</i>	CTR 02N9307	AY304309	Dingle <i>et al.</i> , 2006	Ecuador, Morona-Santiago, PN Sangay
20	<i>H. leucophrys</i>	NA	EU022433	Dingle <i>et al.</i> , 2008	Ecuador, Zamora-Chinchipec, Romerillos
21	<i>H. leucophrys</i>	LSUMZ B-32605	AY304306	Dingle <i>et al.</i> , 2006	Peru, Cajamarca
	<i>Henicorhina leucosticta</i>	CTR 00N0009	AY304334	Dingle <i>et al.</i> , 2006	Belize, Cayo District, Chaa Creek
	<i>Henicorhina leucosticta</i>	CTR 00N0627	AY304331	Dingle <i>et al.</i> , 2006	Ecuador, Loreto, Orellana
	<i>Cyphorhinus aradus</i>	CTR 00N3310	AY304300	Dingle <i>et al.</i> , 2006	Ecuador, Napo, Loreto
	<i>Cantorchilus nigricapillus</i>	NA	AY103284	Dingle <i>et al.</i> , 2006	Panama
	<i>Pheugopedius rutilus</i>	LSUMZ 163699	AY103274	González <i>et al.</i> , 2003	Panamá, Old Gamboa Road
	<i>Troglodytes aedon</i>	NA	AY115237	Ricklefs & Bermingham, 2001	Lesser Antilles

TrN+I+G model of nucleotide substitution, selected as the best-fit to the data according to the Akaike and Bayesian information criteria in JMODELTEST 0.1.1 (Posada, 2008). We ran 100 bootstrap replicates to assess branch support. We also used the program Network 4.5.0.0 (Bandelt *et al.*, 1999) to generate median-joining haplotype networks to visualize relationships within major clades using data for all 100 individuals from the SNSM.

To examine statistical support for predictions of our alternative phylogenetic hypotheses (parapatric divergence predicts populations from the SNSM to be sister taxa, double colonization does not), we implemented

an approximately unbiased test (Shimodaira, 2002) using PAUP*. Because phylogenetic analyses appeared to support the double-colonization hypothesis (see below), we tested whether a tree in which the forms from the SNSM were forced to be sister to each other was significantly less likely than the maximum-likelihood tree.

Testing for phenotypic divergence along the elevational gradient

To examine phenotypic variation along the elevational gradient in fitness-related traits (Smith *et al.*, 2005a;

Milá *et al.*, 2009), we measured standard morphological features with dial callipers (to the nearest 0.1 mm) from all individuals captured. Measurements included tarsus length, wing chord, tail length, exposed culmen length, total culmen length, bill depth and bill width (measured at the proximal edge of nostrils and at the commissures). We also recorded body mass to the nearest 0.25 g using a spring scale. All individuals were banded using numbered aluminium bands. Because we only considered data from adult birds, not all genotyped individuals were included in morphological analyses; accordingly, the sample size was 81 individuals.

We used a principal components analysis (PCA) to reduce morphometric data to an uncorrelated set of variables, and then used factor scores obtained following varimax rotation to characterize variation along the elevational gradient. Visual inspection of patterns of morphometric variation as summarized by the first principal component (PC1) revealed the existence of two groups along the elevational gradient. To formally test the existence of two morphological groups of wood-wrens defined by PC1, we used a maximum-likelihood classification approach based on mixture models implemented in the *mclust* package for R (Fraley & Raftery, 2002; Fraley *et al.*, 2012). Because this analysis confirmed the existence of two separate morphological groups (see Results), we obtained the probability that each individual bird belonged to either group using *mclust* and plotted this probability with respect to elevation.

From March to June 2009, we recorded five to ten 'fast solo' songs (Dingle *et al.*, 2008) for 20 individuals in each of three different elevational zones: the two extremes of the gradient (1100–1360 m and 2270–2810 m) and intermediate elevations (1780–2200 m), where we suspected the two forms of wood-wren would overlap (Hilty & Brown, 1986). Recordings were made using a Marantz PMD661 Portable Solid State Recorder and a ME 67 Sennheiser shotgun microphone with a K6 Sennheiser Power Supply. Song recordings were digitized at a sampling rate of 44 kHz, and sonograms were generated with a 5 ms frame-length using 1 ms time-steps in the software *Luscinia* (Lachlan, 2007). These settings resulted in a spectral resolution of 43 Hz for single recordings and 10.5 Hz for the individual means.

We measured spectral and temporal characteristics of songs on sonograms to examine vocal differentiation. We measured the following parameters for each song (Dingle *et al.*, 2008): maximum and minimum frequency, maximum and minimum peak frequency (frequency with highest amplitude in a note) of songs, overall peak frequency (average of maximum and minimum peak frequency), song duration, duration of within-song silent interval and note duration in order to calculate the delivery rate (number of notes per second). We then reduced these variables using PCA and plotted scores with respect to elevation to characterize vocal variation along the gradient.

Examining patterns of genetic divergence

Because different kinds of genetic markers might introgress to different extents between populations owing to various evolutionary processes and to their different modes of inheritance (Sætre & Sæther, 2010), we examined the variation at multiple molecular markers along the elevational gradient. Specifically, we attempted to obtain allele or sequence data for all individuals sampled for: six unlinked autosomal microsatellite loci (Bowie *et al.*, 2012), mitochondrial DNA (the ATPase 6 and 8 genes; Eberhard & Bermingham, 2004), an autosomal nuclear intron (β -fibrinogen-5; Fuchs *et al.*, 2004) and a sex-linked nuclear intron (CHDZ; Griffiths & Korn, 1997). Because phylogenetic analyses of mtDNA data indicated that some individuals from the SNSM were closely related to individuals from populations in the Serranía de Perijá (see below), we also obtained microsatellite and β -fibrinogen-5 sequences for seven individuals from this area.

Microsatellite analyses were based on six polymorphic loci (10–25 alleles per locus; Bowie *et al.*, 2012). Allele sizes were estimated using GeneMapper 3.7 (Applied Biosystems, Foster City, CA, USA). Estimates of the frequency of null alleles and extent of large allelic dropout events were obtained using Micro-checker (Van Oosterhout *et al.*, 2004). Assumptions of Hardy–Weinberg equilibrium and linkage disequilibrium were tested in Genepop 4.0.10 (Raymond & Rousset, 1995). No evidence of significant null-allele frequency or large allelic dropout events was found, and deviations from Hardy–Weinberg equilibrium and linkage disequilibrium were not significant for any locus (Bowie *et al.*, 2012).

As described above, sequencing the mitochondrial ATPase 6 and ATPase 8 genes was based on standard protocols, which were also used with minor modifications (available upon request) to sequence the β -fibrinogen-5 and CHDZ nuclear introns. It was not necessary to phase sequences because all individuals were homozygous.

To examine whether two genetically distinct groups of wood-wrens exist in the SNSM with no hybridization or whether there was evidence for gene flow, we compared the patterns of variation across markers and related such patterns to elevation. We used *STRUCTURE* 2.3.3 (Pritchard *et al.*, 2000) to determine the number of genetically defined populations existing in the SNSM and to compute the posterior probability of assignment of individuals to each population using microsatellite data. *STRUCTURE* analyses were conducted with an admixture model ($\alpha = 1$) with a run length of 1 000 000 generations following a burn-in of 100 000 steps. To determine the number of populations (i.e. the most likely number of clusters, K), we used a heuristic approach (Evanno *et al.*, 2005) by considering variation in likelihood between $K = 1$ through 10. The results of ten replicate analyses for each value of K were collec-

tively summarized using the Greedy algorithm of CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007).

Based on patterns observed using mtDNA and β -fibrinogen-5 data, which revealed the existence of distinct lineages, we also assigned each sample to a clade for each of these markers. Because nuclear variation was reduced, we used a median-joining haplotype network for this purpose. Owing to the clear population structure observed (see below), we were able to assign each individual to mtDNA and nuclear DNA lineages unambiguously. With these data, we examined membership to different genetic groups along the elevational gradient.

Testing divergence in song perception

To determine whether vocal variation along the elevational gradient was related to patterns of conspecific recognition or aggressive response, we conducted song playback experiments following the design of Dingle *et al.* (2010). We conducted a total of 60 playback trials in three experiments. The first two experiments compared the behavioural response of territorial males of higher elevations (2270–2810 m) and lower elevations (1100–1360 m) to playback of their own songs (i.e. songs recorded in their elevational zone) and to foreign songs (i.e. songs from the other elevational zone). The third experiment compared the behavioural response to songs from high-elevation and low-elevation populations of males occurring at mid-elevations (1780–2200 m), where we expected the two forms to overlap.

We selected the best 10 ‘fast solo’ song recordings, each belonging to a different individual, to prepare 10 playback stimuli of the high-elevation population, and we did the same to prepare 10 playback stimuli of the low-elevation population. We applied a balanced reciprocal design in each of three playback experiments: at low-, mid- and high-elevation populations as defined above. Each experiment consisted of 20 trials, each conducted in one of 20 different territories. Our set-up limited the potential impact of pseudoreplication (Kroodsma, 1989; Slabbekoorn & Bouton, 2008) while optimally exploiting the available recordings. Playback trials lasted 30 min. The first 5 min was devoted to observations of ‘baseline behaviour’, followed by 5 min during which we recorded ‘response behaviour’ during and after a 2-min period of stimulus playback. Then, there was silence for 10 min, followed by the same procedure for the other stimulus. We used a Marantz PMD661 Portable Solid State Recorder and a portable field speaker SME-APS for song playback. Playback levels were standardized at 85 dB (A) at 1 m from the speaker, as measured with a Sphynx digital Sound Pressure Level meter. Speakers were located *c.* 1 m above the ground at locations near to sites where we had previously heard or seen territorial displays during monitoring visits.

We measured the strength of response to playback by evaluating three response measures: the approach-to-speaker distance, the total duration of vocal response and the response delay time. The approach distance was measured as the minimum distance between subject and speaker during the subsequent response periods in a trial. Distance categories were as follows: 0 = >16 m; 1 = 8–16 m; 2 = 4–8 m; 3 = 2–4 m; and 4 = <2 m (Nelson & Soha, 2004; Dingle *et al.*, 2010). The duration of vocal response was measured as the total amount of time singing. The response delay time was defined as the time elapsed between start of playback and the initial response.

To investigate whether there was a difference in response to playback stimuli in the three populations, we first reduced all response variables using PCA to a single variable, indicating the aggressiveness of the response. Wilcoxon signed-ranks tests were used to test for significant differences in response to different stimuli at the different elevations using SPSS (2007).

Results

Testing the geographic pattern of differentiation

Mitochondrial sequence data indicate that two separate, divergent clades of *H. leucophrys* exist in the SNSM: one ranges from *c.* 1100 to 2270 m elevation and the other from *c.* 2270 to 2810 m (Fig.2). Uncorrected genetic distance between these clades is 6%, suggesting that they have been isolated for *c.* 3 million years assuming an overall rate of divergence of *c.* 2% per million years estimated for avian mtDNA (Weir & Schluter, 2008) or for *c.* 1.5 million years assuming a faster rate estimated for ATPase 6 (Lerner *et al.*, 2011). The results obtained using different methods of phylogenetic inference consistently indicate that the two mtDNA clades occurring in the SNSM are not sister to each other: the cluster occurring at lower elevations was strongly supported as sister to populations from the Colombian and Venezuelan slopes of the Serranía de Perijá, whereas the high-elevation cluster appears to be more closely related to a clade from the Colombian, Ecuadorian and Peruvian Andes, but the latter relationship is not strongly supported (Fig.2). However, according to the approximately unbiased test of topology, a tree in which the forms from the SNSM are sister to each other cannot be rejected as significantly less likely (maximum-likelihood tree score = 4907.730, constraint tree score = 4921.710; $P = 0.098$). Although we are therefore unable to firmly reject the hypothesis of parapatric divergence, it appears more likely that divergence of the two populations involved a period of allopatry and that their coexistence with elevational replacement in the SNSM reflects secondary contact following separate colonization events.

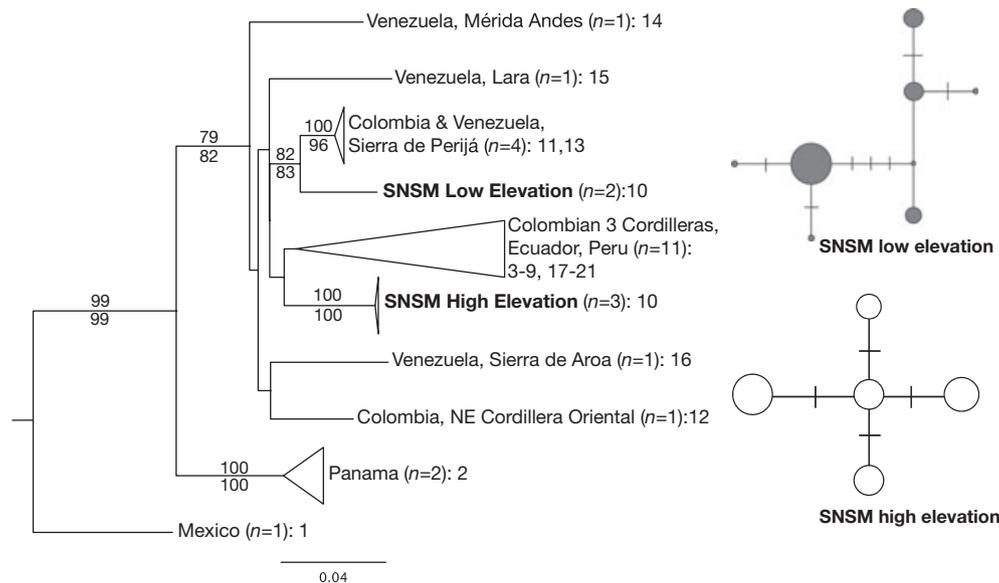


Figure 2 Simplified view of phylogenetic relationships of forms of *Henicorhina leucophrys* occurring in the SNSM with respect to other populations of this species based on sequences of the mitochondrial ATPase 6 and 8 genes analysed using the maximum-likelihood method. Two different clades exist in the SNSM, occur over different elevational ranges (low elevations, 1100–2270 m; high elevations, 2270–2810 m) and appear not to be each other's closest relatives, suggesting they did not diverge as a result of differentiation of a single lineage within the SNSM. Bootstrap values $\geq 75\%$ obtained in parsimony and maximum-likelihood analyses are shown above and below branches, respectively. Numbers correspond to localities mentioned in Table 1. The median-joining haplotype networks show relationships within each of the lineages from the SNSM based on the complete sample of 100 individuals from the study area.

Testing for phenotypic divergence along the elevational gradient

Three principal components explained 70.2% of the variation in the nine morphological features measured for the 81 adult individuals included in analyses. The first principal component (PC1) accounted for 43.6% of the variation and was positively correlated with bill length and depth, wing chord and body mass (Table 2). The second and third principal components explained 14.2% and 12.2% of the phenotypic variation and were correlated with bill width and tail length, respectively (Table 2). PC1 associated negatively with elevation: 68% of the variation in PC1 was explained by elevation (linear regression, $r^2 = 0.685$, d.f. = 79, $P < 0.01$), such that birds occurring at higher elevations tended to be smaller (i.e. to have lower body mass) and to have smaller bills (Fig. 3). However, morphological variation along the elevational gradient was not continuous; instead, there were two distinct groups identified by the model-based clustering analysis (mclust, Fig. S1), within which variation was unrelated to elevation: one ranged from c. 1100 to 2270 m and the other from c. 2270 to 2810 m (Fig. 3).

We recorded a total of 587 'fast solo' songs along the elevational gradient: 207 from 20 territories at low elevations (1100–1360 m), 167 from 20 territories at mid-elevations (1780–2200 m) and 210 from 20 territories at high elevations (2250–2810 m). Individuals living at

high elevations sing songs with a wider bandwidth including higher-frequency notes compared to individuals from low and mid-elevations; regarding the temporal pattern of songs, individuals from either high or low elevations sing faster than individuals from mid-elevation (Table S1, Fig. S2). Two principal components explained 94.6% of variation in seven song features ($n = 60$ territories). PC1 explained 68.4% of the variance and correlated positively with all of the frequency measurements (i.e. higher values of PC1 indicate higher-pitched songs; Table 2). PC2 explained 26.2% of the variation, had lower loading coefficients and correlated most strongly with the delivery rate of songs. Below, we describe vocal variation only based on PC1 because PC2 showed no consistent variation with respect to elevation.

Vocal PC1 was correlated positively with elevation: 86% of the variation in this component was accounted for by elevation ($r^2 = 0.859$, d.f. = 58, $P < 0.01$; Fig. 3). Low-elevation populations sing at lower frequencies and over a narrower bandwidth than high-elevation populations. Compared to morphological variation, which involved sharp replacement of two markedly distinct groups, vocalizations tended to vary more gradually with elevation (Fig. 3).

Because we found that bill morphology and vocal variation were correlated (Fig. 3), we sought to examine vocal variation along the elevational gradient while controlling for the influence of morphology by examining

Table 2 Loading coefficients for principal components used to reduce multivariate morphological, vocal and playback response data.

	Variable	Components		
		1	2	3
Morphological	Tarsus length	-0.46	-0.04	0.48
	Wing chord	0.81	-0.11	0.34
	Tail length	0.21	0.09	-0.83
	Bill length (tip to feathering)	0.89	0.01	0.01
	Bill length (tip to skull)	0.90	0.07	0.02
	Bill depth	0.78	0.16	0.10
	Bill width (proximal edge of nostrils)	0.48	0.64	0.11
	Bill width (where culmen begins)	-0.11	0.93	-0.008
Vocal	Mass	0.68	0.18	-0.26
	Peak maximum frequency	0.98	0.01	N/A
	Peak minimum frequency	0.97	0.12	N/A
	Overall peak frequency	0.98	-0.02	N/A
	Delivery rate	-0.04	0.95	N/A
	Note duration	-0.07	-0.95	N/A
	Mean minimum frequency	0.98	-0.05	N/A
Playback	Mean maximum frequency	0.98	0.01	N/A
	Delay in response	-0.84	N/A	N/A
	Total time singing response	0.73	N/A	N/A
	Minimum approach to speaker distance	0.83	N/A	N/A

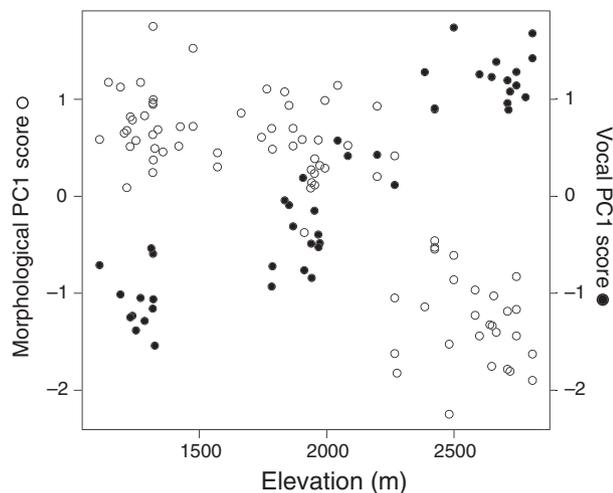


Figure 3 Morphological and vocal variation in wood-wrens along the elevational gradient in the SNSM. The first principal component (PC1; see loading coefficients in Table 2) of morphological variation (open circles) decreases with elevation, indicating a reduction in bill size and body size ($r^2 = 0.68$, $F = 172.9$, d.f. = 79, $P < 0.01$, $n = 81$); however, two clearly distinct groups exist, ranging from 1100 to 2270 m and from 2270 to 2810 m, respectively. Vocal variation as summarized by PC1 (black circles) increases gradually with elevation ($r^2 = 0.85$, $F = 308.9$, d.f. = 54, $P < 0.01$, $n = 56$), indicating songs have higher frequencies at higher elevations (see loading coefficients in Table 2). Note there is greater coverage of the elevational range for morphological data.

the change with elevation in the residuals of a regression between morphometric PC1 and vocal PC1. This analysis indicated a significant but relatively weak correlation

between the residuals and elevation: only 17% of the vocal variation was explained by elevation once the influence of morphology was controlled for ($F = 11.096$, d.f. = 46, $n = 48$, $r^2 = 0.17$, $P = 0.02$). This suggests that vocal variation depends largely, but not entirely, on morphological variation.

Examining patterns of genetic divergence

The STRUCTURE analyses of microsatellite data showed the existence of two clear genetic clusters; other values of K (e.g. 1 or 3 clusters) had much lower likelihood values (Fig S3). The H' value computed using CLUMPP was very high (0.999), which indicates that the results across different STRUCTURE runs were essentially the same. One of the genetic clusters identified based on the multilocus data ranged from 1100 to 2270 m and the other from 2270 to 2810 m (Fig. 4). All individuals had high probabilities of being assigned to either cluster except for one individual from high elevations which seemed to have a mixed genetic composition, indicating that it was likely a hybrid. When analyses included individuals from the Serranía de Perijá, the same two clusters were observed, and individuals from this area were clearly assigned to the low-elevation cluster (Fig. 4).

As described above, mtDNA variation was also clearly structured along the elevational gradient, with divergent clades ranging from 1100 to 2270 m and from 2270 to 2810 m, and a very similar pattern was seen in the β -fibrinogen-5 nuclear DNA data. As with the microsatellite data, samples from the Serranía de Perijá were

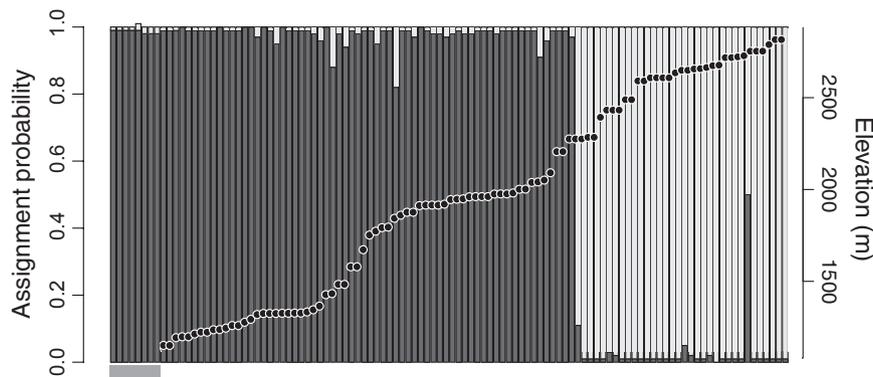


Figure 4 Results of STRUCTURE analysis based on microsatellite data ($K = 2$ populations) showing marked genetic divergence between wood-wren populations replacing each other along the elevational gradient ($n = 96$ individuals from the SNSM). The figure shows a STRUCTURE plot where each bar represents one individual for which the probability of assignment to one of the two populations is shown in different shades of grey. Dots indicate the elevation at which each individual was sampled; the light grey area on the lower left indicates individuals from the Serranía de Perijá ($n = 7$), which are genetically similar to birds from lower elevations in the SNSM. Note the high probability of assignment of most individuals and the abrupt change in assignment probabilities at around 2270 m elevation. A single individual with nearly equal assignment probability to both populations is a likely hybrid. Analyses including only samples from the SNSM (i.e. not considering birds from Perijá) also recovered $K = 2$ as most likely. [Correction added after online publication 11 January 2013: Figure 4 updated to greyscale version with new configuration.]

closely related to the lower-elevation lineage occurring in the SNSM based on mtDNA (Fig. 2) and nuclear β -fibrinogen-5 data (haplotype network not shown). The CHDZ gene showed no variation among individuals.

Concordance in patterns of morphological, vocal and genetic variation

Morphological, vocal, and genetic data varied consistently with elevation, indicating the existence of two distinct populations (Fig. 5). One population ranges from *c.* 1100 to 2270 m, and the other from *c.* 2270 to 2810 m. The former population ranges to lower elevations in San Lorenzo, which we did not sample for this study. The contact zone between both forms is located at *c.* 2270 m (members of both groups were found at this elevation), and the elevational replacement is sharp (Fig. 5), suggesting a stepped transition and the lack of a hybrid zone. Because sampling for vocal variation was not as thorough as for other data sets near the contact zone, we did not attempt to formally test for the existence of two distinct clusters based on vocalizations. However, although song properties appeared to change more gradually than other traits along the gradient, a noticeable change in song characteristics was observed at *c.* 2270 m, generally reflecting patterns of change in other traits.

Testing divergence in song perception

Principal component 1 explained 63% of the variation in the playback response data (Table 2), suggesting that this is an appropriate and simple measure of response to song stimuli. Individuals from both the high- and the low-elevation populations, but not those from mid-elevations, discriminated markedly against foreign

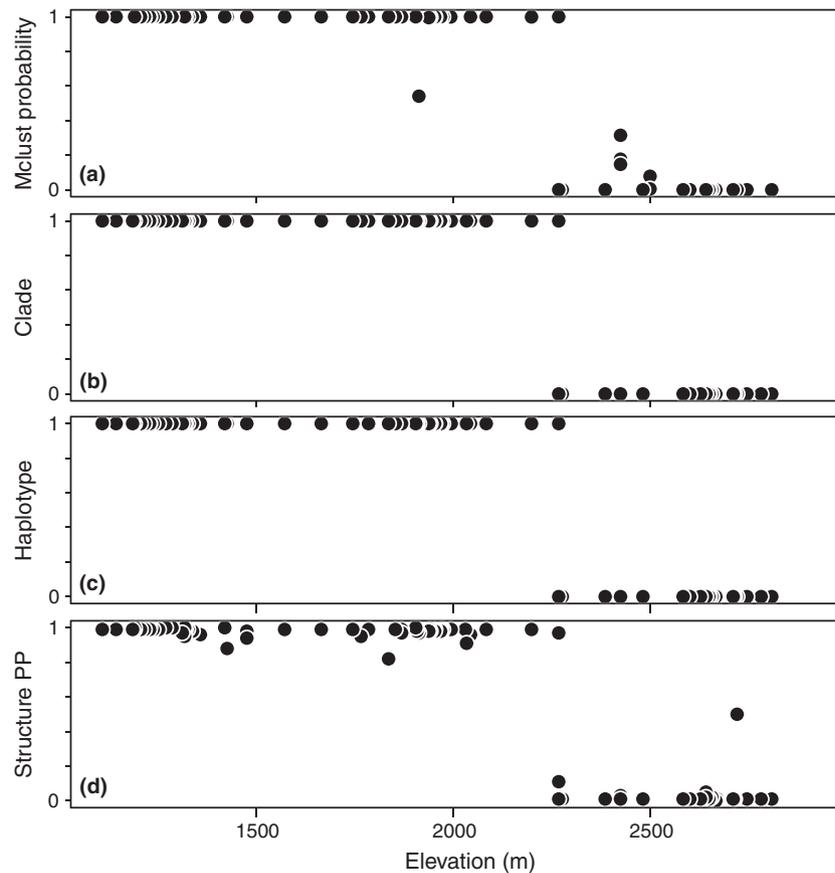
songs (Fig. 6). High-elevation birds responded much less strongly to low-elevation songs than to their own songs ($n = 20$, $Z = -3.81$, $P < 0.01$), and low-elevation birds responded significantly less strongly to high-elevation songs than to their own ($n = 20$, $Z = -2.80$, $P < 0.01$). The individuals from mid-elevations responded equally strong to low-elevation and high-elevation songs ($n = 20$, $Z = -0.037$, $P > 0.05$; Fig. 6).

Discussion

Geographic pattern of differentiation

Phylogenetic analyses revealed that the two forms of *H. leucophrys* replacing each other along the elevational gradient in the SNSM are apparently not sister taxa. This indicates that they likely did not differentiate in parapatry from a single ancestor that colonized this isolated mountain, but instead resulted from separate colonization events. This result is consistent with the hypothesis first proposed by Todd & Carriker (1922), who postulated that the divergence between wood-wren populations in the SNSM likely involved an allopatric phase, with present-day abutting distributions reflecting secondary contact. However, because a phylogenetic tree in which the forms from the SNSM are sister to each other is not significantly less likely than the maximum-likelihood tree (in which they are not), we cannot firmly reject the hypothesis of parapatric divergence. In addition, given the unresolved phylogenetic position of the high-elevation form from the SNSM, we cannot exclude the possibility that it is sister to the clade formed by the low-elevation form and pop-

Figure 5 Concordance in patterns of phenotypic and genetic variation in woodwrens sampled along the elevational gradient in the SNSM. A measure of assignment to different groups is shown for (a) morphometrics, with assignment defined by the probability of belonging to groups estimated by mclust ($n = 81$ individuals); (b) mitochondrial ATPase 6 and 8 sequences, with assignment based on individuals belonging to clades identified by phylogenetic analyses ($n = 100$; see Fig. 2); (c) nuclear β -fibrinogen-5 sequences, with assignment-based individuals carrying either a low-elevation haplotype or one of two high-elevation sister haplotypes ($n = 99$); (d) six microsatellite loci, with assignment determined by STRUCTURE analyses ($n = 96$; see Fig. 4). All the data are consistent in showing the existence of two distinct populations that are sympatric over a narrow elevational range and replace each other abruptly along the gradient.



ulations from the Serranía de Perijá. If the latter were true, then one could not exclude the possibility that the SNSM was indeed colonized only once, with populations from the Perijá being derived from the lower elevations of the SNSM. In sum, although our analyses are more consistent with the hypothesis of double colonization than with a single colonization event followed by parapatric ecological speciation, reaching definitive conclusions would require additional data providing greater phylogenetic resolution (i.e. sequences of multiple loci analysed using species-tree methods incorporating coalescent models to account for incomplete lineage sorting in nuclear genes). We note, however, that the pattern of strong genetic differentiation in presumably neutral microsatellite loci we observed is more consistent with differentiation in allopatry than in parapatry. When populations differentiate in parapatry, one would expect them to differ markedly in functionally important loci and less so in neutral loci owing to the influence of selection, whereas genome-wide divergence is expected when populations differentiate in geographically separate areas in the absence of gene flow.

Although some sister species in a variety of taxonomic groups replace each other along elevational gradients (reviewed by Cadena, 2007), compelling empirical evidence for parapatric speciation in mountains is still

largely lacking (but see Hall, 2005 for a promising example in butterflies and Savolainen *et al.*, 2006 for a case of ecological speciation involving minor differences in elevation in plants). In birds and other organisms, there are well-documented examples of local adaptation of populations along elevational gradients (Angert & Schemske, 2005; Dingle *et al.*, 2008; McCormack & Smith, 2008; Cheviron & Brumfield, 2009; McCracken *et al.*, 2009; Milá *et al.*, 2009; Storz *et al.*, 2012), but we are unaware of conclusive evidence that this mode of divergence has 'scaled up' and led to reproductive isolation and thus to the origin of two distinct species from a single, continuously distributed ancestral lineage as a result of divergent selection.

Multiple analyses, including those in this study, indicate that speciation in tropical montane organisms often involves an allopatric phase and that elevational replacements result from secondary contact between populations (reviewed by Cadena, 2007; see also Fuchs *et al.*, 2011). More generally, relative to the temperate zone, speciation in tropical montane vertebrates often involves comparatively little ecological divergence, such that population differentiation occurs predominantly in allopatry owing to ecological restrictions to dispersal imposed by niche conservatism and not in parapatry via selection along elevational (i.e. thermal) gradients

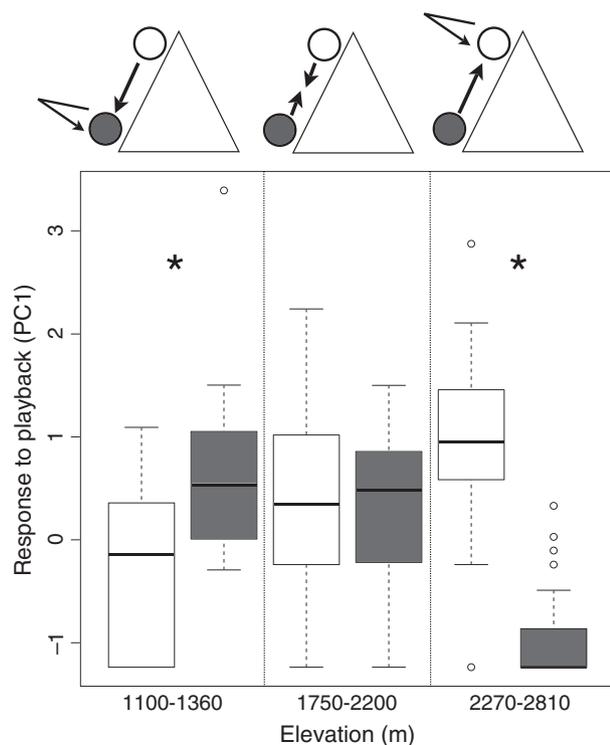


Figure 6 Results of playback experiments conducted at three ranges of elevation along the transect indicating stronger responses by Grey-breasted Wood-wrens to local songs in the highland and lowland populations, but no significant difference in the response to lowland or highland songs at mid-elevations. Playback response is expressed as a combined measure of aggressiveness resulting from a principal components analysis based on several variables measuring behavioural response (see text and Table 2).

(Cadena *et al.*, 2012; *contra* Keller & Seehausen, 2012). We note, however, that our results do not imply that ecological speciation (*sensu* Schluter, 2001, 2009) has not occurred in *Henicorhina* because, regardless of whether they evolved in elevational parapatry or not, selection related to environmental variation is a likely explanation for the origin of morphological and vocal divergence between populations, as we shall describe below.

Phenotypic variation and possible evidence for adaptation

Our analyses indicate that phenotypic variation leading to recognition of two forms of *H. leucorhynchus* in the SNSM based on few specimens (Bangs, 1899; Ridgway, 1903) is corroborated by our larger sample of birds, which identified clear differences in bill morphology, body size and song characteristics. Consistent with our data, earlier workers referred to low-elevation birds as larger-billed (Bangs, 1899; Ridgway, 1903). However, in contrast to Bangs (1899), who described birds from high elevations as larger in size, we found birds from such elevations to be smaller (i.e. of lower mass). Bangs

(1899) based his study on measurements of only eight birds (two males and two females per form) indicating seemingly longer tails, wings and tarsi at higher elevations. Our much larger sample is generally not consistent with Bangs's (1899) appraisal; birds from higher elevations actually have shorter tails and wings, but they do have longer tarsi. Below, we consider the smaller mass and shorter wings of high-elevation birds collectively as indicators of smaller body size.

Because morphology and songs vary in parallel, and because selection for a particular morphology may influence song properties and *vice versa* (Slabbekoorn & Smith, 2000; Podos, 2001; Podos & Nowicki, 2004; Seddon, 2005), divergence of these characters as a result of local adaptation can generate reproductive isolation (i.e. ecological speciation). Our results are consistent with a link between morphological and vocal variation in wood-wrens, as supported by the analysis indicating that much of the variation in songs between populations can be accounted for by morphological differences. Because birds found at higher elevations are smaller and have smaller bills, they are expected to sing at higher frequencies and to be able to sing over a wider frequency range based on biomechanical considerations (Podos, 2001; Podos & Nowicki, 2004; Seddon, 2005; Derryberry *et al.*, 2012). This is exactly what we observed. A difficult question to answer, however, is whether our correlational results may reflect the action of selection on songs, morphology or both.

Bill size can respond to selection driven by the availability of food resources (Grant & Grant, 2007; Badyaev *et al.*, 2008), which may vary with elevation (Schluter, 1982; Kleindorfer *et al.*, 2006). Likewise, body size can respond to selection along elevational gradients; Bergmann's rule predicts larger body size at higher elevations for optimal heat conservation at lower temperatures (Zink & Remsen, 1986), a pattern observed in some birds (Milá *et al.*, 2009). Because we have no information on the diet of wood-wrens in the SNSM nor on variation in food availability along the elevational gradient, we cannot determine whether variation in bill size may reflect adaptation to alternative food resources. The pattern of body-size variation observed (i.e. overall smaller birds at higher elevations) is contrary to that predicted by Bergmann's rule, so it is unlikely to be attributable to selection related to thermal balance in relation to climatic variation. In contrast, the observed reduction in bill size with elevation could be adaptive in the context of reducing thermoregulatory costs in colder environments (i.e. Allen's rule; Symonds & Tattersall, 2010; Greenberg *et al.*, 2012).

We also entertain the possibility that divergent selection has acted directly on songs at different elevations, as documented for populations of *Henicorhina* occurring along another elevational gradient in Ecuador. In agreement with our results, Dingle *et al.* (2008) found that songs of populations from low elevations were

lower-pitched than songs of populations from high elevations in the same mountain range. These authors suggested that vocal variation in Ecuador reflects acoustic adaptation resulting from interference via high-frequency ambient noise at lower elevations caused by the calls of cicadas. Preliminary analyses (Caycedo-Rosales, 2010; P. C. Caycedo-Rosales *et al.*, unpubl. data) indicated that high-frequency ambient noise produced by insects is also prevalent at lower elevations in the SNSM. Thus, we are currently examining whether ambient noise might underlie the elevational variation in songs across the contact zone in the SNSM. Furthermore, because song transmission can be affected by vegetation structure, vocal variation in wood-wrens in the SNSM could also reflect acoustic adaptation related to habitat structure as documented by earlier studies on birds (Wiley & Richards, 1982; Slabbekoorn, 2004; Kirschel *et al.*, 2009) and other animals (Ryan *et al.*, 1990; de la Torre & Snowdon, 2002; Couldridge & van Staaden, 2004). Finally, the arguably more exaggerated acoustic traits within the songs from higher, colder elevations in the SNSM (i.e. notes with a distinct wide-band frequency range) may reflect that sexual selection for more elaborate signals of male quality might be stronger in more demanding and temporally more variable environments (Botero *et al.*, 2009; Weir & Wheatcroft, 2010; but see Prum, 2010).

Phenotypic variation and alternative explanations to adaptation

Our results are consistent with the adaptive scenarios described above, but as with many other field studies, we acknowledge that the morphological and vocal variation we observed could be the result of phenotypic plasticity and not of evolutionary adaptation (cf. Ripmeester *et al.*, 2010). Bill morphology is highly heritable in some birds and it can thus respond to natural selection (Boag, 1983; Badyaev *et al.*, 2008), but no information on the genetic basis of bill morphology is available for our study populations. Although song properties are likely heritable to some extent because they are influenced by morphological and neurobiological variation and because birds can exhibit genetic predispositions to learn songs of their own species or population (Baptista, 1996; Nelson, 2000), learning plays an important role in vocal development in wrens (Kroodsma, 2005) and likely in *Henicorhina* (Jankowski *et al.*, 2010). Therefore, the vocal variation we observed is probably influenced by the social environment experienced by birds during development. This may make the role of song in the emergence and maintenance of species barriers more difficult, but not impossible (Slabbekoorn & Smith, 2002a; Dingle *et al.*, 2010). More generally, although we discussed a variety of adaptive explanations for patterns of variation above, in the

absence of detailed functional studies on the adaptive value of morphological and vocal traits, we cannot rule out the possibility that evolutionary mechanisms other than selection (notably, genetic drift) may be partly or wholly responsible for population differentiation.

Evidence for divergence in signal perception

The differences in songs we observed are not only in terms of song production but also in terms of song perception: both high-elevation and low-elevation birds responded significantly more strongly to songs from their own elevational range. Considering that song differences could be adaptive, our results hint at the possibility of assortative mating with respect to songs, an important component of models of ecological speciation in birds (Slabbekoorn & Smith, 2002a; Price, 2008). However, understanding response patterns in areas where birds of both forms might actually meet is crucial to assess the influence of signal divergence on the origin of reproductive isolation. This is why we also conducted playback experiments at mid-elevations. In contrast to the pattern seen at the extremes of the elevational gradient, mid-elevation birds did not discriminate between songs from high and low elevations. What are the implications of this pattern for our understanding of the consequences of signal divergence in terms of reproductive isolation?

To address the question above, we first note that the range where we conducted mid-elevation playback experiments (1750–2200 m) was not, in retrospect, the most appropriate area because all birds from these elevations were later found to belong to the low-elevation genetic group (Fig. 5). Because we did not know this *a priori*, we chose elevations for experiments in the field based on existing distributional information (Hilty & Brown, 1986). Furthermore, because both high- and low-elevation songs used for playback differed from the local mid-elevation songs (Table S1, Fig. S2), mid-elevation birds may have discriminated between any of the two 'foreign' song types had we tested them against local songs. Consequently, patterns of discrimination at mid-elevation remain somewhat unclear; experiments at slightly higher elevations where both forms meet are necessary.

The above notwithstanding, even if further data confirmed no song discrimination by birds from the contact zone, this need not undermine our inference that the two forms of wood-wrens likely mate assortatively with respect to songs. The mutual responsiveness to songs could also reflect that such responses are learned and that they play a role not only in mate choice but also in intra- and interspecific territoriality. The hypothesis of song-based interspecific territoriality has been tested for two different species of *Henicorhina* replacing each other along an elevational gradient in Central America; the response

to heterospecific song was strong in territories close to the elevational replacement zone, but declined as distance from the replacement zone increased (Jankowski *et al.*, 2010). Therefore, song learning may play a role in interspecific aggression such that individuals close to the replacement zone respond to heterospecific songs because they recognize them as potential threats, whereas those located far from the contact zone do not (Jankowski *et al.*, 2010). It follows that the apparent lack of song discrimination at mid-elevations in the SNSM does not imply that birds do not distinguish songs in the context of mate selection, but rather that their response might also reflect interspecific territoriality near the contact zone. A crucial future step to better understand the function of songs is to conduct playback experiments separately on males and females to evaluate whether females selecting mates discriminate songs more strongly than males interested in both attracting mates and defending their territories against conspecific and heterospecific intruders (Bernal *et al.*, 2007; Seddon & Tobias, 2010; Daner *et al.*, 2011).

Two species of wood-wren in the SNSM

Despite the caveats noted above, our data strongly suggest that the two forms of wood-wren existing in the SNSM have reached reproductive isolation because (1) they are phenotypically, genetically and behaviourally divergent, (2) they are sympatric (albeit over an apparently very narrow elevational range), and (3) hybridization between them is quite limited, such that the elevational replacement is abrupt and all characters (except songs, as discussed above) show strikingly similar patterns of variation along the gradient. Such coincidence in patterns of variation is remarkable relative to results of other studies on contact zones between recently diverged avian populations (e.g. Brelsford & Irwin, 2009), which often reveal contrasting patterns of variation across different traits and high degrees of genetic introgression. Therefore, these forms should be considered different species under the biological species concept (Mayr, 1942). A challenging question for future studies is to establish the taxonomic status of these two populations with respect to the rest of the Grey-breasted Wood-wren complex, which ranges from Mexico to Bolivia and exhibits complicated patterns of phenotypic and genetic variation.

Considering the phylogenetic evidence indicating that the wood-wren populations of the SNSM do not appear to be sister taxa, a likely explanation for the origin of reproductive isolation between them is allopatric divergence in mating signals resulting from elevation-related selection for acoustic adaptation or as a by-product of natural selection acting on morphological variation. Alternatively, it is possible that morphological and song characteristics initially diverged in allopatry and were later reinforced by selection,

completing premating isolation upon secondary contact (Price, 2008). However, as indicated above, we cannot rule out the possibility that selection has played no role in song divergence (Prum, 2010). Regardless of mechanism (i.e. whether speciation could be considered ecological or not; Schluter, 2009), vocal differentiation appears to have resulted in the origin of a premating barrier maintaining the two forms as distinct where they come into contact. That vocalizations are likely influenced by the environment (i.e. learning from neighbours) does not undermine our inference of the existence of a reproductive barrier because reproductive isolation between populations can be maintained by selection even if characters involved in mating are not highly heritable (Svensson *et al.*, 2010; Olofsson *et al.*, 2011; Verzijden *et al.*, 2012).

Conclusion

To our knowledge, this is the first study to examine speciation related to adaptation at different elevational zones that integrates phylogenetic analyses relevant to understanding the geographic context of divergence with detailed characterizations of variation in fitness-related morphological traits, signals involved in mate choice and multiple molecular markers with different modes of inheritance along an elevational gradient. Moreover, our study supplements these data with behavioural experiments to infer the influence of vocal divergence on assortative mating. This integrative approach allowed us to link morphological and vocal differentiation of populations occurring in different elevational zones with the existence of reproductive isolation.

Our study demonstrates that the two forms of Grey-breasted Wood-wren existing in the SNSM are two different species based on morphological, genetic and behavioural divergence. In addition, such divergence is consistent with adaptation along the elevational gradient. Although our results are seemingly (but not conclusively) inconsistent with parapatric speciation because phylogenetic analyses indicate that the elevational replacement reflects secondary contact and not primary differentiation, our data are consistent with a possible role for ecology in the origin of reproductive barriers (i.e. ecological speciation; Schluter, 2001, 2009) and in the maintenance of such barriers. However, understanding the adaptive value of vocal and morphological variation requires further work.

The similarity between the patterns observed in Ecuador (Dingle *et al.*, 2008, 2010) and in our study (i.e. marked vocal and mtDNA differentiation; birds from lower elevations with songs not extending into high frequencies used by insect calls) suggests that parallel evolution in phenotypic traits resulting from adaptation to similar ecological conditions might have occurred in two similar but independent elevational gradients involving Grey-breasted Wood-wrens. How-

ever, detailed analyses of environmental noise and sound transmission are necessary to test whether factors shaping songs in the SNSM coincide with those acting in Ecuador. In turn, a more detailed characterization of patterns of genetic differentiation at multiple loci along the elevational gradient in Ecuador would enable assessment of whether population differentiation has proceeded to the same extent in that region as we have documented for the SNSM. Furthermore, mitochondrial sequence data (J. L. Pérez-Emán *et al.*, unpubl. data) and vocal and phenotypic variation (Salaman *et al.*, 2003) suggest that elevational replacements involving different forms of what is currently treated as a single species, '*H. leucophrys*', are likely commonplace in Neotropical mountains. The existence of replicate replacements along gradients involving similar patterns of presumably adaptive variation at perhaps different stages of differentiation represents an exciting scenario for more detailed analyses aimed at understanding the forces underpinning ecological speciation (Berner *et al.*, 2009; Merrill *et al.*, 2011; Rosenblum & Harmon, 2011).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Summary of measurements of frequency-based and temporal patterns of songs of populations of Grey-breasted Wood-wren from the Sierra Nevada de Santa Marta in three ranges of elevation.

Figure S1 Results of model-based clustering analysis of morphometric data.

Figure S2 Representative spectrograms showing elevational variation in songs.

Figure S3 Results of STRUCTURE analyses of microsatellite data.

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Table S1. Summary of measurements of frequency-based and temporal patterns of songs of populations of Grey-breasted Wood-wren from the Sierra Nevada de Santa Marta in three ranges of elevation. Data shown are means \pm standard deviations ($n = 20$ territories per elevational zone). Means for all measurements were statistically different according to Kruskal-Wallis tests ($P < 0.001$). Superscripts indicate statistically different groups according to post-hoc analyses; when superscripts are not shown, all three means were significantly different from each other.

	Low elevations (1100-1360 m)	Mid elevations (1780-2200 m)	High elevations (2270-2810 m)
Maximum frequency (Hz)	4358.5 \pm 33.6 ^a	4560.4 \pm 132.1 ^a	5779.7 \pm 41.3 ^b
Minimum frequency (Hz)	1134.5 \pm 23.5	1879.5 \pm 129.9	1103.5 \pm 27.8
Maximum peak frequency (Hz)	3022.2 \pm 28.5	3319.4 \pm 43.4	3893.5 \pm 31.4
Minimum peak frequency (Hz)	1757.3 \pm 39.7	2091.8 \pm 47.7	2792.9 \pm 45
Overall peak frequency (Hz)	2672.3 \pm 26.6	2942.5 \pm 32	3426.7 \pm 27.2
Delivery rate (notes s ⁻¹)	7.2 \pm 1.4 ^a	6.4 \pm 1.9 ^b	7.2 \pm 1.2 ^a
Note duration (ms)	87 \pm 2.3 ^a	100 \pm 3.3 ^b	84 \pm 1.5 ^a

Figure S1. Results of model-based clustering analysis of morphometric data (i.e., morphological PC1; see text) conducted using mclust. The top figure shows the support (i.e., the Bayesian Information Criterion [BIC]) for models assuming that the mixture of morphological variation consists of 1-9 separate components, with (E) or without (V) equal variances. Note the strong support for the model assuming two separate clusters with equal variances (log likelihood = -92.211, $n = 81$, d. f. = 4, BIC = -202). The bottom figure shows the probability density function estimated via mixture modeling for morphological PC1, which exhibits clear bimodality. These results clearly support the existence of two morphologically distinct populations of wood-wren along the elevational gradient in the SNSM.

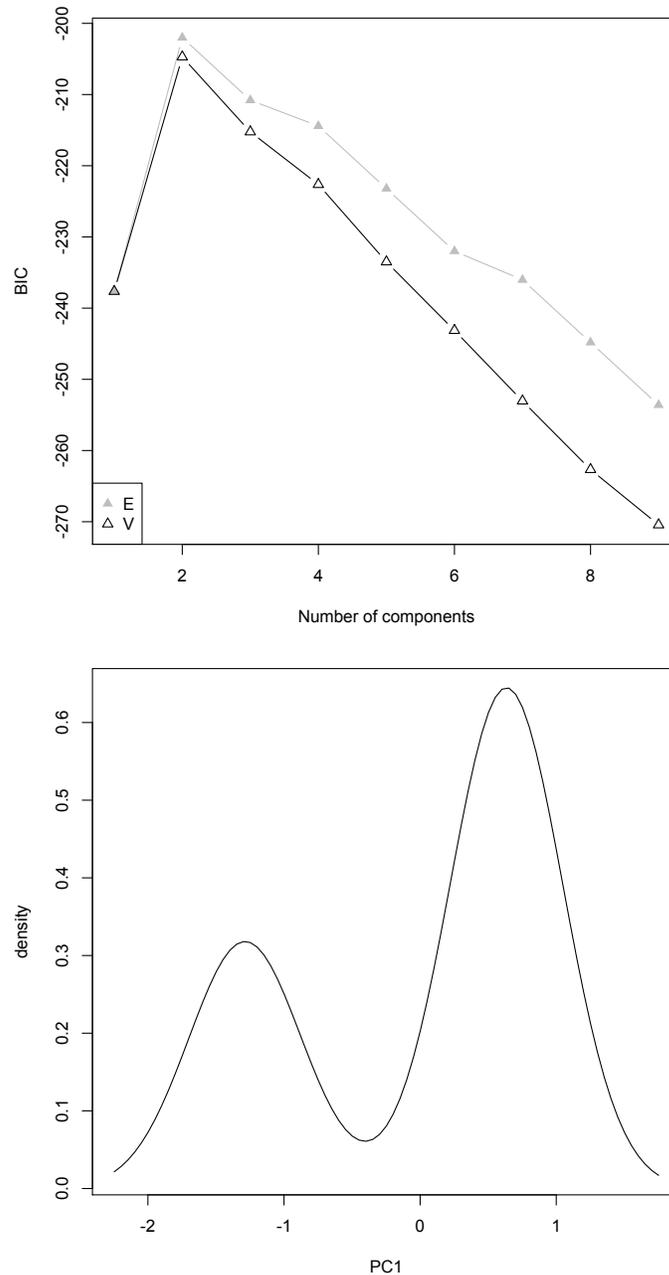


Figure S2. Representative spectrograms (time in seconds vs. frequency in kHz) depicting elevational variation in songs of populations of Grey-breasted Wood-wren from the Sierra Nevada de Santa Marta; (top) low-elevation song recorded at 1210 m; (middle) mid-elevation song recorded at 1970 m; (bottom) high-elevation song recorded at 2710 m. Spectrograms (DFT size: 2048 samples, 50% frame overlap) using Raven Pro version 1.4 (<http://www.birds.cornell.edu/raven>). Note the overall lower frequency and reduced bandwidth of songs from low elevations. The noise evident in the low-elevation spectrogram at above c. 4.5 kHz corresponds to vocalizations produced by a cricket species.

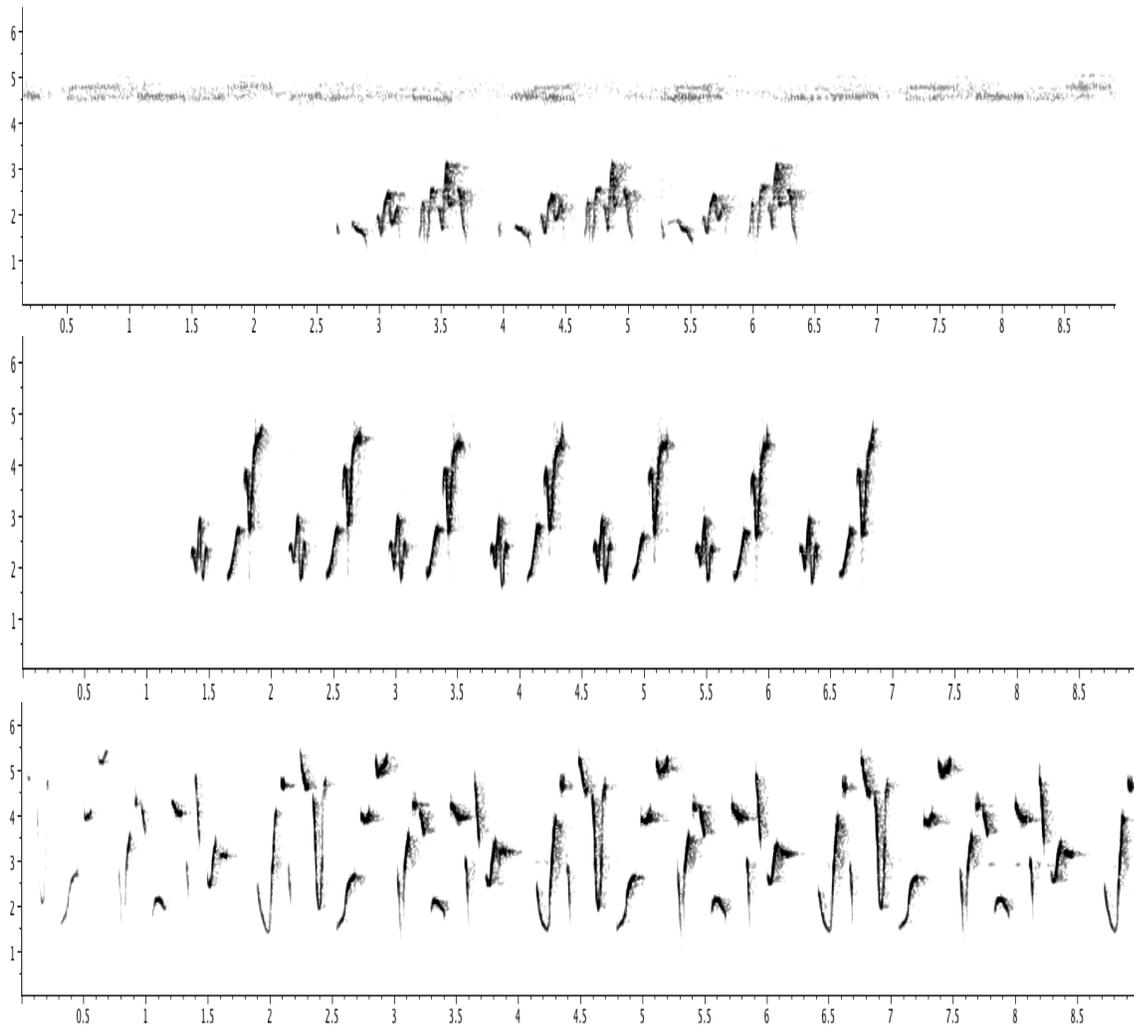


Figure S3. Results of STRUCTURE analyses of microsatellite data showing the likelihood of different values of K (for $K = 1-4$, top) and the Evanno *et al.* (2005) delta K statistic (for $K = 1-10$, bottom). For each K , the program was run ten times; dots in the top figure correspond to results obtained in each of the independent runs. Note the strong support for the existence of two genetic clusters ($K = 2$) along the elevational gradient.

