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Geographic isolation has been proposed as the factor driving subspecific diversity of the Ecuadorian hillstar (*Oreotrochilus chimborazo*), a highland species restricted to the naturally fragmented paramos of Ecuador and southern Colombia. Current taxonomy recognizes three subspecies: *O. c. chimborazo* (from the Chimborazo volcano), *O. c. soderstromi* (from the Quilotoa volcano), and *O. c. jamesonii* (along the paramos of Ecuador and southern Colombia). To understand the origin of this morphological diversity, we explored the genetic variation along the species range based on analyses of two mitochondrial genes (ND2 and ND4), and one nuclear intron (MUSK). Subspecies *O. c. soderstromi* was not included in the analysis, as it was not registered at or around its type locality, the Quilotoa volcano. Instead, only *O. c. jamesonii* was encountered in that area. We found no evidence of genetic structure corresponding to subspecies or physiographic units, aside from some inconclusive evidence in putatively isolated populations. Ecological niche modeling predicted continuous and homogeneous environmental space between the two volcanos, and field expeditions showed evidence of a potential contact zone between *O. c. jamesonii* and *O. c. chimborazo*. Also, our data suggest that the only specimen described as *O. c. soderstromi* may have been an intergrade. We discuss our results in the light of possible range shifts in the past, resulting from climatic fluctuations around the Pleistocene–Holocene transition.

Comprendiendo la historia evolutiva de un ave endémica de los altos Andes: la Estrellita Ecuatoriana (*Oreotrochilus chimborazo*)

El aislamiento geográfico ha sido propuesto como el factor más importante en la generación de diversidad subespecífica en la estrellita ecuatoriana *(Oreotrochilus chimborazo)*, un ave de los altos Andes restringida a los páramos naturalmente fragmentados de Ecuador y el sur de Colombia. La taxonomía actual reconoce tres subspecies: *O. c. chimborazo* (en el volcán Chimborazo), *O. c. soderstromi* (en el volcán Quilotoa) y *O. c. jamesonii* (a lo largo de los páramos de Ecuador y el sur de Colombia). Con el fin de comprender el origen de esta diversidad morfológica, exploramos la variación genética a lo largo del rango de la especie con base en los análisis de dos genes mitocondriales (ND2 y ND4) y un intrón nuclear (MUSK). La subespecie *O. c. soderstromi* no fue incluida en el análisis, porque no se registraron individuos de este taxón en su localidad tipo, el volcán Quilotoa, donde solo confirmamos la presencia de *O. c. jamesonii*. No encontramos evidencia de estructura genética correspondiente a las subspecies o a unidades fisiográficas de páramo, a excepción de una posible estructuración genética probablemente debida a poblaciones presumiblemente aisladas. Análisis de modelamiento de nicho ecológico predijeron que el espacio ambiental entre los dos volcanes es continuo y homogéneo, mientras que los resultados de expediciones de campo sugieren la existencia de una zona de contacto entre *O. c. jamesonii* y *O. c. chimborazo*. Además, nuestros datos apoyan la idea de que el único espécimen descrito como *O. c. soderstromi* puede ser un híbrido. Discutimos nuestros resultados en el contexto de posibles cambios de rango de distribución en el pasado, como resultado de las fluctuaciones climáticas que se dieron en el periodo de transición entre el Pleistoceno y el Holoceno.

Keywords: hummingbird; contact zone; phylogeography; intergradation; dispersal barrier; Andes; paramo; Ecuador

Introduction

Tropical mountain ranges have probably hosted more events of bird diversification in geologically recent times than any other geographic area of comparable extension.[1] By establishing elevational gradients of temperature and precipitation, these landscapes promote a diversity of ecosystems ranging from tropical forests and savannahs, near the base, to tundra-like grasslands, near the top, with each ecosystem having its share of specialized birds. To add another layer of complexity to this process of diversification, the rugged topography of mountains promotes the generation of dispersal barriers, leading to divergence by geographic isolation. In the Andes, for example, deep, dry valleys act as dispersal barriers for birds in the cloud forest, and often, closely related and ecologically similar species replace each other on either side of the barrier.[2–4]

High-elevation ecosystems tend to be more restricted in area, and thus their inhabitants are more likely to diverge in response to habitat fragmentation.[5,6] In extreme cases, habitats are restricted to the slopes of outstanding mountain peaks, as probably happens with the habitat of some populations of the Ecuadorian hillstar (*Oreotrochilus chimborazo*), a hummingbird with one of the highest elevational ranges in the world [7] that seems to be undergoing rapid evolutionary divergence.

Ecuadorian hillstars have a geographic range restricted to the paramos of Ecuador and extreme southern Colombia.[8,9] Within this small range, three subspecies have been described: *O. c. chimborazo*, *O. c. jamesonii*, and *O. c. soderstromi* [8,10,11] (Figure 1(A)). Subspecies *O. c. chimborazo* and *O. c. soderstromi* are described in the literature as being endemic to the volcanoes Chimborazo

and Quilotoa, [8,9,12] respectively (Figure 1(A)). The soil on the slopes and surrounding areas of these volcanoes are barren and consists of fine, volcanic sediment.[13] Among the few scattered bushes that thrive under these conditions is *Chuquiraga jussieui* (Asteraceae), a primary source of nectar for *O. Chimborazo*, which might limit the distribution of the hummingbird.[11] This case of extreme habitat insularity has caught the attention of notable naturalists such as Alfred Russel Wallace, who wrote in his treatise *Island Life* that the ranges of these subspecies were among 'the most wonderfully restricted.'[10]

Since the publication of *Island Life*, however, important findings about the distribution of *O. chimborazo* have challenged the assumption that these hummingbirds are restricted to volcanic peaks. The subspecies with the most widespread distribution, *O. c. jamesonii*, is now known to
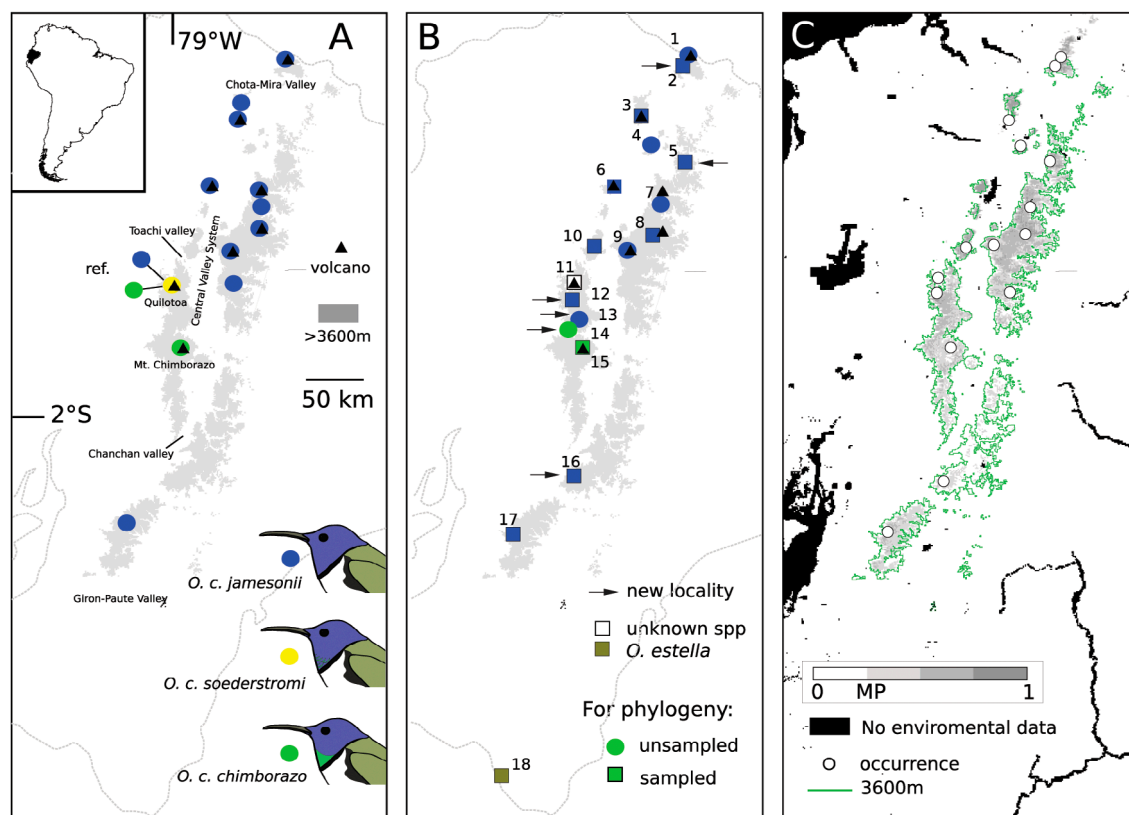

Figure 1. Geographic range of *Oreotrochilus chimborazo*. (A) Range as described in the literature.[8,9,11,14,15,17,18] Recent pictures [20] taken in Quilotoa show individuals clearly identifiable as males of *O. c. chimborazo* and *O. c. jamesonii*, thereby putting in doubt the geographic isolation between these subspecies. (B) Localities visited during this study. Localities 12, 13, and 14 were those visited around Quilotoa and Chimborazo and in the intervening páramo between both volcanoes. (C) Predicted distribution of *O. c. chimborazo* based on ecological niche modeling using data from WorldClim [36] and remote sensing. The localities in which *O. chimborazo* was found between volcanoes Quilotoa and Chimborazo were not used during modeling to test whether occurrence in that region could be predicted using information from the other localities only. MP, Minimum Presence threshold provided by MaxEnt analysis. Localities, numbered according to latitude starting at the northernmost: 1, Mt. El Chiles; 2, El Ángel Ecological Reserve; 3, Mt. Cotacachi; 4, lakes of Mojanda; 5, Mt. Cayambe; 6, Mt. Pichincha; 7, Papallacta Pass; 8, Antisana Ecological Reserve; 9, Mt. Cotopaxi; 10, Mt. Illinizas; 11, Quilotoa crater; 12, Apagua mountain ridge; 13, Mountains around Mula Corral reservoir; 14, Mountains around Simiatug; 15, Cunungyacu (near Mt. Chimborazo); 16, Culebrillas lake; 17, Mountains around Migüir; 18, Lagunillas mountain ridge.

also occur in grassland paramo, outside the slopes of volcanoes but still in places where *Chuquiraga* grows (Figure 1(A)).[11,14–18] Dependence on volcanic slopes is still being proposed as the factor driving divergence between hillstars living in Quilotoa and Chimborazo.[8,9] Nevertheless, grassland paramo stretches between both volcanoes,[19] at elevations above the minimum that at which *O. c. jamesonii* has been recorded elsewhere in Ecuador. Therefore, unless the subspecies at Quilotoa and Chimborazo were restricted to volcanoes, and thus differ ecologically from *O. c. jamesonii*, no reason exists to claim that birds belonging to such subspecies are isolated. In fact, the possibility of contact between subspecies of *O. chimborazo* has been suggested even in the description of subspecies *O. c. soderstromi*,[12] upon the realization that morphological characteristics that define *O. c. soderstromi* are consistent with what would be expected of an intergrade between the other two subspecies.[8,9,12]

All three subspecies are recognized on the basis of a single character, the color of the gorget in males: in *O. c. jamesonii*, it is violet-blue, the same color as that of the rest of the head; in *O. c. chimborazo*, it is bright green; and in *O. c. soderstromi*, the gorget is violet-blue with a few interspersed green feathers [8,9,12] (Figure 1(A)). The intermediate plumage and geographic distribution of *O. c. soderstromi* thus fits with the description of an intergrade, but such hypothesis did not surface because it was assumed that hillstars from Quilotoa were isolated from hillstars elsewhere.[8,9] Moreover, recent photographic evidence shows males of *O. c. jamesonii* and *O. c. chimborazo* in Quilotoa,[20] providing some preliminary evidence that both subspecies occur in the area.

In spite of the possibility of limited contact and intergradation, how did these differentiated forms came into being? Divergence under sympatry is among the scenarios least favored by evolutionary models [21,22]; a more likely scenario is divergence in allopatry caused by a geographic barrier that is non-existent in the present. Furthermore, geographic isolation might be affecting genetic structure within *O. c. jamesonii*. The distribution of this subspecies extends across all grassland paramo in Ecuador (Figure 1(B)), but is intersected by deep dry valleys. Some of these valleys, such as those of the Mira and Chanchán rivers, have been proposed to be barriers of dispersal for birds.[9] To understand how divergence by isolation may occur in relatively short evolutionary times within *Oreotrochilus* in particular and paramo birds in general, it is worth testing whether geographic isolation might be affecting genetic structure within *O. c. jamesonii*.

Phylogeography, as the study of the relation between geography and phylogenetics is often named, may provide important insights on the evolutionary history of *O. chimborazo*. In past studies, phylogeography has been useful in confirming the role of dry Andean valleys as dispersal barriers,[2,3,23,24] and has allowed researchers to uncover cryptic diversity [24–29] and find correlations between sequences of speciation events and emergence of potential dispersal barriers.[24] Most phylogeographic studies in the Andes have focused on the inhabitants of the cloud forests, but the paramo also offers promising case studies, such as that of the Ecuadorian hillstar. Thus, in this study, we used phylogeographic methods to test: (1) whether subspecies *O. c. jamesonii* and *O. c. chimborazo* represent diverging evolutionary lineages, and (2) whether *O. c. jamesonii* shows genetic structure along its geographic distribution. We also aimed to gain a better understanding of the distribution of *O. chimborazo*. To meet this goal, we conducted systematic visits to the most important 'paramo islands' in Ecuador, in addition to visits to volcanoes Quilotoa and Chimborazo, and three sites in between these two volcanoes, to explore whether subspecies of *O. chimborazo* are in contact. We expect this study to pioneer phylogeographic research of birds in the paramos of the northern Andes, and to present a model organism for the study of geographic isolation and divergence at its earliest stages.

Materials and methods

Methods

Fieldwork and specimen collection

We visited 17 localities to test for occurrence of *O. chimborazo* (Figure 1(B)). We included three localities situated between Quilotoa and Chimborazo, which we hypothesized to have suitable habitat for the species, against some claims from the literature.[8,9] In selecting the 17 localities, we tried to visit at least one locality from each 'paramo island' in Ecuador (Figure 1(B)). A 'paramo island' is defined here as a set of regions topographically continuous between 3600 and 4800 m of elevation and separated from other paramos by more than 15 km. This last criterion of separation was chosen because it is the minimum width of valleys that have been hypothesized to be barriers for the dispersal of birds.[30]

Collection of samples for genetic analysis was possible in 11 of the above-mentioned localities. Additionally, we visited one locality in southern Ecuador (Lagunillas mountain range) (Figure 1(B)) to obtain samples from *O. estella*, a closely related species to *O. chimborazo*.[31,32] Specimens of *Oreotrochilus* were collected around patches of *Chuquiraga jussieui* in the paramo. Samples of pectoral muscle were used for genetic analysis (stored in 95% ethanol). Voucher specimens for most individuals are housed in the zoology museums of Pontificia Universidad Católica del Ecuador (QCAZ) and Universidad Tecnológica Indoamérica (MZUTI) (list of specimens provided in Table 1). Pectoral muscle samples were frozen at −20 or −80 °C, and deposited at the same institutions.

Table 1.   Samples of *Oreotrochilus chimborazo* and *Oreotrochilus estella* collected for this study. Museum abbreviations: QCAZ, Museo de Zoología de la Pontificia Universidad Católica del Ecuador; MZUTI, Museo de Zoología de la Universidad Tecnológica Indoamérica; UTI, Universidad Tecnológica Indoamérica (only for specimens for which no voucher is available). Sex codes: F, female; M, male; U, undetermined. Gene accession numbers are given in each gene column.

Voucher	Subspecies	Sex	Locality	ND2	ND4	MUSK	mtDNA haplotype
QCAZ 4162	*O. c. jamesonii*	F	Antisana Ecological Reserve	KU600139	KU587749	KU600191	A
QCAZ 4164	*O. c. jamesonii*	M	Antisana Ecological Reserve	KU600145	KU587755	KU600186	E
QCAZ 4171	*O. c. jamesonii*	M	Antisana Ecological Reserve	KU600167	KU587777	KU600215	A
QCAZ 4182	*O. c. jamesonii*	F	Antisana Ecological Reserve	KU600171	KU587781	KU600198	A
QCAZ 4184	*O. c. jamesonii*	F	Antisana Ecological Reserve	KU600142	KU587752	KU600181	J
QCAZ 4188	*O. c. jamesonii*	U	Antisana Ecological Reserve	KU600137	KU587746	KU600201	B
QCAZ 4189	*O. c. jamesonii*	U	Antisana Ecological Reserve	KU600130	KU587739	KU600200	B
QCAZ 4190	*O. c. jamesonii*	M	Antisana Ecological Reserve	KU600151	KU587761	KU600196	A
MZUTI 105	*O. c. jamesonii*	M	Apagua mountain ridge	KU600132	KU587741		A
MZUTI 109	*O. c. jamesonii*	F	Apagua mountain ridge	KU600170	KU587780		A
MZUTI 112	*O. c. jamesonii*	F	Apagua mountain ridge	KU600155	KU587765		I
QCAZ 3789	*O. c. jamesonii*	M	Apagua mountain ridge	KU600163	KU587773	KU600214	A
QCAZ 3790	*O. c. jamesonii*	M	Apagua mountain ridge	KU600159	KU587769	KU600210	A
QCAZ 4169	*O. c. jamesonii*	F	Apagua mountain ridge	KU600147	KU587757	KU600182	A
UTI 57	*O. c. jamesonii*	M	Apagua mountain ridge	KU600135	KU587744		A
UTI 58	*O. c. jamesonii*	U	Apagua mountain ridge	KU641486	KU587748		A
UTI 63	*O. c. jamesonii*	M	Apagua mountain ridge	KU600153	KU587763		G
QCAZ 4148	*O. c. jamesonii*	F	Culebrillas lake	KU600129	KU587738	KU600224	A
QCAZ 4149	*O. c. jamesonii*	M	Culebrillas lake	KU600148	KU587758	KU600209	A
QCAZ 4150	*O. c. jamesonii*	F	Culebrillas lake	KU600158	KU587768	KU600218	A
QCAZ 4152	*O. c. jamesonii*	F	Culebrillas lake	KU600122	KU587731	KU600177	A
QCAZ 4153	*O. c. jamesonii*	M	Culebrillas lake	KU600166	KU587776	KU600178	A
QCAZ 4155	*O. c. jamesonii*	M	Culebrillas lake	KU600120	KU587729	KU600188	C
QCAZ 3783	*O. c. chimborazo*	M	Cunungyacu (near Mt. Chimborazo)	KU600131	KU587740	KU600204	B
QCAZ 3784	*O. c. chimborazo*	M	Cunungyacu (near Mt. Chimborazo)	KU600168	KU587778	KU600202	B
QCAZ 3791	*O. c. chimborazo*	M	Cunungyacu (near Mt. Chimborazo)	KU600140	KU587750	KU600205	H
QCAZ 4146	*O. c. chimborazo*	F	Cunungyacu (near Mt. Chimborazo)	KU600156	KU587766	KU600197	A
QCAZ 4156	*O. c. chimborazo*	F	Cunungyacu (near Mt. Chimborazo)	KU600126	KU587735	KU600193	A
QCAZ 4157	*O. c. chimborazo*	M	Cunungyacu (near Mt. Chimborazo)	KU600162	KU587772	KU600187	C
QCAZ 4165	*O. c. chimborazo*	F	Cunungyacu (near Mt. Chimborazo)	KU600123	KU587732	KU600183	A
QCAZ 4166	*O. c. chimborazo*	F	Cunungyacu (near Mt. Chimborazo)	KU600125	KU587734	KU600185	A
QCAZ 4168	*O. c. chimborazo*	F	Cunungyacu (near Mt. Chimborazo)	KU600124	KU587733	KU600184	A
QCAZ 4170	*O. c. chimborazo*	M	Cunungyacu (near Mt. Chimborazo)	KU600161	KU587771	KU600190	C
QCAZ 4173	*O. c. chimborazo*	F	Cunungyacu (near Mt. Chimborazo)	KU600150	KU587760	KU600194	A
QCAZ 4178	*O. c. chimborazo*	F	Cunungyacu (near Mt. Chimborazo)	KU600160	KU587770	KU600189	C
QCAZ 4179	*O. c. chimborazo*	M	Cunungyacu (near Mt. Chimborazo)	KU600128	KU587737	KU600208	A
QCAZ 4180	*O. c. chimborazo*	M	Cunungyacu (near Mt. Chimborazo)	KU600134	KU587743	KU600195	A
QCAZ 4159	*O. c. jamesonii*	F	El Ángel Ecological Reserve	KU600169	KU587779	KU600221	D
QCAZ 4160	*O. c. jamesonii*	M	El Ángel Ecological Reserve	KU600121	KU587730	KU600219	D
QCAZ 4161	*O. c. jamesonii*	M	El Ángel Ecological Reserve	KU600138	KU587747	KU600220	D
QCAZ 3782	*O. c. jamesonii*	F	Mountains around Migüir	KU600144	KU587754	KU600223	F
QCAZ 4177	*O. c. jamesonii*	M	Mountains around Migüir	KU600127	KU587736	KU600222	F
MZUTI 30	*O. c. jamesonii*	M	Mt. Cotacachi	KU600136	KU587745		B
QCAZ 4147	*O. c. jamesonii*	M	Mt. Cotacachi	KU600165	KU587775	KU600179	E
QCAZ 4175	*O. c. jamesonii*	M	Mt. Cayambe	KU600173	KU587783	KU600216	A
QCAZ 4176	*O. c. jamesonii*	M	Mt. Cayambe	KU600141	KU587751	KU600203	B
QCAZ 4183	*O. c. jamesonii*	M	Mt. Cayambe	KU600133	KU587742	KU600212	A
QCAZ 4191	*O. c. jamesonii*	M	Mt. Cayambe	KU600146	KU587756	KU600217	A
QCAZ 4158	*O. c. jamesonii*	F	Mt. Cotacachi	KU600164	KU587774	KU600180	A
QCAZ 4167	*O. c. jamesonii*	F	Mt. Cotacachi	KU600157	KU587767	KU600199	A
QCAZ 4187	*O. c. jamesonii*	M	Mt. Illinizas	KU600174	KU587784	KU600207	A
QCAZ 4163	*O. c. jamesonii*	M	Mt. Pichincha	KU600149	KU587759	KU600206	A
QCAZ 4172	*O. c. jamesonii*	M	Mt. Pichincha	KU600172	KU587782	KU600211	A
QCAZ 4174	*O. c. jamesonii*	F	Mt. Pichincha	KU600152	KU587762	KU600192	A
QCAZ 4181	*O. c. jamesonii*	M	Mt. Pichincha	KU600143	KU587753	KU600213	A
UTI 74	*O. chimborazo*	F	Quilotoa crater	KU600154	KU587764		A
QCAZ 3868	*O. estella*		Las Lagunillas, Zamora	KU600118	KU587727	KU600175	
QCAZ 3901	*O. estella*		Las Lagunillas, Zamora	KU600119	KU587728	KU600176	

Species distribution analysis

We built an ecological niche model (ENM) for *O. chimborazo* and projected it back on to geography using MaxEnt.[33,34] Occurrences used to build the model were localities visited during fieldwork and additional localities retrieved from the literature [8,9,14,18] (Figure 1(C)). The database eBird [35] was consulted to find additional localities, but we found none that were not already covered by the literature and our field work. In calibrating the ENM, we excluded the localities situated between Quilotoa and Chimborazo to test whether environmental information retrieved from other localities was sufficient to predict occurrence of these birds between the two volcanoes. Predicting occurrence in the left-out localities would provide evidence that the hillstars that we found in those localities, rather than being vagrant individuals, belong to populations thriving under suitable environmental conditions. To avoid spatial auto-correlation, we included only localities situated more than 5 km apart, a distance large enough to overpass small mountain ridges that may set boundaries to local climate. Upon applying this filter, 15 occurrences remained for the analysis (Figure 1(C)).

The environmental data-set used to build the ENM consisted of raster grids containing data from WorldClim [36] on temperature and precipitation. The geographic area covered by the grids was defined by a continuous area that encompassed a 120-km buffer around presence localities: an area large enough to cover the known distribution of the species and to provide enough contrast between environmental conditions characterizing presence localities and background areas. We did not use a larger area representing a biogeographic zone,[see [37]] because wide topographic depressions at the north and south of the hummingbird's known distribution preclude the presence of immediately accessible areas to the species.[8]

The resolution of the grids was of 1 km per pixel. Temperature was used in the ENM because of its strong correlation to elevation, and because using temperature, in contrast to using elevation, allows constructing a biologically meaningful description of potential occurrence. Precipitation was used because it was hypothesized to explain the occurrence of arid environments, which may be preferred by the hillstars.[11] WorldClim provided 19 variables derived from temperature and precipitation, but based on results from a preliminary run of MaxEnt we selected only six of those variables, which were those that had a relative contribution of at least 1% to the ENM in preliminary runs.

In addition to WorldClim data, six data-sets from remote satellite sensors were used to build the ENM: NDVI, the normalized vegetation index from NOAA-AVHRR [38]; LAI1, LAI2, and LAI3, which are different measurements of leaf area index derived from the remote sensor MODIS [39]; and two layers from the scatterometer QSCAT (http://www.scp.byu.edu/) containing data on microwave reflectance of land surface, which is informative on aridity of soils.[40] These MODIS and QSCAT data were the same as the ones used in a previous study [40] to model the potential distribution of South American birds. Measurements derived from MODIS corresponded to averages of monthly data taken between 2000 and 2004. Measurements derived from QSCAT corresponded to the average and standard deviation of three-day composite images for the year 2001.

We used MaxEnt 3.3.3 k to model the species distribution, with the following settings: convergence threshold set to 1×10^{-5}, maximum number of background points to 10^4, maximum iterations to 500, and the regularization parameter set to 'auto.' Equal training and minimum presence thresholds were used to filter the ENM. A map of potential occurrence of *O. chimborazo* was created by projecting the filtered ENM back on to geography. Support for the ENM was tested using a Pearson cross-validation test, which is recommended for ENMs built with fewer than 25 known occurrences.[41] (see [42] for a justification of not using AUC curve).

Sample preparation for genetic analysis

DNA was extracted following a protocol based on guanidine thiocyanate lysis and isopropanol precipitation.[43] Three regions were amplified: subunits 2 and 4 of the mitochondrial DNA (mtDNA) gene Nicotinamide Dehydrogenase (ND2 and ND4, respectively), and the third intron of the Muscle-specific Kinase gene (MUSK). The amplification product for ND4 also included flanking tRNA-coding regions. Amplification primers were used as follows: for ND2, primers 5219 and 6313 [44]; for ND4, primers ND4 and LEU [45]; and, for MUSK, primers MUSK I3F and MUSK I3R.[46] A standard protocol was followed for amplification of ND2, a step-down protocol for that of ND4, and a touch-down protocol for that of MUSK (details available upon request). Unincorporated primers and dNTPs were degraded with Exo-Sap-IT® (Affimetrix). PCR products and PCR primers were processed in Macrogen Inc. (South Korea) for sequencing using BigDye® Terminator chemistry (Applied Biosystems). In Macrogen Inc., sequencing reaction products were purified through precipitation with ethanol and resolved on an ABI 3730XL (Applied Biosystems) genetic analyzer. Back in our lab, sequence chromatograms were edited using Sequencher 4.1 (Gene Codes Corporation 2007) to correct for discrepancies between the forward and reverse chains. The forward and reverse chains were aligned automatically using CLUSTAL X.[47]

Phylogenetic analyses and hypothesis testing

Genetic variability was summarized using haplotype (h) and nucleotide (π) diversity indicators in DNAsp.[48] To visualize genetic variability in geographic space, a haplotype network was built in TCS 2.1 [49] under maximum parsimony and 95% of connectivity (gaps coded as missing data). Haplotype frequency pie charts for mtDNA data were drawn over corresponding localities. Phylogenetic trees were inferred from a data-set consisting of the sequences generated here, plus outgroup sequences retrieved from GenBank. The outgroup included other species of *Oreotrochilus* (accession numbers: *O. estella,* AY830507.1,[50] EU042327.1 [32]; *O. melanogaster*, GU166854.1,[51], GU166866.1 [51]), and *Ramphomicron microrhynchum* (accession number: *R. microrhynchum*, EU042266.1,[32] EU042587.1 [32]).

Phylogenetic inference was obtained by maximum likelihood (ML) and Bayesian inference (BI) using the software GARLI 2.01 [52] and Phycas,[53] respectively. The partitioning scheme and models of sequence evolution used in both ML and BI were selected using PartitionFinder [54] under the Bayesian Information Criterion. The best-fit partitioning scheme (BIC = 7290.7421) consisted of the following subset partitions: (1) codon 2 of ND2 + codon 1 of ND4, (2) codon 3 of ND2 + codon 2 of ND4, and (3) codon 1 of ND2 + codon 3 of ND4 + tRNAs flanking ND4. The best-fit models of molecular evolution for these subsets were, respectively: (1) F81,[55] (2) K81uf,[56] and (3) HKY [57] + I.

In ML and BI, data-sets of mtDNA (ND2 + ND4) and of MUSK were first analyzed separately and then combined. Support for the ML trees was estimated from 100 bootstrap repetitions performed in GARLI. The bootstrap trees were then used to build a 50% majority-rule consensus tree in Mesquite.[58] A clade was considered to be supported if it was found in 70% of the bootstrap trees. BI proceeded with priors set to uninformative values, except for the inclusion of a polytomy prior, which is recommended to avoid the 'Bayesian star tree paradox' problem.[53] For each data-set, a Markov chain Monte Carlo (MCMC) was run for 10 thousand generations, with sampling occurring once every 100 generations. The first 1000 trees were discarded as burn-in. Stationarity of the MCMC chain was confirmed by visual inspection (plotting functions in R [59]) of the marginal likelihood at each sampled generation. The remaining trees were summarized into a 50% majority rule consensus tree in Mesquite.[58] A clade was considered to be supported if it was found within 95% of the trees drawn from the posterior distribution.

To explore whether our data support the hypothesis of genetic differentiation between individuals identified as *O. c. chimborazo* and *O. c. jamesonii*, we applied a hypothesis test based on the results of the Bayesian analysis. In PAUP, we took the post burning-in trees from the posterior probability distribution and filtered all trees that portrayed: (1) both *O. c. chimborazo* and *O. c. jamesonii* as monophyletic (not necessarily as sister taxa), or (2) any of them as monophyletic. The total of trees retained by the filter divided by the total number of post burn-in trees indicated the posterior probability that any of these hypotheses is correct (conditional on the model, data, prior probabilities, and convergence of the MCMC). [62]

Molecular analysis of variance

We used an molecular analysis of variance (AMOVA) [60] to test for genetic structure among subspecies and populations living across paramo islands. The analysis was conducted using Arlequin 3.5.[61] In the AMOVA hierarchy, groups were the subspecies *O. c. chimborazo* and *O. c. jamesonii* and demes were paramo islands. Because *O. c. chimborazo* is found in only one island, Quilotoa-Chimborazo, the test for genetic differences among islands within subspecies is effectively a test of differences among islands within *O. c. jamesonii*. Probability values were estimated from permutation analyses, as in standard AMOVA.

Results

New records

We recorded *O. chimborazo* in six localities where it was previously unreported (Figure 1(B), see arrows). At each of these localities, the hillstars were found visiting *Chuquiraga jussieui* bushes. Three of these localities were situated between Quilotoa and Chimborazo, thereby confirming that hillstars occur in the paramo extending between both volcanoes. Males in each of the three localities were identified as either *O. c. jamesonii* or *O. c. chimborazo*; however, we never registered both subspecies in the same site. In Apagua, the northernmost of the three localities, 10 adult males (observed) and the 5 males (captured) belonged to *O. c. jamesonii*. In the mountains around Mula Corral, intermediate in latitude between the other two, we identified five males as *O. c. jamesonii*. In the mountains around Simiatug, the southernmost locality (closer to Chimborazo volcano), the only adult male for which we had good views of its gorget belonged to *O. c. chimborazo*. In Mula Corral strong winds precluded the collection of specimens. In Simiatug, only a juvenile male was captured, which could not be identified to subspecies because of its age.

We could not confirm the presence of either *O. c. chimborazo* or *O. c. jamesonii* in Quilotoa. We captured a female Ecuadorian hillstar in that site, but as discussed previously, females cannot be identified to either

subspecies. *Chuquiraga* was abundant at the site, but the bushes were mostly visited by other species of hummingbirds (*e.g. Lesbia victoriae*) which tend to live at lower elevations than *O. chimborazo*. Quilotoa, however, was visited for fewer than 2 days, and given previous reports of the occurrence of both subspecies,[20] the site clearly deserves longer visits in future studies.

Ecological niche modeling

Percentages of contribution of each environmental variable to the ENM were as follows: mean temperature of the coldest quarter, 50.8%; mean temperature of the warmest quarter, 22.5%; mean temperature of the driest quarter, 16.7%; minimum temperature of the coldest month, 3.1%; precipitation of the warmest quarter, 2.2%; NDVI7, 1.8%; maximum temperature of the warmest month, 1.5%; QSCAT standard deviation, h sensor, 0.7%; QSCAT arithmetic mean, h sensor, 0.5%; and Leaf Area Index 3, 0.2%.

The potential distribution of *O. chimborazo*, as predicted by the ENM, covers most areas between 3600 and 4600 m of elevation within the geographic extent of the model (Figure 1(C)). The potential distribution of the hummingbird clearly extends beyond arid volcanic slopes. The distribution is discontinuous because of the presence of deep valleys that break topographical continuity above 3600 m (Figure 1(C)). This model was strongly supported by the Pearson's small sample test ($D = 10.760$, $p < 0.001$). The ENM also predicted suitable habitat between Quilotoa and Chimborazo, suggesting that environmental conditions in that area are not different from conditions in the rest of the range of the species. In fact, within this geographic region of suitable conditions, are the three intermediate localities between Quilotoa and Chimborazo where we recorded Ecuadorian hillstars were located (Figure 1(B)).

Specimen attributes

In total, we collected 51 samples of *Oreotrochilus chimborazo* and two of *O. estella stolzmanni* (Table 1). Of the 51 specimens of *O. chimborazo*, 15 belonged to subspecies *O. c. chimborazo,* 34 to *O. c. jamesonii*, one was an unidentified female from Quilotoa, and another an unidentified male juvenile from Simiatug. All males observed and collected at each locality belonged to only one of the subspecies; females were therefore assumed to belong to the subspecies to which the males from the same locality belonged.

Sequence attributes

With the exception of the juvenile male from Simiatug, DNA sequences were retrieved for all other specimens. In those birds, sequencing was generally successful, with the exception of the first nucleotide of ND2 in 42 specimens and the entire MUSK sequence in 4 cases. Variable/parsimony-informative sites within fragments were distributed as follows: 4/5 for ND2; 4/3 for ND4; and 0/0 for MUSK (data for two variable sites in MUSK were missing for some sequences and thus those sites were excluded from the DNAsp analysis). No indels were found. With inclusion of outgroup sequences, these parameters changed as follows: 113/26 for ND2; 97/21 for ND4; and 2/1 for MUSK (no MUSK sequences were retrieved for *O. estella estella*, *O. melanogaster*, or *R. microrhynchum*). Haplotypic and nucleotide diversity were low across the species (ND2 + ND4, $h/\pi \pm SD$: $h = 0.660 \pm 0.067$, $\pi = 0.0064 \pm 0.0034$). All sequences are available from Genbank (accession numbers available in Table 1).

Phylogenetic trees and hypothesis testing

MUSK sequences were uninformative regarding tree topology; the BI and ML trees resulting from using this gene were completely unresolved within *O. chimborazo* (data not shown). The mtDNA and total combined data analyses produced parallel topologies. For the rest of the paper, then, we will refer only to the trees inferred using mtDNA.

Topologies of the majority-rule consensus ML and BI trees were similar. The BI tree, however, resolved additional clades, albeit with low support. In both majority-rule consensus trees *O. chimborazo* was monophyletic (mtDNA data-set trees: $Pp = 1.00$; bootstrap ML = 95%). The clade most closely related to *O. chimborazo* comprised *O. melanogaster* and the three individuals of subspecies *O. estella stolzmanni* that were collected at Lagunillas (southern Ecuador) (Figure 1(A)). The other individual of *O. estella*, the one for which DNA sequences were retrieved from GenBank, was sister to all other individuals of *Oreotrochilus* (Figure 1(A)), which mirrors recent claims that *O. estella* is not a monophyletic group.[31] The clade of *O. chimborazo* was an almost completely unresolved polytomy. It was not possible to resolve subspecies (Figure 2(A)); excluding females (data not shown) did not improve the resolution of the trees. No clade in the Bayesian tree showed geographical structure. In the Maximum Likelihood tree, the birds from El Cajas were grouped in their own clade, albeit with no statistical support (bootstrap value below 70; data not shown). Finally, the test of hypothesis in the Bayesian framework revealed that no tree from the posterior probability distribution showed *O. c. chimborazo* or *O. c. jamesonii* as monophyletic.

Haplotype and genetic differentiation

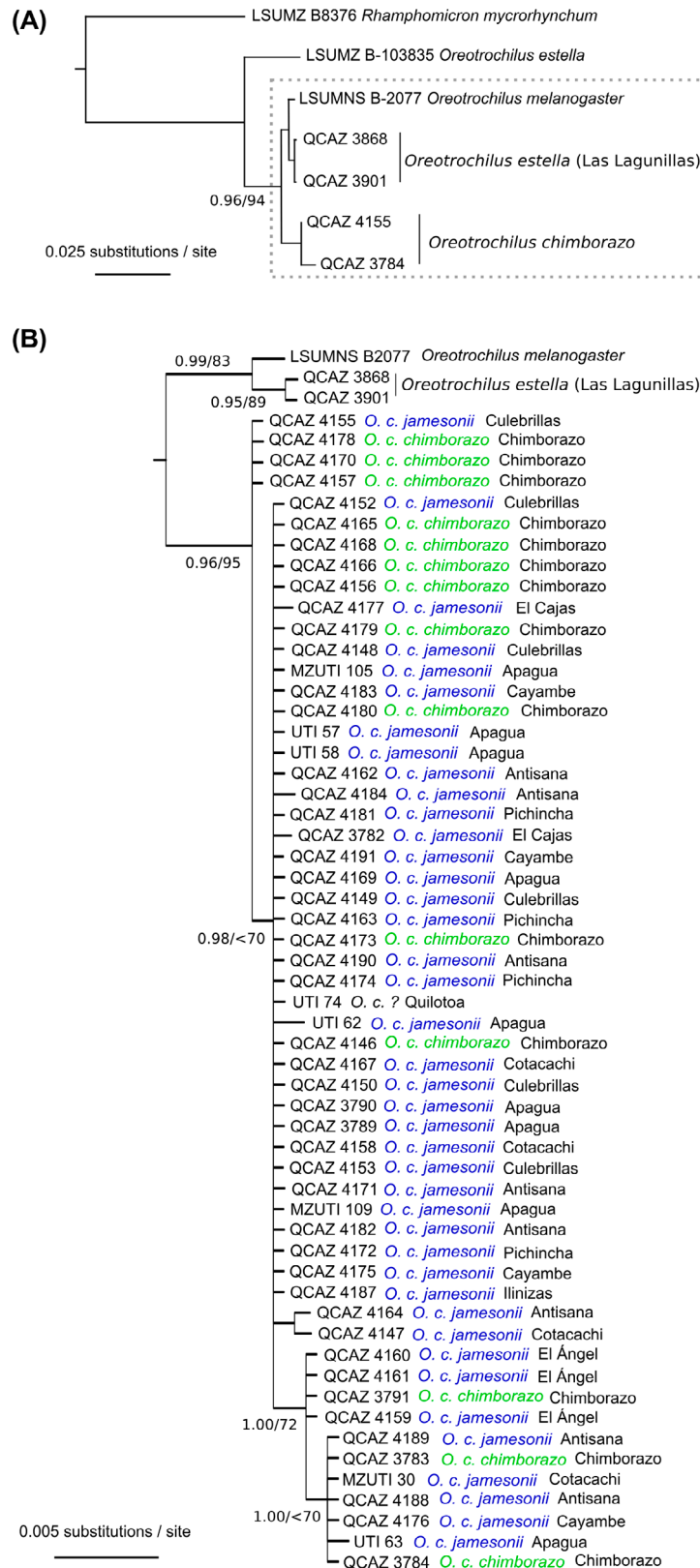Among the 50 individuals analyzed, 10 mtDNA haplotypes were found. The haplotype network was almost

Figure 2.   Bayesian analysis tree (50% majority-rule consensus) of the mitochondrial data-set (ND2 and ND4; 1730 bp). (A) Phylogenetic relationships of *Oreotrochilus chimborazo* and closely related taxa. The clade inside the grey box, (*O. melanogaster* + *O. estella* (Lagunillas)) + *O.* chimborazo, is expanded in (B) to show all sampled individuals within *O. chimborazo*. Values of clade support are shown close to the clade root: Posterior Probabilities (Bayesian Infenrence)/bootstrap values (maximum likelihood.

star-like, with the same haplotype being present in most individuals of both *O. c. jamesonii* and *O. c. chimborazo* (Figure 3(A)). Most haplotypes were distanced from their closest haplotype by just one evolutionary step. Only two haplotypes showed some degree of geographic aggregation, one at El Cajas Plateau and the other at El Ángel. Individuals from these regions had private alleles (Figure 3(B)), removed from the common haplotype by one and two steps, respectively (Figure 3(A)). However, the low sample size from El Ángel or El Cajas precludes estimating the prevalence of these haplotypes within their respective populations.

Using AMOVA we did not detect genetic differences between subspecies ($F_{CT=}0.177$, $p = 0.70 \pm 1.54E-2$). We did detect genetic differences among paramo islands within

subspecies ($F_{SC=}0.255$, $p = 0.004 \pm 1.85E-3$) and among paramo islands within the whole species ($F_{ST=}0.123$, $p = 0.003 \pm 1.64E-3$). Because *O. c. chimborazo* is found in only one island, Quilotoa-Chimborazo, differences among islands within subspecies are driven by variation among populations of *O. c. jamesonii*.

Discussion

The subspecific diversity of the Ecuadorian hillstar has often been cited as a remarkable example of how restricted ecological requirements can combine with habitat insularity to generate evolutionary divergence.[8–11,14] According to the literature, the species comprises three geographically isolated subspecies, two of them restricted
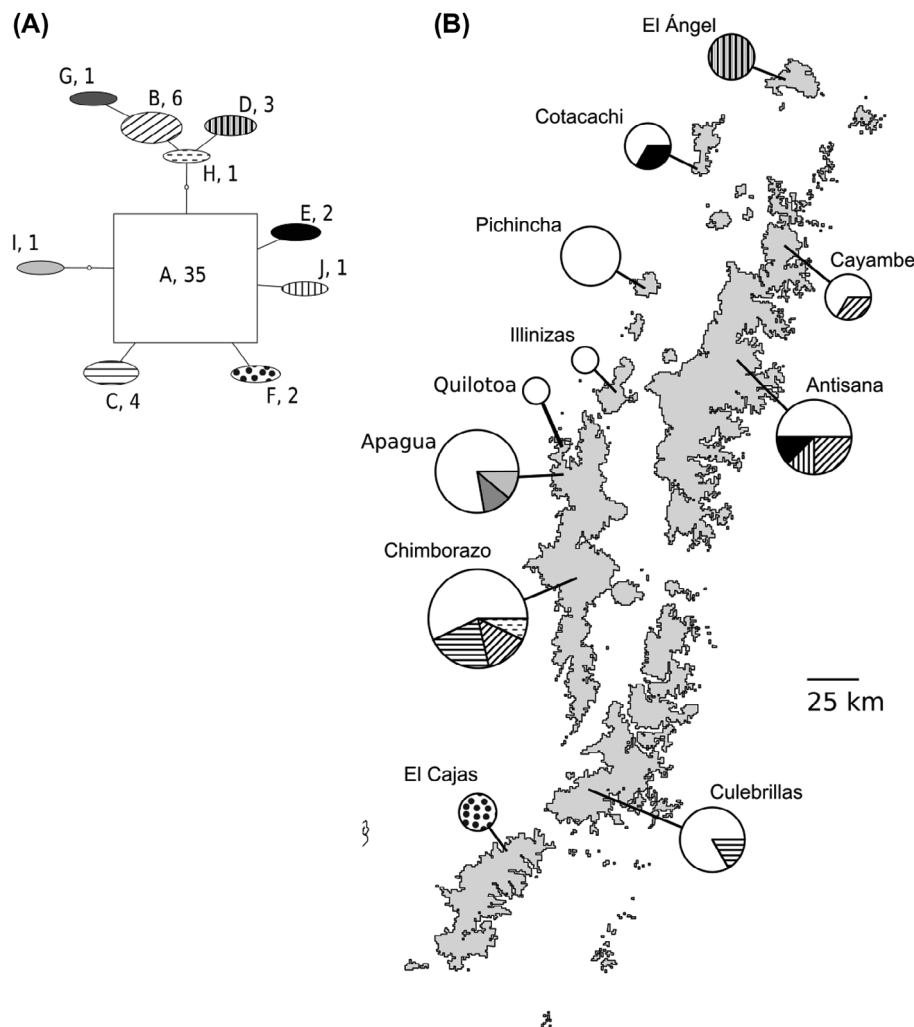

Figure 3. Data from Haplotype network based on the combined mitochondrial regions ND2 and ND4. (A) Transition network between haplotypes according to maximum parsimony. Haplotypes are named with a unique letter code. Following the haplotype name, the number of individuals bearing the haplotype is given. Box sizes are proportional to number of individuals bearing the haplotypes; (B) Frequency pies for the haplotypes sampled at each locality. The area of the pie is proportional to the sample size at each locality. A map of surface area above 3600 m in elevation is provided for showing the distribution of haplotypes within the Ecuadorian Andes.

to the volcanoes Chimborazo and Quilotoa.[8,9,12] Careful review of the literature, however, suggests that populations of Chimborazo and Quilotoa may not be isolated from each other. Elsewhere in Ecuador, *O. chimborazo* has been recorded at localities that are not particularly close to volcanoes,[11,14,17,18] in grassland paramo, which is the habitat that occurs between Quilotoa and Chimborazo.[19] Recently, photographs taken in Quilotoa presented preliminary evidence that populations of both subspecies occur in the area.[20] In this study, we confirmed that Ecuadorian hillstars occur between Quilotoa and Chimborazo. We found subspecies *O. c. jamesonii* 15-km south of Quilotoa and, further south, we found individuals of *O. c. jamesonii* and *O. c. chimborazo* living at most 15 km apart from each other (Figure 1(B)). Our ENM (Figure 1(C)) predicted occurrence of *O. chimborazo* in this region, despite not using the localities between Quilotoa and Chimborazo to build the model. Taken together, our results strongly suggest that the populations of both subspecies are in contact.

Our results also suggest that subspecies *O. c. soderstromi* is probably an intergrade between the other two subspecies. A visual inspection of the holotype (NRM 569802, Naturhistoriskariksmuseet, Royal Museum of Natural History, Sweden) reveals characteristics that are intermediate between *O. c. chimborazo* and *O. c. jamesonii*; namely, a purple gorget interspersed with scattered green feathers. Even in the original description of the holotype,[12] the intergrade hypothesis was already stated, but it was dismissed on the basis of rather weak claims. It was mentioned that hummingbirds of *O. c. soderstromi*, have 'a known local name' and that they are ecologically different from other Ecuadorian hillstars because *O. c. soderstromi* supposedly occured at lower elevations.[12] Ecological differences are unlikely because *O. c. jamesonii* has been recorded within the same elevational range at which *O. c. soderstromi* was recorded in Quilotoa.[8] However, to be fair to the authors of the account, those records of *O. c. jamesonii* were obtained several years after the description of *O. c. soedrestromi* was published. The validity of *O. c. soerstromi* has also been defended on the assumption that hillstars in Quilotoa are geographically isolated from other hillstars.[8,9] However, as we have discussed, considerable evidence supports the contrary. Arguments in favor of *O. c. soderstromi* as a valid subspecies are therefore unconvincing; consequently, we suggest that the demotion of this taxon is in place.

According to our ENM, the potential distribution of *O. chimborazo* is widespread along the Ecuadorian paramo, with no higher probability of occurrence in drier regions. In the literature, some places that are predicted by our ENM have been stated to be too humid for *O. chimborazo*. Such places include, for example, Llanganates National Park [8] and Cayambe paramo.[11]

However, there is a relatively recently published record of the hillstar in Llanganates,[18] and this occurrence is predicted by our ENM. Though this locality was used to build the ENM, removal of the locality during Pearson validation tests did not affect the outcome of the ENM with regards to predicting occurrence in this area (data available upon request). In Cayambe, we confirmed the occurrence of *O. chimborazo* and collected voucher specimens; also, this locality is in a region of high likelihood of occurrence according to our ENM (Figure 1(C)).

Although *O. chimborazo* may occur throughout the Ecuadorian paramos, we do not reject a possible preference for arid paramos. During our fieldwork, *O. chimborazo* seemed to be more abundant at sites with barren, sandy soil, where *Chuquiraga* was among the few yet abundant plants growing there. Cunungyacu, our locality closest to Mt. Chimborazo, was one of those sites. Hillstars were so abundant at that locality that we managed to capture 15 individuals in just two days of field work. In other sites, it would take us about three days to catch up to five individuals. Strong preference for arid environments may effectively isolate populations, which may have resulted in at least one morphological character (*i.e.* gorget color) being different between populations of the hillstar. However, studies aimed to characterize local abundances, specific habitat preferences, and patterns of individual movement between sites are required to test this hypothesis.

Arid conditions in the paramo may be promoted by anthropogenic pressure. Burning is a common and widespread regime in the Ecuadorian paramos that leads to desertification and erosion.[63] In the past, grassland paramos, generally associated with drier conditions than those of other paramos, may have not been as extensive as they are now. Indeed, ecological studies suggest that forests in the Ecuadorian Andes may have been more extensive than they are today, before burning and grazing practices begun,[64,65] and may have reached elevations close to 4100 m.[65] If this pattern holds true, paramo degradation may be masking the original environmental factors that originally limited the distribution of the hummingbird.

Every site where *O. chimborazo* was found showed some evidence of degradation, whether in the way of regular fires or cattle grazing (personal observations). By benefiting paramos over original forest, habitat disturbance may have extended the distribution of *Chuquiraga* and consequently that of the Ecuadorian hillstar.[11,15] A restricted geographic range in the recent past and expansion of such range is consistent with the star-like haplotype network (Figure 3) and low genetic diversity within *O. chimborazo*. Interestingly, the area where the presumed contact zone is located has a long record of human activity.[66] Before human intervention, both subspecies may have been isolated to islands of arid

paramo, separated by a barrier of undisturbed humid habitat where neither *Chuquiraga* nor *Oreotrochilus* may have occurred. In our visits to the field we found, amid the grassland paramo, patches of *Polylepis* forest (in Mulá Corral creek) restricted to hilltops along very steep slopes where the degree of human intervention is low, suggesting that the area was in a good climatic zone for forest. On the other hand, slash and burn agriculture has also been hypothesized to threaten *Chuquiraga* and, therefore, *O. chimborazo*.[14] Accordingly, in some mountains we did not find large patches of *Chuquiraga* below 4100 m, presumably because of intense degradation of the paramo.

Moderate anthropogenic pressure in the recent past is not the sole possibility that may explain secondary contact between subspecies *O. c. chimborazo* and *O. c. jamesonii*. Natural fluctuations in climate, possibly during the early Holocene,[65] may have restricted the range of suitable habitats for *O. chimborazo* in the recent past. For example, after glaciers retreated from the Last Glacial Maximum, global temperature increased to levels slightly higher than those of today, which has been suggested to have favored the growth of forests and displaced paramos to higher elevations.[65,67,68] Paramos, therefore, may have been more insular, presenting more opportunities for divergence promoted by geographic isolation.

Regardless of the factors driving morphological divergence, we hypothesized that *O. c. chimborazo* and *O. c. jamesonii* constitute distinct genetic lineages. Our analyses, however, did not support our hypothesis: (1) we did not recover the two subspecies in the phylogeny, (2) the probability of monophyly of either subspecies was close to zero (according to the Bayesian hypothesis test), and (3) we failed to reject the null hypothesis that *O. c. chimborazo* and *O. c. jamesonii* are genetically similar (see AMOVA). Our results, however, cannot be used to conclude that genetic divergence is inexistent among subspecies of *O. chimborazo*. Their distinct morphology and geographic ranges suggest some degree of genetic divergence that arose at some point in their evolutionary history. Such divergence, however, may have been too recent to be reflected in mtDNA. In fact, if current geographic isolation has played any role in the separation between subspecies, it may not have started to act until very recently, because before 13,000 years ago, during the last glaciation, the paramo probably occupied a lower, more continuous zone.[65,69,70] In addition, slow molecular evolution, which has been suggested to exist in high-elevation hummingbirds,[71] may have hindered accumulation of informative mtDNA substitutions in this relatively short time.

Is not uncommon for mtDNA to be similar between populations classified as different subspecies.[72,73] Fast accumulation of plumage characters could be promoted, for example, by sexual selection [22] when novel characters increase the attractiveness of males. The diversity of plumages among males of the different species of *Oreotrochilus* [7] is in sharp contrast with the similar plumages among females, suggesting a role for sexual selection in shaping the evolution of male plumage in this genus. Distinct plumage characters between the subspecies of *O. chimborazo* may hint at incipient divergence resulting from geographic isolation in the recent past; the populations may have been isolated for enough time for one population to start diverging on plumage characters under sexual selection, but not enough time to leave traces of the divergence in mtDNA. In cases like these, the use of faster evolving markers, such as those based on microsatellites [74] or SNPs, is recommended.

Finally, because the range of *O. c. jamesonii* is highly fragmented, we hypothesized that the population genetics of this subspecies would show geographic structure. Using AMOVA, we found significant differences between hummingbirds living in different paramo islands. However, results from phylogenetic trees and haplotype networks were not supportive for such differences (Figure 3(A)); only two populations seemed to have genetic structure in the haplotype network, those from El Cajas plateau and from El Ángel (Figure 3(A) and (B)), but this separation is not conclusive. Still, these paramos are notorious for the presence of several endemic species of plants,[19] insects, [74] and distinct forms of other Andean birds.[30] Such endemism might be related to the age of the valleys that separate these paramos from other paramos, which are ~2 million years older than most other valleys in the Ecuadorian Andes.[75] Thus, we think that it would be worthwhile using larger sample sizes to test if hillstars from El Cajas and El Ángel belong to diverging lineages.

Disclosure statement

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