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Local Genetic Structure on Breeding Grounds of a Long-Distance Migrant Passerine: The Bluethroat (*Luscinia svecica*) in Spain

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Abstract

Breeding site fidelity can be determined by environmental features, which depending on their heterogeneous distribution may shape the genetic landscape of a population. We used 10 microsatellite loci to study the genetic variation of 83 bluethroats (*Luscinia svecica azuricollis*) across 14 localities within the Spanish breeding population and assess the relative influence of different habitat characteristics (physiography and vegetation) on genetic differentiation. Based on the genetic variation of this population, we identified 3 geographically consistent genetic clusters that on average showed a higher genetic differentiation than among other north European populations, even those belonging to different subspecies. The inferred genetic clusters occurred in geographic areas that significantly differed in elevation. The highest genetic differentiation was observed between sites at different mountain ranges, as well as between the highest altitude sites in the northeastern locale, whereas vegetation type did not explain a significant percentage of genetic variation. The lack of correlation between geographic and genetic distances suggests that this pattern of genetic structure cannot be explained as a consequence of isolation by distance. Finally, we discuss the importance of preserving areas encompassing high environmental and genetic variation as a means of preserving evolutionary processes and adaptive potential.

Key Words: *breeding site selection, environmental factors, genetic structure, Luscinia svecica, microsatellites, Spain*

The interplay between gene flow and local habitat selection and its influence on species diversification constitutes a long-lasting research topic in evolutionary biology (Wright 1940; Felsenstein 1976; Hedrick 1986; Hedrick 2006). The occurrence of a species at a particular site largely depends on environmental variability, which is ultimately determined by the range of suitable habitats according to their spatial configuration and seasonal variation (Bell et al. 1993; Dufour et al. 2006). The spatial variation of ecological factors, linked both to habitat heterogeneity and quality, may also shape levels of genetic variability in wild populations (Frankham 1995; Foll and Gaggiotti 2006; Pitra et al. 2011). As a consequence, genetic differentiation among populations depends not only on the strength of habitat selection on each local population but also on the relative importance of dispersal. Therefore, it is expected that if habitat preferences are stronger than dispersal among local populations, local adaptation may arise in such populations even if this geographic scale is much smaller than the scale of dispersal (Wright 1940; Blondel et al. 2006). Strong habitat selection in heterogeneous landscapes may cause local populations to evolve traits that provide advantages under their local habitat characteristics (Kawecki and Ebert 2004). However, several factors may hamper local adaptation. In this context, gene flow is the most important factor, since the exchange of genes between populations homogenizes allele frequencies and thus prevents genetic differentiation (Balloux and Lugon-Moulin 2002). Therefore, it is generally assumed that at small spatial scales, intraspecific variation does not occur in highly vagile organisms such as birds. This assumption would be valid if gene flow was spatially random, but evidence suggests that birds may show dispersal biases with respect to habitat (Davis and Stamps 2004; Blondel et al. 2006; Hull et al. 2008; Alda et al. 2011).

Birds breeding in heterogeneous landscapes may choose territories with different environmental qualities, which can

affect demographic parameters and genetic diversity of populations (Penteriani et al. 2004; Porlier et al. 2009). For example, birds with migratory behavior might differ in their degrees of fidelity to their breeding and wintering sites (i.e., migratory connectivity; Esler 2000). This philopatric behavior has been associated with key features of the environment that are patchily distributed or difficult to locate, such as specialized breeding locations or food resources (Van Bekkum et al. 2006; Clark et al. 2008; Hull et al. 2008). Hence, migratory connectivity is directly related to gene flow, which in turn determines the geographical pattern of genetic variation within a species. Consequently, it would be expected that high levels of genetic and morphological variation among populations with strong migratory connectivity are due to low gene flow and local adaptations (Webster et al. 2002).

The bluethroat *Luscinia svecica* (Linnaeus 1758) is a long-distance migratory passerine that breeds throughout Europe, Asia, and Alaska. There are 10 subspecies that constitute a subspecies complex described on the basis of body size and plumage coloration of males and on differences of their breeding habitats, migration routes, and wintering areas (Cramp 1988). However, these subspecies are not recognized according to mitochondrial DNA differentiation and only a shallow divergence exists between the northern and southern subspecies, suggesting a recent divergence of these populations (Questiau et al. 1998; Zink et al. 2003). In addition, faster evolving microsatellite markers indicate restricted gene flow among some subspecies in *L. svecica*, particularly among southern populations, which generally are more differentiated than northern populations. Furthermore, the southern group of subspecies, which includes the Spanish and French subspecies, is morphologically distinct in showing white or no throat spots, in contrast with the northern group of chestnut-spotted populations. Thus, because the Spanish subspecies *L. s. azuricollis* is clearly genetically differentiated, it and the French *L. s. namnetum* populations are proposed to be ancestral to the other European subspecies (Johnsen et al. 2006). In general, bluethroats show high fidelity to their migratory routes between wintering and breeding areas (Markovets and Yosef 2005; Hellgren et al. 2008), so the observed genetic heterogeneity among regions in Europe could be either due to isolation processes or a consequence of local adaptations of southern populations (Johnsen et al. 2006).

Spanish bluethroats are believed to winter south of the Sahara (Arizaga et al. 2006) and breed in the northwestern mountains of Iberian Peninsula (Tellería 1999; Gómez-Manzaneque 2003). In the Iberian mountains, *L. s. azuricollis* occurs in a variety of habitat types greatly differing in vegetation structure and composition, altitude, and orientation. These differences can be observed at a very small spatial scale (only a few kilometers apart), providing a framework for habitat choice and some degree of local genetic divergence (Guschanski et al. 2008). However, there is limited knowledge of the genetic variation among bluethroat populations at such small geographic scales, with the exception of *L. s. svecica* in Scandinavia (Hellgren et al. 2008). Thus, the bluethroat breeding population in Spain constitutes a good model to evaluate the relationships between this site fidelity and the environmental features shaping the genetic structure at a local scale in a wide-ranging species.

The main aim of this study is to examine the genetic variation of bluethroats within the Spanish breeding population, in order to determine: 1) the extent of genetic differentiation at the local scale and 2) whether landscape features have a direct influence on the genetic structure of local populations. Different habitat characteristics (physiography and vegetation) might imply different adaptations or selection patterns for breeding individuals. Thus, we would expect to observe significant genetic differentiation among breeding sites if bluethroats are preferentially selecting certain habitat conditions. If this selection is strong, it might imply a low capability of adaptation to different environments. On the other hand, a lack of genetic differentiation could be a consequence of extensive gene flow and therefore suggest a lack of habitat selection.

Materials and Methods

Study Sites and Sampling

Breeding bluethroats were sampled across the species distribution range in northwestern Spain, from the southern slope of the Cantabrian Mountains to the Mountains of León (León province), ranging from 800 to 1900 m above sea level (Figure 1A). This area spans the putative limit of 2 major European biogeographic regions, the Atlantic and the Mediterranean, and features a wide diversity of habitats. Fourteen localities were sampled during the breeding season between April 2009 and August 2010 and classified on the basis of the main environmental characteristics that could directly or indirectly influence the selection of breeding sites by bluethroats (Table 1).

Localities were assigned to the mountain range where they were sampled (Cantabrian Mountains and Mountains of León). The Cantabrian Mountains run on an east–west axis and are on average higher in altitude than the Mountains of León. They are also more influenced by the Atlantic climate and have higher precipitation than the Mountains of León. Most sampling localities were found along valley bottoms and foothills (800–1200 m) and mountain ridges (1500–1900 m) (Figure 1B) and were further differentiated into low- and high-altitude sites, respectively. Three main habitats were defined according to their vegetation type: brooms, mainly composed by *Cytisus* spp. and *Genista* spp.; heathlands, constituted by *Erica* spp. and *Calluna vulgaris*; and holm oak shrublands, consisting of *Quercus rotundifolia* and *Cistus* spp. (Table 1, Figure 1A, B).

Bluethroats were captured with tape-lured mistnets and clap-traps baited with mealworms. Blood samples from all individuals were obtained by venipuncture of the brachial vein and stored in absolute ethanol until they were analyzed. All animals were released unharmed.

DNA Extraction and Microsatellite Genotyping

Total genomic DNA was extracted from blood using a standard ammonium acetate precipitation protocol (Perbal

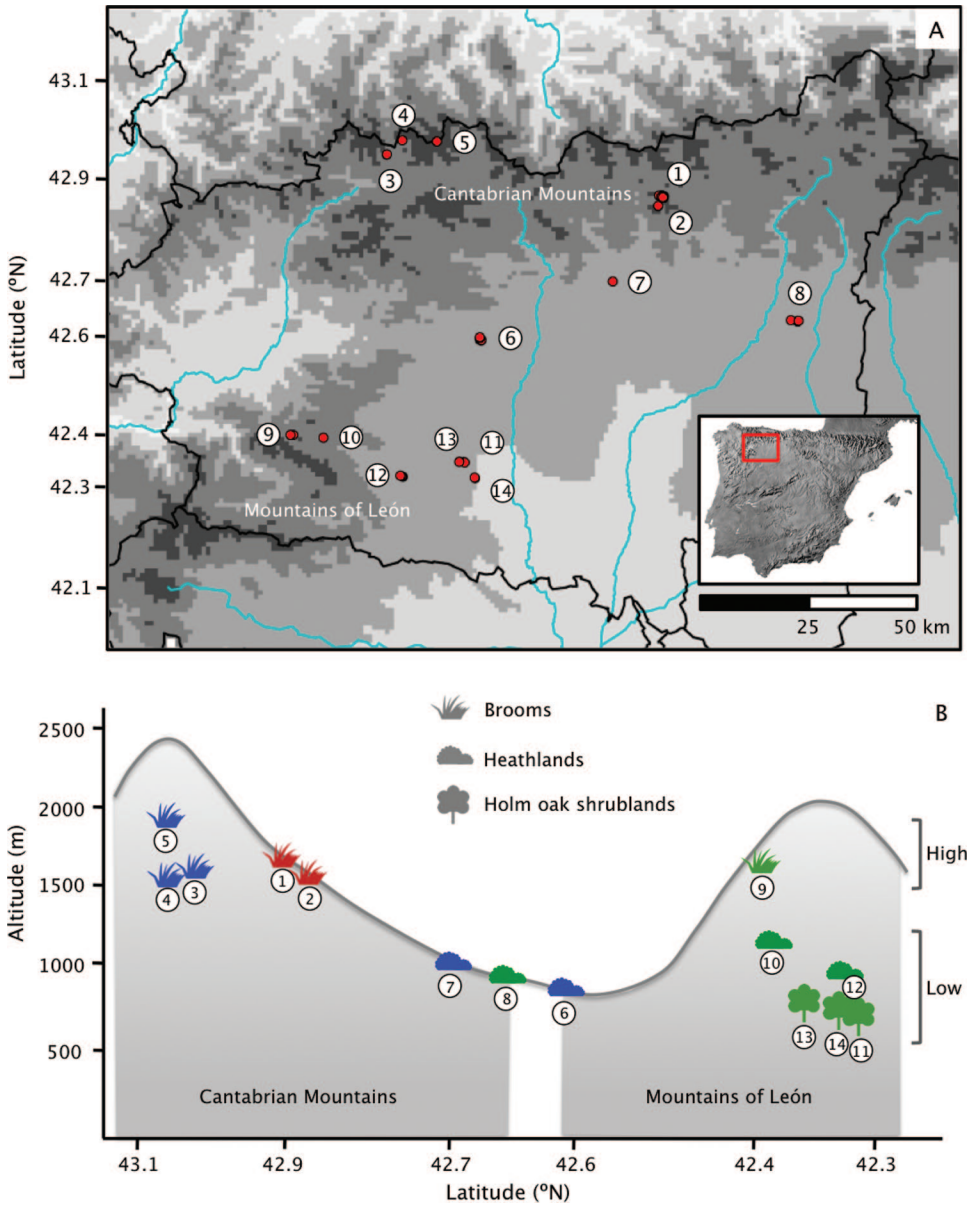

Figure 1. (**A**) Map illustrating the 14 bluethroat localities sampled in northwestern Spain. Gray layers, from light to dark, correspond to elevations 400–800 m, 800–1200 m, 1200–1600 m, and 1600–2600 m. Black lines represent province limits and blue lines are main rivers in the area. Numbers refer to localities in Table 1. (**B**) Schematic representation of the relief profile of the study region. Mountain range, altitude classes, and vegetation type for each locality is indicated. Colors represent genetic clusters to which localities were assigned; black (red): cluster K-NE, northeastern localities; medium gray (blue): cluster K-NW, northwestern and central areas; and light gray (green): K-S, southern sites. Colors between parentheses refer to the color version of the figure.

1988) following Proteinase *K* digestion. All samples were genotyped for 12 microsatellite loci: Aar8, Ase19, Cuμ4, Cuμ10, Fhu2, Hru7, Mcy4, PAT MP 2-43, Pdo5, Phtr2, PmaC25, and Ppi2 (Ellegren 1992; Primmer et al. 1996; Double et al. 1997; Fridolfsson et al. 1997; Otter et al. 1998; Gibbs et al. 1999; Martínez et al. 1999; MacColl et al. 2000; Richardson et al. 2000; Saladin et al. 2003). The microsatellites were co-amplified in 4 multiplex polymerase chain reactions (PCRs; Mix1: Fhu2, PmaC25, Ptc2; Mix2: Ase19, Cuμ4, PAT MP 2-43; Mix3: Cuμ10, Hru7, Mcy4; Mix4: Aar8, Pdo5, Phtr2), following the QIAGEN Multiplex PCR kit protocol for 30 cycles and 3 different annealing temperatures (60 °C for Mix1, 57 °C for Mix2 and 48 °C for Mix3 and 4). Reactions were prepared in a final volume of 7 μL including: 3.5 μL of Qiagen 2X PCR Master Mix, 0.7 μL of 10X primer mix (2 μM each), 1 μL DNA (ca. 25 ng/μL) and 1.8 μL of RNase-free H_2O. Fluorescently labeled PCR products were analyzed on an ABI3130*xl* DNA Analyzer

Table 1 Sampling localities of bluethroat (*Luscinia s. azuricollis*)

	Locality	*n*	Mountain range	Altitude class	Vegetation	Altitude (m)	Latitude	Longitude
1	Genicera	14	Cantabrian Mountains	High	Brooms	1777.9	42.95°	−5.49°
2	Rodillazo	2	Cantabrian Mountains	High	Brooms	1640.5	42.92°	−5.51°
3	Meroy	2	Cantabrian Mountains	High	Brooms	1592.0	42.97°	−6.22°
4	La Cueta	5	Cantabrian Mountains	High	Brooms	1566.0	43.01°	−6.18°
5	La Majúa	2	Cantabrian Mountains	High	Brooms	1895.0	42.98°	−6.02°
6	Ferreras de Cepeda	17	Mountains of León	Low	Heathlands	973.1	42.65°	−6.03°
7	La Seca	1	Cantabrian Mountains	Low	Heathlands	1122.0	42.74°	−5.60°
8	Corcos	9	Cantabrian Mountains	Low	Heathlands	1012.7	42.67°	−5.08°
9	Pobladura de la Sierra	2	Mountains of León	High	Brooms	1676.5	42.42°	−6.44°
10	Molinaferrera	1	Mountains of León	Low	Heathlands	1138.0	42.39°	−6.36°
11	Palacios de la Valduerna	13	Mountains of León	Low	Holm oak shrublands	809.4	42.33°	−5.94°
12	Villar de Golfer	3	Mountains of León	Low	Heathlands	974.3	42.35°	−6.19°
13	Bustos	8	Mountains of León	Low	Holm oak shrublands	834.0	42.38°	−6.02°
14	Toralino de la Vega	4	Mountains of León	Low	Holm oak shrublands	834.0	42.37°	−5.97°

Number of individuals sampled in each locality, classes based on physiographic and ecological characteristics, mean altitude and coordinates are indicated.

(Applied Biosystems) and allele sizes were determined using GeneMapper 3.7 software (Applied Biosystems).

Data Analysis

Data were checked for null alleles and genotyping errors using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). We estimated the following genetic diversity parameters: number of alleles (N_A), allelic richness permuted by the lowest number of individuals genotyped in a locality (A_R), observed and expected heterozygosity (H_o and H_e) and inbreeding coefficient (F_{IS}) using FSTAT 2.9.3 (Goudet 1995). Departures from Hardy–Weinberg equilibrium were assessed in GenoDive 2.0b20 (Meirmans and Van Tienderen 2004).

To investigate the genetic structure and spatial location of genetic discontinuities within the breeding population, we first employed a Bayesian clustering method without prior assignment to their locations of origin. For that purpose, we used GENELAND 3.2.2 (Guillot et al. 2005; Guillot et al. 2008), which utilizes both genetic information and geographic coordinates from each individual to infer population structure. We initially ran 10 independent Markov Chain Monte Carlo (MCMC) simulations using the following parameters: 5×10^5 iterations, maximum rate of Poisson process fixed at 50, maximum number of nuclei in the Poisson–Voronoi tessellation fixed at 150, and the Dirichlet model for allele frequencies. Since the number of genetic populations was unknown, we allowed the number of clusters (K) to vary on a wide range from $K = 1$ to $K = 10$. Next, we determined the best number of clusters from the highest likelihood number of K obtained from these runs and ran the MCMC 20 times with K fixed to the value identified in the first step. We then computed the posterior probability of population membership for each pixel of the spatial domain (150×150 pixels) and for each individual for each of the 20 runs (with a burn-in of 5×10^4 iterations).

Spatial patterns of genetic differentiation across the full landscape were visualized using the "Genetic Landscape Shape interpolation" analysis implemented in Alleles in Space 1.0 (Miller 2005). This analysis infers a genetic surface based on interindividual distances of sampled individuals and on interpolated distances in areas where individuals were not sampled. Across the genetic landscape, the peaks and troughs indicate high and low genetic distances between individuals, respectively.

To test genetic differentiation among all sampling localities and to assess whether the inferred genetic clusters, the physiographic or habitat characteristics (i.e., mountain range, altitude, and vegetation) explained a higher percentage of the genetic variance, we performed an analysis of molecular variance (AMOVA) in GenoDive 2.0b20. Moreover, we calculated the genetic diversity parameters previously explained for each group of localities obtained from the best partition in AMOVA.

In addition, we tested the effect of geographic distance on the observed genetic differentiation of the bluethroat. We calculated Euclidean and altitudinal distances between localities and individuals, and tested their correlation with their genetic distance (pairwise $F_{ST}/1-F_{ST}$ between localities and Smouse & Peakall distances between individuals; Smouse & Peakall 1999, using Mantel tests; Mantel 1967). We used partial Mantel tests (Smouse et al. 1986) to assess the association between altitudinal and genetic distances while controlling for the influence of Euclidean geographic distances and vice versa (i.e., the association between geographic and genetic distances controlled by altitudinal distances). These analyses were performed in GenoDive 2.0b20 and their statistical significance was assessed by 10 000 randomizations.

Further relationships of altitude of sampling localities with genetic diversity parameters (N_A, A_R, H_o, H_e) were tested by Pearson correlations. Statistical support for the hypothesis that localities with different habitat features differ in genetic diversity was tested using a type-III analysis of variance (ANOVA), with altitudinal block (high or low) and mountain range (Cantabrian Mountains or Mountains of León) as factors and each of the genetic diversity parameters as response variables. Finally, to address if the assignment of

Table 2 Genetic diversity of bluethroat based on microsatellite loci for the whole population and for each of the three genetic clusters (K-NE, K-NW and K-S) inferred in GENELAND

Locus	Ase19	Cuμ4	Cuμ10	Hru7	Mcy4	PAT MP 2-43	PmaC25	Ppi2	Ptc2	Phtr2	Pdo5*	Aar8*	Mean (SD)
K-NE (n = 16)													
N_A	4	5	3	7	5	4	3	5	2	8	5	1	4.636 (1.747)
A_R	3.597	4.818	2.988	6.613	4.812	3.682	2.786	5.000	2.000	7.316	4.734	1.000	4.395 (1.604)
H_o	0.500	0.938	**0.250**	0.750	0.688	0.750	0.286	0.545	0.286	0.857	**0.214**	0.000	0.551 (0.079)
H_e	0.606	0.729	0.425	0.760	0.644	0.631	0.508	0.773	0.516	0.835	0.541	0.000	0.634 (0.039)
F_{IS}	0.212	−0.183	0.600	−0.027	−0.123	−0.122	0.19	0.231	0.323	−0.007	0.508	na	0.090 (0.081)
K-NW (n = 27)													
N_A	5	4	3	7	6	4	3	5	3	11	7	1	5.273 (2.412)
A_R	4.390	3.963	2.394	6.496	5.227	3.344	2.984	4.963	2.653	8.729	5.729	1.000	4.625 (1.890)
H_o	0.556	0.593	0.115	0.923	0.852	0.593	0.500	0.731	0.519	0.889	**0.200**	0.000	0.588 (0.078)
H_e	0.652	0.706	0.245	0.814	0.748	0.607	0.520	0.795	0.520	0.875	0.562	0.000	0.640 (0.054)
F_{IS}	0.050	0.097	0.053	−0.060	−0.092	−0.091	0.021	0.192	0.046	0.144	0.695	na	0.033 (0.042)
K-S (n = 40)													
N_A	5	6	3	10	8	6	3	7	4	11	6	1	6.273 (2.611)
A_R	4.074	5.274	2.579	7.307	6.582	3.952	2.983	5.228	2.769	8.504	4.622	1.000	4.897 (1.927)
H_o	0.650	**0.625**	0.250	0.895	0.850	0.450	0.579	0.605	0.462	0.775	**0.176**	0.000	0.574 (0.069)
H_e	0.637	0.751	0.267	0.850	0.793	0.421	0.596	0.763	0.535	0.864	0.491	0.000	0.634 (0.058)
F_{IS}	−0.025	0.356	−0.04	−0.111	−0.081	0.180	0.149	0.111	0.117	−0.037	0.683	na	0.052 (0.036)
ALL (n = 83)													
N_A	6	6	3	10	8	6	3	7	4	13	8	1	6.727 (3.003)
A_R	4.185	5.025	2.638	6.911	5.996	3.692	2.977	5.173	2.587	8.577	5.179	1.000	4.813 (1.870)
H_o	0.590	0.675	**0.207**	0.875	0.819	0.554	0.500	**0.640**	0.450	0.827	**0.192**	0.000	0.571 (0.070)
H_e	0.635	0.740	0.289	0.833	0.755	0.563	0.561	0.783	0.527	0.876	0.517	0.000	0.636 (0.049)
F_{IS}	0.070	0.089	0.282	−0.051	−0.085	0.016	0.109	0.182	0.146	0.056	0.629	na	0.058 (0.039)

n: number of samples, N_A: number of alleles, A_R: allelic richness standardized to the minimum sample size, Ho: observed heterozygosity, He: expected heterozygosity, F_{IS}: inbreeding index. Bold values indicate significant departures from Hardy–Weinberg equilibrium ($P < 0.05$). *indicates loci that were not included in the analyses.

birds to each of the inferred genetic clusters was independent of altitude, vegetation, and mountain range of their sampling localities, a log-linear analysis of frequencies was performed. The log-linear analysis is considered an ANOVA-like design of frequency data. Specifically, it is used to test the different factors that are used in a cross-tabulation with categorical factors and their interactions for statistical significance (StatSoft-Inc. 2007). All these analyses were performed in STATISTICA 8.0 (StatSoft-Inc. 2007).

Results

Eighty-three bluethroats were captured and genotyped for 12 microsatellite loci. Evidence of null alleles was found for locus Pdo5 and consequently it was not included in further analyses. Also, Aar8 turned out to be monomorphic and was removed. Overall, the number of alleles ranged from 3 for loci PmaC25 and Cuμ10 to 13 for locus Phtr2 (average $N_A = 6.727 \pm 3.003$ standard deviation [SD]). Observed heterozygosity per locus ranged from 0.207 to 0.875 with an average value of $H_o = 0.571 \pm 0.070$ SD (Table 2).

The Bayesian clustering analysis performed with GENELAND suggested an optimum structure of three genetic clusters in over 85% of the MCMC iterations. One cluster (K-NE) consisted of the individuals from northeastern localities of Genicera and Rodillazo. The second cluster (K-NW) was formed by the northwestern and central localities: Meroy, La Cueta, La Majúa, Ferreras de Cepeda, and La Seca. The third cluster (K-S) included the southernmost localities (Pobladura de la Sierra, Molinaferrera, Villar de Golfer, Bustos, Toralino, and Palacios de la Valduerna) but also the most eastern one (Corcos) (Figure 1B and Figure 2). The three clusters showed similar and significant pairwise F_{ST} values, such as $F_{ST} = 0.025$ ($P = 0.007$) between K-NE and K-NW, $F_{ST} = 0.024$ ($P = 0.004$) between K-NE and K-S, and $F_{ST} = 0.020$ ($P = 0.000$) between K-NW and K-S. All individuals were assigned with high probabilities (>80%) and none of the sampled localities contained individuals assigned to more than one genetic cluster.

The genetic surface obtained in the Genetic Landscape Shape interpolation analysis showed sharper "ridges" in the southwestern part of the range, indicating the greatest genetic distances between localities from Mountains of León and western Cantabrian Mountains (Figure 3). Furthermore, this analysis indicated that genetic distances decreased in areas to the east of the main genetic discontinuity, with the exception of the localities in the northeastern Cantabrian Mountains, which also indicated high genetic differentiation. Qualitatively similar results were obtained regardless of the grid size or distance weighting parameters chosen. Likewise, use of raw genetic distances or residual genetic distances had no effect on the relative shape of the landscape surface.

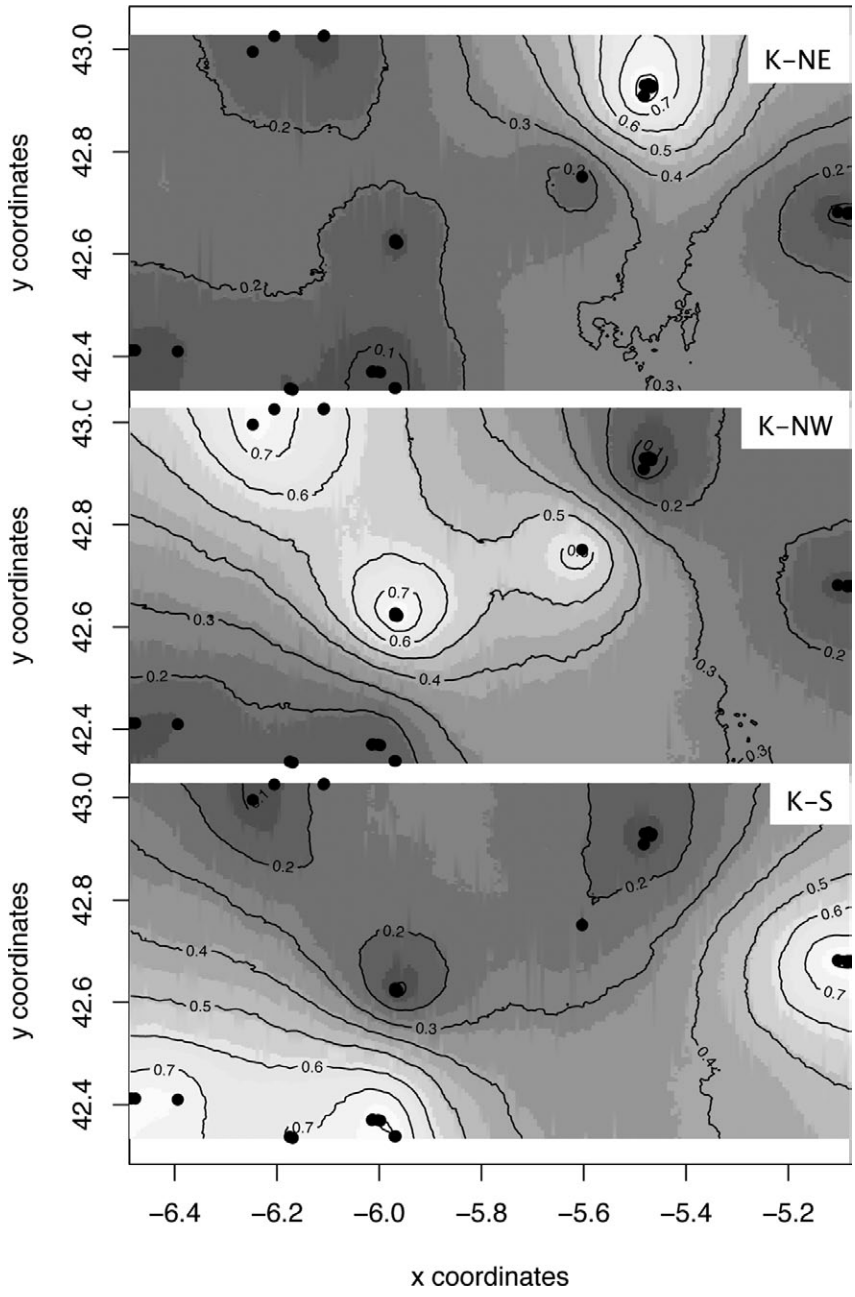

Figure 2. Maps of the posterior probabilities to belong to each genetic cluster inferred in GENELAND. Color gradient represents high (white) to low (gray) posterior probabilities.

The AMOVA analyses indicated that most of the molecular variation resided among individuals within the breeding population (F_{IT} = 0.919). The remaining genetic variation was best explained by differences among the three genetic clusters inferred in GENELAND (F_{CT} = 0.026, $P < 0.001$), and no significant differences were found among localities within clusters (Table 3). Partitions according to altitude classes and mountain ranges explained significant although lower percentages of genetic variation, but vegetation was nonsignificant (Table 3).

Genetic diversity parameters were very similar among the 3 inferred clusters (ANOVA, all $P > 0.104$) and compared with the whole population, although lower genetic variability was found in cluster K-NE (Table 2). Furthermore, none of the genetic diversity parameters were significantly correlated with the altitude of the sampling localities (all P values > 0.148) or were significantly different between mountain ranges (all P values > 0.157).

On the other hand, H_o values were almost significantly different between altitude classes (ANOVA $F_{1,11}$ = 3.488, P = 0.088), suggesting a tendency for lower genetic diversity in localities at a higher altitude. Furthermore, the altitude at which individuals were sampled was significantly different among the 3 genetic clusters, after controlling for

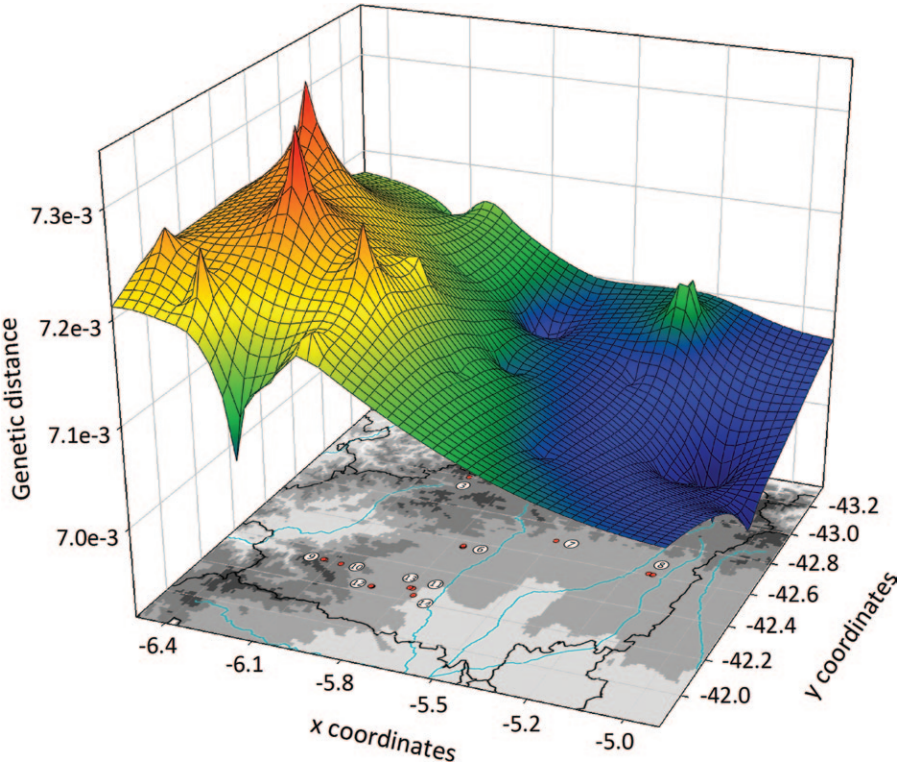

Figure 3. Genetic Landscape Shape interpolation based on a 50 × 50 grid and a distance weighting value (a) of 0.2. Surface plot heights are proportionate to genetic distances.

Table 3 Analysis of molecular variance performed between the bluethroat localities analyzed

Partition tested	% variation among groups	F_{CT}	F_{SC}	F_{ST}	F_{IS}
Among localities (All) (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14)	2.2			0.022**	0.054*
Between mountain ranges (Cantabrian Mt.) vs (Mt. León) (1, 2, 3, 4, 5, 7, 8) vs (6, 9, 10, 11, 12, 13, 14)	0.1	0.001*	0.025**		0.089**
Between altitude classes (High) *vs* (Low) (1, 2, 3, 4, 5, 9) *vs* (6, 7, 8, 10, 11, 12, 13, 14)	1	0.010*	0.020*		0.089**
Among vegetation types (Brooms) vs (Heathlands) vs (Shrublands) (1, 2, 3, 4, 5, 9) vs (6, 7, 8, 10, 12) vs (11, 13, 14)	0	0	0.025*		0.089**
Among genetic clusters (K-NE) vs (K-NW) vs (K-S) (1, 2) vs (3, 4, 5, 6, 7) vs (8, 9, 10, 11, 12, 13, 14)	2.6	0.026**	0.004		0.054*

F_{IS}: variation among individuals within localities, F_{ST}: variation among localities within the population, F_{SC}: variation of localities within groups, F_{CT}: variation among groups within the population.
*values indicate significant probabilities at $P < 0.05$ and **values indicate significant probabilities at $P < 0.01$. Numbers correspond to locality codes in Table 1.

their geographic position (i.e., latitude and longitude; ANOVA $F_{2,78} = 116.252$, $P < 0.001$), with K-NE at the highest altitude (post-hoc Tukey Test: $P = 0.0002$ for K-NE vs. K-NW and $P = 0.0002$ for K-NE vs. K-S) and K-S at the lowest (post-hoc Tukey Test: $P = 0.0002$ for K-S vs. K-NW). The log-linear analysis indicated that the best model for sample distribution did not include any interaction involving the variable genetic cluster (all P values > 0.501). Only the interaction between the variables genetic cluster and mountain range was close to significance ($\chi^2_2 = 5.457$, $P = 0.065$), indicating a trend for samples from cluster K-S to be more frequent in the Mountains of León than in the Cantabrian Mountains. As expected for these highly correlated variables, the interaction between vegetation and altitude was significant in the model ($\chi^2_2 = 6.306$, $P = 0.043$), indicating that samples belonging to broom-type vegetation were more frequent at high altitudes and samples in shrub lands were more frequent at low altitudes.

The Mantel test found a nonsignificant correlation between geographic or altitudinal distances and genetic distances between bluethroat localities (Mantel's $r = 0.061$, $P = 0.319$ and $r = 0.007$, $P = 0.456$, respectively), indicating that geographic distance between localities has no effect on their genetic differentiation. On the other hand, correlations were significant when individuals instead of localities

were considered (Mantel's $r = 0.051$, $P = 0.017$ for the geographic distances and $r = 0.060$, $P = 0.025$ for the altitudinal distances). However, when the effect of altitude was controlled by Euclidean geographic distances and vice versa, correlations were not significant (Partial Mantel's $r = 0.014$, $P = 0.317$ and $r = 0.023$, $P = 0.239$).

Discussion

Higher Genetic Structure but Lower Diversity in Spanish Than in European Bluethroat Populations

Three genetic clusters were identified within the Spanish breeding range of *L. s. azuricollis* (Figure 1B and Figure 2), which were almost equally divergent from each other, indicating the existence of well-delimited genetic groups at a local spatial scale and restricted effective dispersal (gene flow) (Clark et al. 2008). Our work provides additional evidence for a significant and much stronger genetic structure in Spain than in northern Europe, considering that the observed values were one order of magnitude greater than those found among all bluethroat populations in Scandinavia ($F_{ST} = 0.002$; Hellgren et al. 2008). Furthermore, the levels of genetic differentiation within the Spanish subspecies were in the range of those obtained among distinct bluethroat subspecies across Europe (significant pairwise $F_{ST} = -0.004$ to 0.174, average pairwise $F_{ST} = 0.044 \pm 0.043$ SD). Indeed, at the continental scale, the highest values of genetic differentiation between bluethroat subspecies were those involving comparisons with *L. s. azuricollis*, whereas the lowest were those comparing the subspecies with a northern distribution (Johnsen et al. 2006; Hellgren et al. 2008).

Our data were congruent with previous studies, with 9 out of 10 microsatellite loci in common but lower sampling size, indicating that *L. s. azuricollis* is the subspecies with the lowest genetic variability. On average, the Spanish population holds 38.6% ± 21.6 SD of all the species alleles, although ranging from 76.9% to 16.6% depending on the locus considered (Johnsen et al. 2006). One possibility is that the low genetic diversity of bluethroats breeding in Spain is a consequence of their geographic and genetic isolations, because the associated effects of genetic drift may both decrease genetic diversity and increase differentiation (Frankham et al. 2002).

In addition, the apparently high philopatry and low gene flow at local scales compared with northern European populations (Hellgren et al. 2008), and the fact that *L. s. azuricollis* is basal to the remaining European subspecies (Johnsen et al. 2006), might also support an isolation of Spanish breeding bluethroats and suggest a relatively independent evolution for this subspecies. This might explain their pattern of greater genetic differentiation, because besides the effect of geographic distance, the isolation of local populations would promote more rapid evolutionary change within the breeding population, and thus more rapid differentiation from the European populations from which it is isolated (Wright 1940). Furthermore, this pattern of genetic variation agrees with a nonmutually exclusive hypothesis proposing an inverse relationship between population differentiation and latitude (Martin and McKay 2004). Our results support the arguments of several authors that increased seasonal variation in climatic conditions at higher latitudes may result in broader tolerance of northern organisms to environmentally changing conditions. Thus, a greater adaptation capability could reduce costs of dispersing between populations, resulting in relaxed philopatric behavior and also in higher levels of gene flow and reduced genetic differentiation among high latitude populations (Martin and McKay 2004; Croteau et al. 2007; Berg et al. 2010). In contrast, strong fidelity to breeding sites at lower latitudes would prevent gene flow among different populations and might reduce genetic variation for dispersal behavior (Both and Visser 2001).

Environmental Factors Shaping Genetic Structure and Diversity

Our study helps identify some of the key factors conditioning species dispersal and distribution, and contributes to a growing body of work that suggests that landscape features influence dispersal and gene flow among bird populations (Bruggeman et al. 2010; Coulon et al. 2010; Milá et al. 2010; Thomassen et al. 2010; Alda et al. 2011). As has been described in previous studies, we found that geographic distance by itself is not a factor determining genetic differentiation in the bluethroat, neither at a local nor at a continental scale (Johnsen et al. 2007). In this case, altitude and mountain range of the localities explained significant percentages of genetic variance (Table 3) and were likely responsible for the observed genetic differentiation, as revealed by the significant differences in altitude among clusters, as well as the almost significant association observed between mountain ranges and the inferred genetic clusters. Indeed, these factors were clearly reflected in the landscape analyses of genetic structure, which showed genetic differentiation of the localities in Mountains of León, as well as those in the highest northeastern localities (Figures 2 and 3). Moreover, these areas that encompass high environmental and genetic variations are particularly important for maximizing adaptive diversity and consequently should be prioritized for conservation (Thomassen et al. 2010). In the end, we must be aware that the variables defined for this study are correlated with ultimate factors, such as climate, which will condition phenology and habitat availability. Therefore, we must keep in mind the combined effect of multiple factors on avian habitat selection that consequently gives rise to the observed genetic structure (Milá et al. 2010).

Limited or differential availability of those features selected by a species across its range distribution may not only explain genetic structure but also differences in population sizes and consequently in genetic diversity (Salvi et al. 2009). We observed a general, although nonsignificant, tendency for lower genetic diversity at high altitude localities. Such patterns of differentiation in altitude are expected in organisms with low dispersal abilities but are remarkable in species with high potential for dispersal, especially given the small geographic

scale of our study (Martínez-Solano and González 2008; Milá et al. 2010). Although our limited sampling size precludes drawing definite conclusions regarding this issue, we might deduce, based on this trend and the genetic differentiation of some high-altitude sites (e.g., cluster K-NE), that a limited number of individuals reach these regions. We further hypothesize that climate variables, such as time differences in the melting of snow at increasing altitudes, might limit habitat availability and thus hinder colonization of breeders and eventually gene flow (Santos González et al. 2010). Our results suggest that the environmental differences across the range explain the putatively neutral genetic variation, rather than by isolation by distance, which further indicates that this pattern of genetic structure might likely be shaped by adaptive differentiation (Salvi et al. 2009; Thomassen et al. 2010). However, the mechanisms underlying the observed genetic structure remain unknown. In our case, genetic differentiation between low- and high-altitude sites could be associated with differences in life-history traits. These differences could be the result of divergent selection pressures, which could have a role in restricting gene flow and leading to local adaptations and differentiation (Milá et al. 2010). On the other hand, under a high migration connectivity scenario, birds arriving from different wintering areas or at different times could select different breeding sites depending on their ecological characteristics. In other species, this pattern has been detected on the basis of genetic differences in birds arriving or breeding at different times in the same place (Moore et al. 2005; Casagrande et al. 2006; Porlier et al. 2009). Nevertheless, for the bluethroat, it is still unknown whether Spanish breeding birds show a pattern of temporal genetic differentiation or originate from different wintering areas (Arizaga et al. 2006). Further research with broader geographical sampling and additional genetic and morphological markers would be necessary to test these hypotheses, as adaptive changes in morphology often evolve at a faster rate than neutral genetic markers and may reflect noncongruent patterns of differentiation (Marthinsen et al. 2007; Milá et al. 2009).

Implications for Conservation

The strength of local selection informs how a species might react in diverse and dynamic environments and influences its potential for adaptation in the face of future climate change (Walther et al. 2002; Thomassen et al. 2010). In this respect, it is necessary to bear in mind that in the Iberian Peninsula, there is no suitable habitat for the bluethroat further north of the Cantabrian Mountains. Consequently, under a global warming scenario, the northward expansion of the Spanish subspecies would be limited (Walther et al. 2002; Förschler et al. 2011). It remains unclear if the proposed site selection and philopatry is strong enough to hamper the adaptation of individuals from clusters K-NE and K-NW to a southern and more Mediterranean habitat under a global warming scenario. On the contrary, if lowland Mediterranean habitats were to expand under such climatic scenario, bluethroats might expand their populations from those already extant in those regions (K-S). Ultimately, all of the above strengthen the importance of preserving the evolutionary potential held in these areas encompassing both high environmental and genetic variations.

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