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The influence of landscape configuration and environment on population genetic structure in a sedentary passerine: insights from loci located in different genomic regions

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Abstract

The study of the factors structuring genetic variation can help to infer the neutral and adaptive processes shaping the demographic and evolutionary trajectories of natural populations. Here, we analyse the role of isolation by distance (IBD), isolation by resistance (IBR, defined by landscape composition) and isolation by environment (IBE, estimated as habitat and elevation dissimilarity) in structuring genetic variation in 25 blue tit (*Cyanistes caeruleus*) populations. We typed 1385 individuals at 26 microsatellite loci classified into two groups by considering whether they are located into genomic regions that are actively (TL; 12 loci) or not (NTL; 14 loci) transcribed to RNA. Population genetic differentiation was mostly detected using the panel of NTL. Landscape genetic analyses showed a pattern of IBD for all loci and the panel of NTL, but genetic differentiation estimated at TL was only explained by IBR models considering high resistance for natural vegetation and low resistance for agricultural lands. Finally, the absence for IBE suggests a lack of divergent selection pressures associated with differences in habitat and elevation. Overall, our study shows that markers located in different genomic regions can yield contrasting inferences on landscape-level patterns of realized gene flow in natural populations.

Introduction

The study of the factors structuring genetic variation can help to infer the neutral and adaptive processes shaping the demographic and evolutionary trajectories of natural populations (Nosil *et al.*, 2005; Freedman *et al.*, 2010). Genetic differentiation between populations has often been found to increase with geographical distance due to migration–genetic drift equilibrium in organisms with restricted dispersal (i.e. isolation by distance, IBD; Wright, 1945; Slatkin, 1993; Hutchison & Templeton, 1999). However, landscape configuration can also have profound effects on gene flow and may explain spatial patterns of genetic differentiation better than Euclidean geographical distances. This has led to the emerging concept of isolation by resistance (IBR) (McRae, 2006; McRae & Beier, 2007). IBR is defined as the correlation between genetic differentiation and resistance distances that summarize information on the probability of an individual dispersing from one population to another across all potential paths weighted by the 'friction' or 'resistance' to movement exerted by discrete (e.g. roads, rivers; Coulon *et al.*, 2006; Quéméré *et al.*, 2010) or continuous landscape elements (e.g. habitat suitability; He *et al.*, 2013; Seymour *et al.*, 2013; Ortego *et al.*, 2015). The growing interest to understand the influence of landscape composition on the genetic

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structure of populations has led to the development of the field of landscape genetics, which has successfully integrated molecular tools, geographical information systems and different analytical techniques to identify the specific landscape features favouring or impeding gene flow among populations (Manel *et al.*, 2003; Storfer *et al.*, 2007, 2010). Landscape genetics has been proved a valuable approach to predict the responses of organisms to habitat alteration and infer unexpected migration pathways for species with cryptic life-history traits or for which dispersal movements are difficult to track due to different technical or logistic reasons (e.g. Wang *et al.*, 2009; Jha & Kremen, 2013).

Geographical distances, landscape composition and the dispersal potential of organisms are not the only factors shaping population genetic structure (Wang *et al.*, 2013; Wang & Bradburd, 2014). Dispersal is a complex process and immigrant individuals arriving to new habitat patches also have to cope with new environmental conditions, achieve a territory and mate and reproduce in an unfamiliar place to which they may not be well adapted (Wheelwright & Mauck, 1998; Hansson *et al.*, 2004; Nosil *et al.*, 2005; Postma & van Noordwijk, 2005; Garant *et al.*, 2007). As a result, natural and sexual selection against immigrants is expected to reduce rates of realized dispersal and gene flow among populations experiencing contrasting environmental or socioecological conditions (Shafer & Wolf, 2013; Sexton *et al.*, 2014; Wang & Bradburd, 2014). Accordingly, different studies have found remarkable genetic differentiation between nearby populations established in different habitat types (Andrew *et al.*, 2012; Porlier *et al.*, 2012a), altitudinal ranges (Murphy *et al.*, 2010) or environmental conditions (Wang *et al.*, 2013). In the case of animals, dispersers may also prefer to move through and settle in areas similar to their natal sites, that is previous experience shapes later preference, which can also result in disrupted gene flow among populations experiencing different environmental conditions (Davis & Stamps, 2004; Stamps *et al.*, 2009; Wang & Bradburd, 2014). Thus, isolation by environment (IBE) can shape patterns of genetic differentiation irrespective of the spatial location of populations due to the interaction between landscape heterogeneity, natural/sexual selection and/or biased dispersal (Wang & Bradburd, 2014). However, IBD, IBR and IBE are not mutually exclusive processes so that landscape elements constraining individual movement and different selective and behavioural processes can all contribute to shape patterns of gene flow in natural populations (Shafer & Wolf, 2013; Wang *et al.*, 2013; Wang & Bradburd, 2014). In this sense, incorporating ecological theory into landscape genetic studies can help to get a more comprehensive view of the neutral and adaptive microevolutionary processes shaping the distribution of genetic variability at small spatiotemporal scales (Garroway *et al.*, 2013; Coster *et al.*, 2015; Peterman *et al.*, 2015).

In this study, we analyse the relative role of geography, landscape composition and environment in structuring genetic variation in the blue tit (*Cyanistes caeruleus*), a small passerine (~10 g) with a short lifespan that has a widespread distribution in forested habitats of the western Palaearctic (Illera *et al.*, 2011). The low rates of dispersal of this species in our study area (mean natal dispersal distance based on a capture–mark–recapture study: 330 and 650 m for males and females, respectively; Ortego *et al.*, 2011) offer an interesting scenario to examine the factors structuring genetic variation at a small spatial scale (García-Navas *et al.*, 2014). Further, previous studies on blue tits have shown the presence of fine-spatial genetic structure (Foerster *et al.*, 2003; Olano-Marin *et al.*, 2010; Ortego *et al.*, 2011) and reduced gene flow among geographically close populations due to local adaptation processes (Porlier *et al.*, 2012a,b; García-Navas *et al.*, 2014). Taking advantage of extensive genotypic data (1385 individuals typed at 26 microsatellite markers), we explore the landscape-level processes shaping gene flow among 25 Mediterranean populations of blue tits experiencing different environmental conditions and degree of geographical isolation. Mediterranean environments provide a fascinating framework to address this question because they are generally characterized by a fine-grained mosaic and an extreme spatial heterogeneity resulting from natural (e.g. fire) and human disturbances (e.g. agriculture, forestry, grazing) (Blondel & Aronson, 1999; Blondel, 2006). We used circuit theory to generate different isolation-by-resistance scenarios (McRae, 2006; McRae & Beier, 2007) and test their importance in shaping contemporary patterns of gene flow relative to geographical distance and environmental dissimilarity (Wang, 2013; Wang *et al.*, 2013). Further, the employed loci were classified into two groups by considering whether they are located in genomic regions that are actively (12 loci, hereafter referred as TL) or not (14 loci, hereafter referred as NTL) transcribed to RNA (e.g. Olano-Marin *et al.*, 2011a,b; Laine *et al.*, 2012; Ferrer *et al.*, 2014). Comparisons between the two sets of markers can help to elucidate whether the factors (e.g. neutral vs. adaptive processes) shaping observed patterns of genetic differentiation can generate distinct signatures across genomic regions with different functions and architecture. Even though this question has recently received considerable attention in the context of landscape genetics (Shafer & Wolf, 2013; Wang & Bradburd, 2014), it has been rarely tested (see Freedman *et al.*, 2010 for an exception).

In particular, we (i) hypothesize that geography (IBD), landscape composition (IBR) and heterogeneous ecological conditions (IBE) contribute to shape patterns of genetic differentiation in the studied populations. The role of IBD in structuring natural populations has been documented in previous studies on less mobile species, whereas a positive correlation between genetic

and geographic distances has been demonstrated (e.g. Temple *et al.*, 2006; Ortego *et al.*, 2011). Effective distance (i.e. habitat-based distance and dispersal barriers) rather than Euclidean distance *per se* is also expected to largely impact patterns of genetic differentiation in populations of sedentary woodland birds like blue tits, which depend on landscape tree cover (Amos *et al.*, 2012, 2014; see also Adams & Burg, 2015; Barr *et al.*, 2015). Divergent selection stemming from environmental dissimilarity (IBE) can induce local adaptation and result in reduced gene flow between populations inhabiting different habitat types (Blondel, 1999; Edelaar *et al.*, 2012; Porlier *et al.*, 2012a,b; ; Langin *et al.*, 2015), which may be a particularly important driver for population differentiation in heterogeneous landscapes like those of the Mediterranean region. Finally, given that our TL are located in multiple genomic regions that are likely subjected to differential selective pressures on different traits (Olano-Marin *et al.*, 2011a), we expect that they (ii) will show more subtle patterns of genetic structure than NTL and (iii) will be less impacted by the different processes contributing to shape contemporary patterns of gene flow in the studied populations.

Materials and methods

Study system

During 2008–2013, we studied 25 blue tit populations located in fragmented woodlands scattered within an area of 1200 km^2 in Montes de Toledo, central Spain (Fig. 1). Seventeen populations were located in San Pablo de los Montes (Toledo Province, 39°31′N 4°21′W), six in Quintos de Mora (Toledo Province, 39°25′N 4°04′W) and two in Cabañeros National Park (Ciudad Real and Toledo provinces, 39°24′N, 3°35′W) (Table 1; see Fig. 1). These three areas are mainly separated by farmlands dominated by extensive cereal crops and olive groves (Fig. 1). Accordingly, the study area exhibits considerable habitat heterogeneity resulting in a mosaic-like pattern, a typical landscape configuration of the Mediterranean region (Blondel & Aronson, 1999). Within forested areas, we can discern three habitat types: (i) deciduous forests of Pyrenean oak (*Quercus pyrenaica*), which predominate in the most humid environments (shaded slopes and gullies); (ii) evergreen forests of holm oak (*Quercus rotundifolia*) widely distributed in the sunny slopes and plains; and (iii) coniferous plantations (*Pinus pinea* and *Pinus pinaster*) derived from afforestation policies carried out in the 1960s. All studied blue tit populations are established in woodlots supplied with nestboxes, of which 21 are located in deciduous Pyrenean oak forests, three in evergreen holm oak woodlands and one in a pine plantation (see Table 1 for more details). Some forest patches show a high degree of isolation as they are separated by scrublands, croplands and grazing land for livestock. Therefore, forested areas in this region do not constitute a continuous habitat; rather, these can be defined as small woodland remnants into a matrix of kaleidoscopic quality (García-Canseco, 1997; Tornero, 2003).

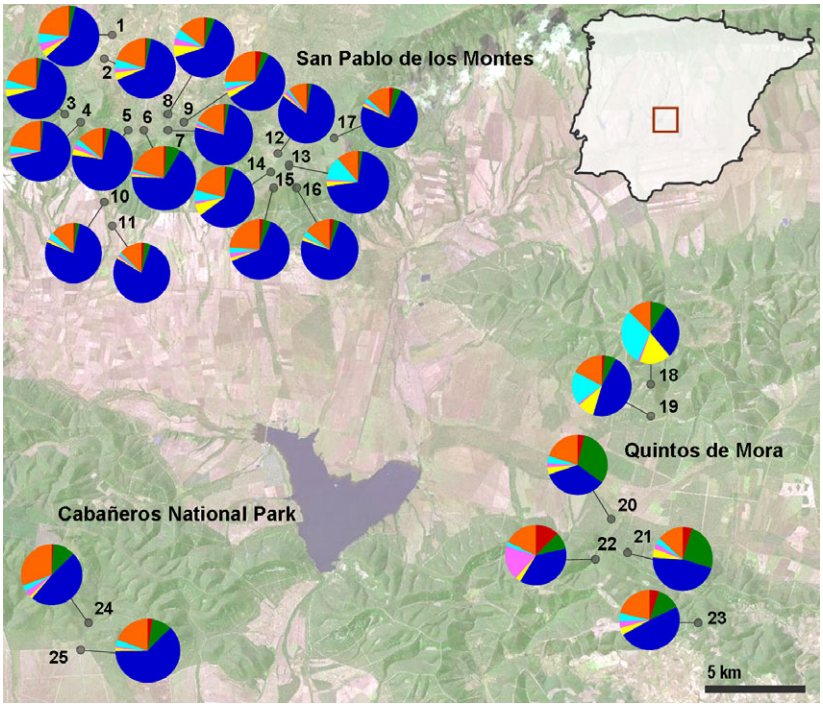

Fig. 1 Map of the study area showing the location of the studied blue tit populations and their genetic assignment based on the Bayesian method implemented in the program STRUCTURE considering seven genetic clusters and all typed markers. The admixture proportions generated by STRUCTURE were represented using pie charts, with each colour indicating a different genotypic cluster. Population codes are indicated in Table 1. The inset map shows the location of the study area in the Iberian Peninsula.

Table 1 Attributes of the 25 blue tit populations analysed in the present study. The following information is given: location of the populations in the main study localities (SPM: San Pablo de los Montes; QM: Quintos de Mora; CNP: Cabañeros National Park), population codes, geographical coordinates, number of individuals analysed in each population (*n*), elevation and habitat type (*Qr*: holm oak forest; *Qp*: Pyrenean oak forest; *Pp*: pinewood).

Area	Population	Code	Latitude	Longitude	*n*	Elevation (m)	Habitat
SPM	NVI	1	39.564	−4.363	16	836	*Qr*
SPM	NAH	2	39.556	−4.362	10	856	*Qp*
SPM	MAR	3	39.534	−4.377	159	953	*Qp*
SPM	MAJ	4	39.538	−4.368	58	1014	*Qp*
SPM	BAN	5	39.529	−4.354	13	1075	*Qp*
SPM	CAS	6	39.528	−4.344	45	1104	*Qp*
SPM	MOR	7	39.528	−4.325	92	1220	*Qp*
SPM	ERM	8	39.532	−4.327	9	1068	*Qp*
SPM	FRI	9	39.531	−4.318	10	1100	*Qp*
SPM	ROB	10	39.509	−4.359	18	866	*Qp*
SPM	FCA	11	39.496	−4.355	10	820	*Qp*
SPM	PLA	12	39.521	−4.280	15	1057	*Qp*
SPM	FCE	13	39.519	−4.277	11	1052	*Qp*
SPM	CAN	14	39.518	−4.257	11	1022	*Qp*
SPM	ARR	15	39.512	−4.288	10	962	*Qp*
SPM	AVE	16	39.512	−4.277	52	1068	*Qp*
SPM	FUB	17	39.523	−4.257	93	1033	*Qp*
QM	VAL	18	39.442	−4.094	211	908	*Qp*
QM	POS	19	39.422	4.092	11	842	*Qr*
QM	NAV	20	39.391	−4.116	75	746	*Qp*
QM	CAZ	21	39.385	−4.107	76	768	*Pp*
QM	GIL	22	39.379	−4.124	237	826	*Qp*
QM	FCO	23	39.363	−4.080	32	919	*Qp*
CNP	BRE	24	39.357	−4.351	78	764	*Qp*
CNP	ANC	25	39.342	−4.366	33	711	*Qr*

Orographically, the study area (Montes de Toledo) constitutes one of the main mountain systems of the Iberian Peninsula. The most important ranges of this system are located in north-western Ciudad Real Province and south-western Toledo Province, where they form a denuded highland with low–medium elevations (max. 1447 m). The studied populations are located at different altitudes (ranging from 700 to 1200 m), depending on whether they are sited in flood plains or in mountain ranges (see Table 1), which could lead to local adaptation processes and genetic isolation (e.g. Milá *et al.*, 2009; Thomassen *et al.*, 2010). Blue tit is a resident and common species in woodlands of the study area, and we assume that our study populations have reached equilibrium.

Sample collection

Adult blue tits were captured when feeding 8-day-old chicks by means of spring traps. Birds were individually marked with aluminium rings for further identification and bled by puncturing the brachial vein. Blood samples were stored on FTA cards (Whatman Bioscience, Florham Park, NJ, USA) or in Eppendorf tubes with 96% ethanol until needed for genetic analyses.

Microsatellite genotyping and basic genetic statistics

A total of 1385 individuals were genotyped across 26 polymorphic microsatellite markers (see Table S1). These markers were classified into two groups by considering whether they are located or not in genomic regions that are actively transcribed to RNA in the zebra finch (*Taeniopygia guttata*) (for more details, see Olano-Marin *et al.*, 2011a,b; see also Olano-Marin *et al.*, 2010; Ferrer *et al.*, 2014) (Table S1). We used NucleoSpin Blood Kits (Macherey-Nagel, Duren, Germany) to extract and purify genomic DNA from the blood samples. Approximately 5 ng of template DNA was amplified in 10-μL reaction volumes containing 1× reaction buffer (67 mM Tris–HCl, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, EcoStart Reaction Buffer; Ecogen, Barcelona, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of Taq DNA EcoStart Polymerase (Ecogen). The PCR programme used was 9 min denaturing at 95 °C followed by 40 cycles of 30 s at 94 °C, 45 s at the annealing temperature (Table S1) and 45 s at 72 °C, ending with a 10-min final elongation stage at 72 °C. Amplification products were electrophoresed

using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and genotypes were scored using GENEMAPPER 3.7 (Applied Biosystems).

We tested for linkage disequilibrium (LD) between pairs of loci and deviations from Hardy–Weinberg equilibrium (HWE) using GENEPOP web version 4.2 (Raymond & Rousset, 1995). Probabilities of significance were assessed applying a Markov chain method (Guo & Thompson, 1992) using 200 batches, 1000 iterations per batch and 1000 dememorization steps as implemented in GENEPOP. The LD correlation coefficient (*r*LD) between alleles at different loci was used to estimate the magnitude of LD. *r*LD was computed with the program LINKDOS (http://genepop.curtin.edu.au/linkdos.html; Garnier-Gere & Dillmann, 1992). Sequential Bonferroni corrections were applied to account for multiple comparisons (Rice, 1989).

Neutrality tests

We used two methods to detect outlier loci across all populations. First, we used the method developed by Beaumont & Nichols (1996) and implemented in LOSITAN (Antao *et al.*, 2008). We conducted an initial run of 100 000 simulations assuming a stepwise mutation model and then computed the distribution of F_{ST} for each microsatellite loci using putatively neutral loci derived from the simulations. The F_{ST} values of all loci were compared with the neutral expectation at the 95%, 99% and 99.5% confidence levels. Second, we used BAYESCAN 2.1 to detect outlier loci, which implements a Bayesian method to estimate population- and locus-specific F_{ST} coefficients and is suspected to be more adequate to detect outlier loci under complex demographic scenarios (Foll & Gaggiotti, 2008). The parameters were set to 10 pilot runs of 5000 iterations and additional burn-in of 50 000 iterations. Outlier loci were identified based on the 90%, 95% and 99% posterior probabilities.

Spatial genetic structure

We investigated the genetic differentiation between the studied blue tit populations calculating pairwise F_{ST} values and testing their significance with Fisher's exact tests after 10 000 permutations as implemented in ARLEQUIN 3.1 (Excoffier *et al.*, 2005). We calculated global F_{ST} values across all populations in FSTAT 2.9.3, and 95% confidence intervals (95% CI) were estimated by bootstrapping over loci (10 000 randomizations; Goudet, 1995). Global and pairwise F_{ST} values were calculated for all markers and separately for the subsets of TL and NTL. We also analysed the spatial genetic structure using the Bayesian clustering method implemented in STRUCTURE 2.3.4, which assigns individuals to populations based on their multilocus genotypes (Pritchard *et al.*, 2000; Falush *et al.*, 2003). We ran STRUCTURE assuming correlated allele frequencies and admixture (Pritchard *et al.*, 2000; Falush *et al.*, 2003) and using the 'prior population information' option to take sampling locations into account (Hubisz *et al.*, 2009). First, we conducted five independent runs for each value of $K = 1–25$ with a burn-in of 100 000 MCMC steps followed by 100 000 iterations. This allowed us to determine basic parameters and the most likely number of genetic clusters (K) and avoid very time-consuming runs for large values of K. Given that the most likely number of genetic clusters inferred in these preliminary analyses was always largely below $K = 10$ (see Results), we performed five additional runs for each value of $K = 1–10$ using the same settings. The most likely value of K was determined using log probabilities [$\Pr(X|K)$] (Pritchard *et al.*, 2000) and the ΔK method (Evanno *et al.*, 2005). We used STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to compile and visualize the results from STRUCTURE, CLUMPP to align multiple runs for the same value of K (Jakobsson & Rosenberg, 2007) and DISTRUCT (Rosenberg, 2004) to visualize the resulting Q matrix. Analyses in STRUCTURE were also performed for all markers together and considering TL and NTL separately.

Landscape genetic analyses

We examined the effects of geographical distance (IBD), resistance distances defined by landscape configuration (IBR) and environmental dissimilarity (IBE) on the genetic differentiation among the studied populations. To test the effect of IBD, we calculated a matrix of Euclidean geographical distances between all pairs of populations. We applied circuit theory to model gene flow across the highly heterogeneous studied landscape and determined the impact of different isolation-by-resistance (IBR) scenarios on observed patterns of genetic differentiation (McRae, 2006; e.g. McRae & Beier, 2007; McRae *et al.*, 2008). We used ARCMAP 10.0 (ESRI, Redlands, CA, USA) and the CORINE land cover map (1 : 100 000) from the Spanish National Centre of Geographic Information (CNIG; https://www.cnig.es) to create raster layers with a spatial resolution standardized to a 50-m grid cell size. We reclassified land uses into three landscape element classes that *a priori* are likely to determine the distribution and dispersal patterns in forest passerines: (i) woodlands; (ii) nonforested natural or semi-natural habitats, mostly constituted by scrublands and meadows; and (iii) non-natural habitats, mostly including open agricultural lands and, in a much lesser extent, reservoirs, villages and other developed areas (e.g. Desrochers & Hannon, 1997; Bélisle *et al.*, 2001; Matthysen *et al.*, 2001; Pavlova *et al.*, 2012). We generated different IBR scenarios assigning a range of resistance values to each of the three habitat classes (Table 2). This allowed us to identify the resistance values for each habitat class that best fit our data on genetic differentiation (e.g. Andrew *et al.*, 2012; Seymour *et al.*,

Table 2 Multiple matrix regressions with randomization for genetic differentiation (F_{ST}) in relation with 25 isolation-by-resistance (IBR) scenarios considering different resistance values (1 = lowest resistance; 100 = highest resistance) for the three land cover types considered in this study: forests, nonforested natural vegetation habitats and lands mostly devoted to agriculture. F_{ST} values were estimated for all markers and two subsets of loci that were classified by considering whether they are located (TL) or not (NTL) in regions of the genome that are being actively transcribed to RNA.

				All loci			NTL			TL		
Model	Forest	Nonforested vegetation	Agricultural lands	R^2	β	P	R^2	β	P	R^2	β	P
Null model	1	1	1	**0.047**	**0.329**	**0.031**	**0.044**	**0.318**	**0.036**	0.008	0.141	0.408
Model 1	1	1	50	0.003	0.054	0.681	0.048	0.218	0.168	0.007	−0.088	0.457
Model 2	1	1	100	0.002	0.048	0.694	0.045	0.210	0.172	0.008	−0.088	0.460
Model 3	1	50	1	0.000	−0.022	0.9	0.005	−0.075	0.660	0.003	0.060	0.756
Model 4	1	50	50	0.000	−0.023	0.883	0.002	−0.054	0.739	0.001	0.043	0.819
Model 5	1	50	100	0.000	−0.024	0.86	0.002	−0.046	0.617	0.001	0.036	0.852
Model 6	1	100	1	0.001	−0.032	0.834	0.006	−0.078	0.636	0.002	0.049	0.806
Model 7	1	100	50	0.001	−0.034	0.823	0.004	−0.066	0.674	0.001	0.039	0.851
Model 8	1	100	100	0.008	0.081	0.836	0.003	−0.062	0.711	0.001	0.032	0.877
Model 9	50	1	1	0.006	0.085	0.601	0.036	0.204	0.228	0.009	−0.101	0.618
Model 10	50	1	50	0.007	0.09	0.597	0.051	0.242	0.161	0.011	−0.117	0.525
Model 11	50	1	100	0.007	0.091	0.544	0.054	0.249	0.123	0.012	−0.118	0.510
Model 12	50	50	1	0.044	0.342	0.089	0.000	−0.046	0.831	0.064	0.412	0.053
Model 13	50	50	100	0.034	0.264	0.064	0.042	0.294	0.054	0.003	0.089	0.570
Model 14	50	100	1	0.035	0.285	0.185	0.000	0.002	0.990	0.063	0.385	0.061
Model 15	50	100	50	0.033	0.265	0.132	0.007	0.126	0.480	0.029	0.252	0.129
Model 16	50	100	100	0.027	0.232	0.14	0.010	0.144	0.348	0.019	0.195	0.239
Model 17	100	1	1	0.005	0.078	0.64	0.035	0.197	0.260	0.009	−0.105	0.597
Model 18	100	1	50	0.006	0.082	0.632	0.046	0.229	0.161	0.012	−0.118	0.521
Model 19	100	1	100	0.006	0.083	0.61	0.050	0.237	0.158	0.012	−0.121	0.526
Model 20	100	50	1	0.037	0.263	0.151	0.040	0.275	0.167	0.001	0.055	0.802
Model 21	100	50	50	0.03	0.228	0.18	0.054	0.307	0.084	0.000	0.008	0.969
Model 22	100	50	100	0.026	0.208	0.234	0.055	0.303	0.072	0.000	−0.007	0.973
Model 23	100	100	1	0.043	0.335	0.097	0.001	−0.063	0.465	**0.066**	**0.416**	**0.048**
Model 24	100	100	50	0.053	0.372	0.027	0.020	0.228	0.180	0.025	0.258	0.131

Values in bold indicate the most significant model for each group of loci, β is the regression coefficient, and R^2 is the coefficient of determination.

2013). We used CIRCUITSCAPE 3.5.8 to calculate resistance distance matrices between all pairs of populations considering an eight-neighbour cell connection scheme (McRae, 2006). We also created a null model with all cells having equal resistance (value = 1), which is expected to yield similar results as Euclidean geographical distance but has been suggested to be more appropriate for comparisons against models of IBR generated by CIRCUITSCAPE (Lee-Yaw *et al.*, 2009; Munshi-South, 2012; Jha & Kremen, 2013). Finally, we tested two isolation-by-environment (IBE) scenarios based on differences in elevation and habitat type between the studied populations, which have been previously found to be involved in processes of local adaptation and to influence patterns of genetic differentiation in a range of organisms (reviewed in Shafer & Wolf, 2013; Sexton *et al.*, 2014). We used a digital elevation model from the Spanish National Centre of Geographic Information to calculate a matrix of differences in elevation between each pair of studied populations (Table 1). We generated a pairwise matrix of habitat differences considering whether the populations were located in deciduous forests, evergreen sclerophyllous forests or coniferous forests (Table 1). All comparisons within the same habitat type were assigned a value of 0, whereas deciduous vs. evergreen forest, coniferous vs. evergreen forest and coniferous vs. deciduous forest comparisons were assigned a value of 1 (e.g. Andrew *et al.*, 2012).

The relationships between genetic differentiation (F_{ST}) and matrices of geographical distance (IBD), landscape resistance (IBR) and environmental dissimilarity (IBE) were examined using multiple matrix regressions with randomization (MMRR) using the MMRR function script (Wang, 2013) implemented in R 3.0.2. These analyses were performed for pairwise F_{ST} values calculated for all markers and considering TL and NTL separately.

Results

Microsatellite data

Genetic diversity was higher for the panel of NTL in comparison with the subset of TL, but these differences

were only statistically significant in terms of allelic richness (one-way ANOVAS, H_E: $F_{1,24} = 2.60$, $P = 0.119$; H_O: $F_{1,24} = 2.08$, $P = 0.161$; allelic richness: $F_{1,24} = 4.90$, $P = 0.036$; Table S1). We observed significant departures from HWE in 18 of 650 population–locus combinations after correcting for multiple comparisons. Such deviations involved loci *Pca2* (FCO and MAR populations), *Pca3* (NAV population), *Pca8* (GIL and NAV populations), *Pdoμ5* (FCA, FUB and VAL populations), *Pocc6* (GIL population), *CcaTgu11* (CAS, GIL, MAR and VAL populations) and *CcaTgu15* (CAZ, FCO, GIL, MAR, and VAL populations). We only found evidence of significant LD between some pair of loci located within the same chromosome for GIL population (*Mcyμ4-Tg053*, *Pca3-Pdoμ5*, *Pca8-Tgu07*, *Pca9-Pocc1*, *PK12-TG05-053*, *PK12-CcaTgu15*, *Pocc6-Tgu07*), VAL population (*Mcyμ4-Tg053*, *Mcyμ4-Tgu14*, *PK12-CcaTgu15*, *Tgu15-Tgu14*), BRE population (*Pca3-Pdoμ5*) and MAR population (*PK12-Mcyμ4*). Given that LD and departures from HWE were not consistent across loci and populations, all markers were used in subsequent analyses.

Neutrality tests

Analyses of neutrality based on the F_{ST} outlier tests implemented in LOSITAN (Beaumont & Nichols, 1996; Antao *et al.*, 2008) and BAYESCAN (Foll & Gaggiotti, 2008) indicated that no locus deviated significantly from neutral expectations (data not shown). Thus, TL do not seem to be under strong selection despite they are located inside of flanking coding gene sequences that are being actively expressed to RNA (Olano-Marin *et al.*, 2010, 2011a).

Spatial genetic structure

After sequential Bonferroni corrections, we found that 10 pairwise F_{ST} values were significant for analyses combining genotypic data from NTL and TL markers (Table S2). When the two subsets of loci were analysed separately, we found 17 and 5 significant pairwise F_{ST} values for the panels of NTL and TL, respectively (Table S3). Pairwise F_{ST} values estimated for NTL and TL were not significantly correlated (Mantel test: $r = -0.018$, $P = 0.396$). Global F_{ST} values were significantly higher than zero for all markers ($F_{ST} = 0.004$, 95% CI: 0.003–0.005) and the subsets of NTL ($F_{ST} = 0.003$, 95% CI: 0.003–0.004) and TL ($F_{ST} = 0.005$, 95% CI: 0.004–0.007) (Fig. S1). Global F_{ST} values were slightly higher in TL than in NTL, but these differences were not significant (i.e. 95% CI overlap; Fig. S1).

STRUCTURE analyses considering all typed loci and the statistic ΔK indicated that the most likely number of genetic clusters was $K = 7$ (Figs 2a, S2 and S3). STRUCTURE analyses for the subset of NTL showed an optimal value of $K = 4$ according to the statistic ΔK (Figs 2b, S2 and S4). However, when the panel of TL was analysed separately, the value of $\Pr(X|K)$ reached a maximum at $K = 1$ (Fig. S2). Overall, STRUCTURE analyses for all loci and the panel of NTL revealed high levels of genetic admixture and subtle levels of genetic differentiation among populations from the three main study areas. Despite the high number of clusters supported by STRUCTURE analyses for all markers and NTL, a detailed inspection of barplots for different values of K suggests that the most biologically relevant clustering is probably $K = 3$ (Figs S3 and S6). Probabilities of population membership for $K = 3$ indicate that VAL and GIL

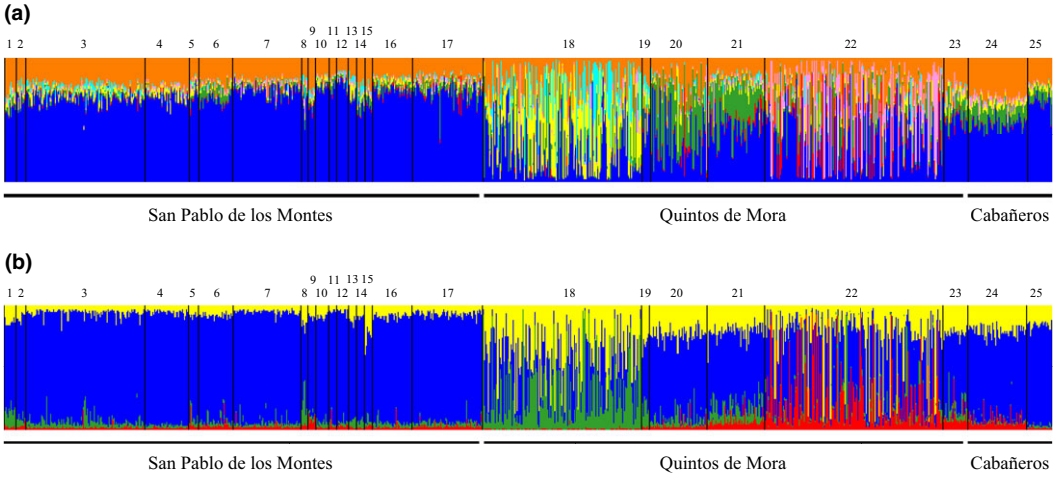

Fig. 2 Results of genetic assignments of blue tits ($n = 1385$ individuals) based on the Bayesian method implemented in the program STRUCTURE considering (a) the seven genetic clusters inferred for all typed markers and (b) the four genetic clusters inferred for the subset of loci not located in regions of the genome that are being actively transcribed to RNA. Each individual is represented by a thin vertical line, which is partitioned into coloured segments that represent the individual's probability of belonging to the cluster with that colour. Individuals are grouped according to their populations of origin using vertical black lines. Population codes are indicated in Table 1.

populations show the highest levels of genetic differentiation both between them and with all other studied populations, a pattern also supported by pairwise F_{ST} values (Tables S2 and S3). Higher values of K resulted in the assignment of low probabilities of membership to different genetic clusters that are not highly represented in any population (Figs 1, 2, S3 and S6).

We performed additional analyses to ascertain whether the contrasting patterns of genetic structure inferred with the subsets of NTL and TL were due to the higher genetic diversity of NTL and/or the different number of typed loci in each group (14 NTL vs. 12 TL; Table S1). For this purpose, we removed the two most diverse loci (*Pca3* and *Pca8*; Table S1) from the panel of NTL, recalculated F_{ST} values and reran STRUCTURE analyses. After the removal of loci *Pca3* and *Pca8*, genetic diversity did not significantly differ between the subsets of NTL and TL (one-way ANOVAS, H_E: $F_{1,22} = 1.34$, $P = 0.258$; H_O: $F_{1,22} = 1.08$, $P = 0.309$; allelic richness: $F_{1,22} = 2.84$, $P = 0.105$). For this subset of 12 NTL, we found 19 significant pairwise F_{ST} values and STRUCTURE analyses showed that $\Pr(X|K)$ increased up to $K = 6$ and the statistic ΔK indicated that the most likely number of genetic clusters was $K = 2$ (Figs S5 and S6).

Landscape genetic analyses

Genetic differentiation (F_{ST}) estimated at all loci and the panel of NTL was associated with geographical distances (all loci: $t = 3.55$, $P = 0.037$; NTL: $t = 3.94$, $P = 0.032$). However, IBE analyses based on either habitat type (all loci: $t = 0.75$, $P = 0.733$; NTL: $t = 2.55$, $P = 0.309$) or elevation distance matrices (all loci: $t = 1.11$, $P = 0.464$; NTL: $t = 1.32$, $P = 0.397$) were not significant. Genetic differentiation estimated at the subset of TL was not associated with geographical distance ($t = 0.06$, $P = 0.575$) or environmental dissimilarity (habitat: $t = -0.05$, $P = 0.978$; elevation: $t = 0.13$, $P = 0.926$). Different models of IBR explained genetic differentiation estimated based on all markers and on the subsets of NTL and TL (Table 2). The null model (i.e. the same resistance value assigned to all cells), which is similar to Euclidean geographical distance ($r = 0.96$, $P = 0.001$), explained genetic differentiation estimated at all loci and NTL (Table 2; Fig. 3a,b), and no other resistance-based distance remained significant after

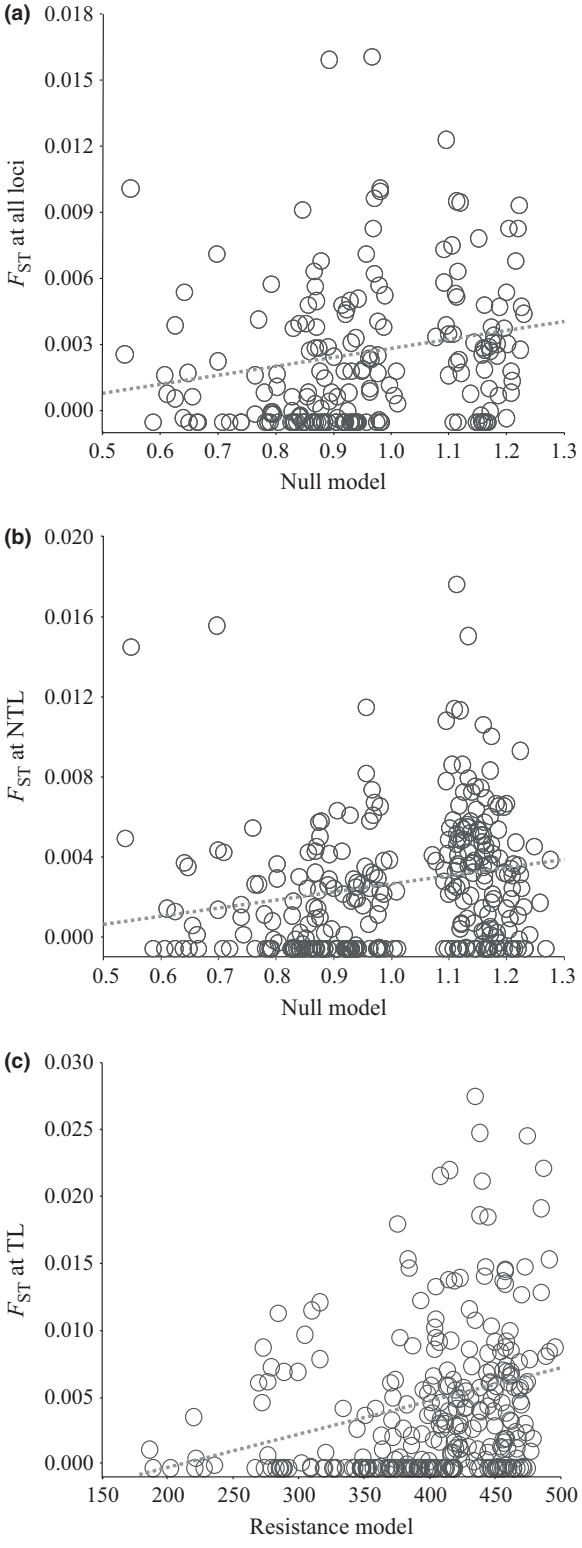

Fig. 3 Relationship between genetic differentiation (F_{ST}) and the best explanatory model of resistance obtained using CIRCUITSCAPE. Models were run for F_{ST} values estimated for all markers and two subsets of loci that were classified by considering whether they are located (TL) or not (NTL) in regions of the genome that are being actively transcribed to RNA. The best explanatory model for (a) all markers and (b) the subset of NTL was the null model (i.e. resistance = 1 for all habitat classes), whereas (c) the model considering high resistance for forest and nonforested natural vegetation (resistance = 500) and low resistance for lands mostly devoted to agriculture (resistance = 1) was the best explaining genetic differentiation at TL.

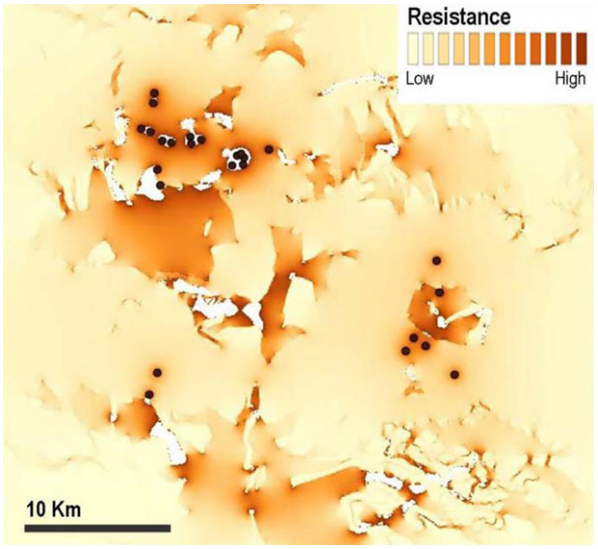

Fig. 4 Landscape resistance model for which we obtained the highest correlation with genetic differentiation (F_{ST}) for markers located in regions of the genome that are being actively transcribed to RNA (resistance = 500 for forest and nonforested natural vegetation and resistance = 1 for lands mostly devoted to agriculture). Lighter colours represent low resistance and darker colours represent high resistance.

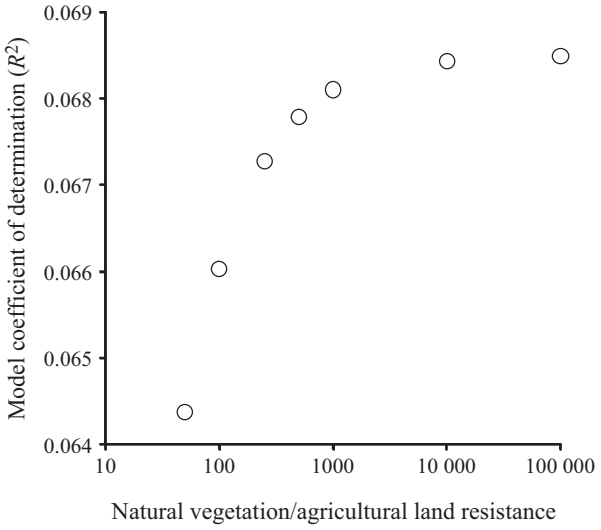

Fig. 5 Coefficient of determination (R^2) for models analysing genetic differentiation (F_{ST}) estimated at markers located in regions of the genome that are being actively transcribed to RNA in relation with isolation-by-resistance (IBR) distance matrices calculated with CIRCUITSCAPE considering different resistance ratios for natural vegetation (forests and nonforested natural habitats) and lands mostly devoted to agriculture. All models considered the minimum resistance (resistance = 1) for agricultural land and increasing resistance values (from 10 to 100 000) for forests and nonforested natural habitats. Resistance ratios are log-transformed for illustrative purposes.

this matrix was included (all P-values > 0.1). However, genetic differentiation estimated at TL was better explained by IBR models considering high resistance values for woodlands and nonforested natural vegetation and the minimum resistance value (i.e. resistance = 1) for lands mostly devoted to agriculture (Table 2; Figs 3c and 4). The null model considering equal resistance to all habitat classes was not significantly associated with genetic differentiation estimated at TL (Table 2). We generated further IBR models progressively increasing resistance for woodlands and nonforested natural vegetation habitat classes (10, 25, 50, 100, 250, 500, 1000, 10 000 and 100 000) to evaluate whether this contributes to increase the goodness of fit of the model (e.g. Andrew *et al.*, 2012). Model goodness of fit increased with the 'forested and nonforested habitats'/'agricultural land' resistance ratio, but the strength of the relationship between genetic differentiation and IBR stabilized beyond a ratio 500 : 1 (Fig. 5; Table S4).

Discussion

We found that TL and NTL yielded contrasting patterns of genetic structure for the studied blue tit populations. Population genetic differentiation was subtle and mostly detected using the panel of NTL. Landscape genetic analyses showed a pattern of IBD for all loci and the panel of NTL, but genetic differentiation estimated at the subset of TL was better explained by IBR models considering high resistance values for natural vegetation and low resistance to managed areas mostly devoted to agriculture. Finally, we did not find any support for IBE in our study system, suggesting a lack of adaptive microevolutionary changes and divergent selection pressures associated with differences in the two environmental components analysed.

Population genetic structure

STRUCTURE analyses and pairwise F_{ST} values indicated the presence of a subtle pattern of genetic structure across all the studied populations (Fig. 1). All individuals and populations showed a high degree of genetic admixture for any value of K considered, and most significant pairwise F_{ST} values involved comparisons between populations from different areas (Fig. 1). The only exception was the significant genetic structure observed within Quintos de Mora, which was mostly due to the high genetic differentiation between VAL and GIL and some comparisons between these two and some of the other populations (Fig. 1). A previous study showed that divergent natural selection has resulted in phenotypic divergence between these two populations, which may have reduced realized gene flow despite the fact that they are located < 7 km apart and sporadic

exchange of individuals has been recorded (García-Navas *et al.*, 2014). Moreover, the same study found that VAL and GIL receive immigrants from genetically differentiated source populations, a fact that could have also contributed to their high genetic differentiation in comparison with other population pairs located at similar or larger geographical distances (García-Navas *et al.*, 2014) (Fig. 1).

When data were analysed separately for the subsets of TL and NTL, STRUCTURE analyses revealed that the most optimal clustering solution for TL was the presence of a single genetic cluster, whereas analyses focused on NTL showed an optimal value of $K = 4$. These differences were not due to the different number of markers employed or their degree of polymorphism, suggesting that the distinct patterns of genetic structure observed for the two subsets of loci may be a consequence of their location in genomic regions subjected to different evolutionary pressures. Loci under directional or disruptive selection are expected to show more marked genetic structure than neutral loci if incipient local adaptive processes have not yet reduced genomewide gene flow, but our results point to the opposite direction (Shafer & Wolf, 2013; see Oetjen & Reusch, 2007; Zhao *et al.*, 2013). Analyses of neutrality indicated that the subset of TL employed in this study do not seem to be under strong selection despite being located inside (or at flanking regions of) coding gene sequences that are actively expressed to RNA. One possibility to explain the observed pattern is that most TL are under stabilizing selection, which is more difficult to detect by outlier tests particularly when populations are weakly differentiated (Beaumont & Balding, 2004). Thus, the presence of functional constraints and the effects of purifying selection may explain the lower levels of genetic differentiation and polymorphism in the subset of TL in comparison with NTL (Oetjen & Reusch, 2007; Olano-Marin *et al.*, 2011a; Agostini *et al.*, 2013).

Isolation by resistance

Pairwise genetic differentiation at TL and NTL was not significantly correlated, suggesting that the two kinds of loci may capture different aspects of the same demographic phenomena (Freedman *et al.*, 2010; Zhao *et al.*, 2013). Accordingly, landscape genetic analyses based on circuit theory showed that the null model considering equal resistance for all habitat classes (equivalent to Euclidean geographical distance) was the one that best explained genetic differentiation estimated at all loci and the panel of NTL. However, contrary to our expectations, interpopulation gene flow estimated for the subset of TL was not significantly associated with geographical distance; the best-fitting model was the one considering the lowest resistance (i.e. resistance = 1) for open croplands and a high resistance (resistance > 100) for natural vegetation (woodlands, scrublands and meadows). Model goodness of fit reached a plateau when considering resistance values for natural vegetation ~500 times higher than those assigned to open agricultural lands (Fig. 5). Studies comparing genetic structure in fragmented and continuous landscapes have often shown lower dispersal and gene flow between patches separated by a matrix of unsuitable habitat (Broquet *et al.*, 2006; Martínez-Cruz *et al.*, 2007; Coulon *et al.*, 2008; Dutta *et al.*, 2013). However, habitat fragmentation can also induce the opposite process if individuals are forced to disperse longer distances across unfavourable habitats to find a suitable breeding site, a fact that may explain the obtained results for the subset of TL (Wiens, 2001; Veit *et al.*, 2005; Mylecraine *et al.*, 2008; Harrisson *et al.*, 2013). The different results found for TL and NTL may be caused by endogenous (e.g. incompatibilities between groups of alleles due to selection) and exogenous (e.g. alleles adapted to different habitats) barriers that are not acting in the same way across different genomic regions (Bierne *et al.*, 2011; Corbett-Detig *et al.*, 2014). Given that TL have higher chances to be involved in the evolution of a reproductive barrier and that genetic incompatibilities arisen at the population level tend to correlate with landscape-level migration barriers, this may explain why IBR is the best-fit model for genetic differentiation estimated at TL (Bierne *et al.*, 2011). However, our results should be interpreted with caution. First, it should be kept in mind that we found subtle genetic differentiation among the studied populations, which indicates widespread gene flow and a limited impact of landscape composition in contemporary patterns of genetic structure at the spatial scale here considered. This makes difficult to rule out that stochastic differences between the two subsets of markers are driving the observed patterns. Second, neutrality tests indicated that none of the employed loci are subjected to selection, which reduces the chances that our TL are actually involved in reproductive isolation or local adaptation in the study populations. Finally, the fact that our TL are located in regions that are being actively transcribed in the zebra finch does not make them necessarily linked to functional regions in other passerines. Thus, there is some uncertainty in the classification of the employed loci made in both our study and previous studies on blue tits (Olano-Marin *et al.*, 2011a,b; Ferrer *et al.*, 2014).

Isolation by environment

Our analyses based on habitat and elevation dissimilarity did not reveal the presence of IBE in our study system, a pattern that may be explained by different reasons. Elevational gradients have been found to be important drivers of genetic and phenotypic

divergence in different small-sized bird species (e.g. Milá *et al.*, 2009; Thomassen *et al.*, 2010), but we did not find any signal of isolation by altitude. Although populations located at different elevations show significant differences in the timing of breeding in our study area (V. García-Navas & J. J. Sanz, unpublished data), our results suggest that such differences in phenology may represent a purely plastic response to temporal differences in prey availability associated with local environmental conditions (García-Navas & Sanz, 2011) and that these populations do not exhibit different reaction norms, which are only possible under reduced gene flow. Alternatively, the lack of association between elevation dissimilarity and genetic differentiation may have resulted from our set of markers that does not include any locus linked to genes related with phenology. Another possibility is that such unsampled loci linked with phenology do not have a considerable impact on disrupting gene flow among populations located at different elevational ranges and, thus, their effect may not be reflected on neutral patterns of genetic differentiation (Shafer & Wolf, 2013). Regarding the second environmental factor, that is habitat type, a previous study carried out on Corsican populations of blue tit (*C. caeruleus ogliastrae*) at a larger spatial scale (~150 km) revealed that genetic differentiation was related to local habitat type but not to the geographic distance or the presence of physical barriers among populations (Porlier *et al.*, 2012a). This suggests that local adaptation might reduce gene flow among populations from different habitat types (deciduous vs. evergreen oak forests) and/or that divergent selection (in response to habitat-specific selection regimes) is strong relative to gene flow (Porlier *et al.*, 2012a). Therefore, the observed differences in morphological and reproductive traits among Corsican blue tit populations inhabiting deciduous and evergreen forests can be explained as a consequence of asymmetrical gene flow resulting from source–sink dynamics between these two habitat types (Dias *et al.*, 1996; Blondel, 2007; Blondel *et al.*, 2006). In our study populations, habitat type (deciduous vs. evergreen) does not seem to impose important selective pressures as indicated by ecological and behavioural studies in which both forest types were compared (García-Navas & Sanz, 2012; García-Navas *et al.*, 2012). Such studies do not support the existence of local maladaption to the less suitable habitat (evergreen forests) in contrast with that reported in Corsican blue tits (cf. Dias & Blondel, 1996). At this point, we must acknowledge that the low number of localities sampled in evergreen forests may have reduced our statistical power to detect such effects. However, no single significant pairwise F_{ST} value involved populations from different habitats, which suggests that the lack of isolation by habitat may be genuine or that considerable gene flow is preventing local adaptation to persist over time.

Despite our number of markers and sample sizes are both way far larger than those employed in most previous studies exploring the role of environment in structuring genetic variation, it should be also considered that IBE generally only explains ~5% of total genetic differentiation (Shafer & Wolf, 2013), which could have reduced the ability of our loci to detect incipient or ephemeral adaptation processes that may be taking place in some of our study populations (Shafer & Wolf, 2013; e.g. García-Navas *et al.*, 2014). Finally, it should be also taken into account that we cannot totally reject the hypothesis of IBE given that other factors not analysed in this study (e.g. food supply: García-Navas & Sanz, 2011; calcium availability: García-Navas *et al.*, 2011; disease risk: Garroway *et al.*, 2013) could potentially lead to micro evolutionary changes and cryptic processes of local adaptation (e.g. Garroway *et al.*, 2013; McDevitt *et al.*, 2013).

Conclusions

Overall, our study shows that markers located in different genomic regions can yield contrasting inferences about the patterns of population genetic structure. The low genetic differentiation observed for the subset TL in comparison with the panel of NTL is particularly remarkable. The subtle pattern of genetic structure and the lack of IBE indicate widespread gene flow in our study system, which may be explained by long-distance dispersal movements through unsuitable habitat matrix as suggested by the best explanatory IBR model for TL. This study is one of the few to incorporate local conditions at each sampling locality in conjunction with landscape factors describing matrix quality to examine how environment influences realized dispersal and gene flow (Murphy *et al.*, 2010; Pflüger & Balkenhol, 2014). Future studies performed at larger spatial scales, considering other potential drivers of local adaptation, and based on genomewide data obtained using high-throughput sequencing technology will help to get a better understanding of the neutral and adaptive processes determining effective dispersal and the resulting patterns of genetic structure in natural populations (Andrews & Luikart, 2014; e.g. Garroway *et al.*, 2013; Orsini *et al.*, 2013; Geraldes *et al.*, 2014).

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Supporting information

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

Figure S1 Global F_{ST} values and 95% confidence intervals for all markers and the two subsets of loci that were classified by considering whether they are located (TL) or not (NTL) in regions of the genome that are being actively transcribed to RNA.

Figure S2 Results of Bayesian clustering analyses in STRUCTURE.

Figure S3 Results of genetic assignments of blue tits based on the Bayesian method implemented in the program STRUCTURE considering all typed markers and $K = 2$–6.

Figure S4 Results of genetic assignments of blue tits based on the Bayesian method implemented in the program STRUCTURE considering $K = 2$–3 and the subset of markers not located in regions of the genome that are being actively transcribed to RNA.

Figure S5 Results of Bayesian clustering analyses in STRUCTURE for the subset of markers not located in regions of the genome that are being actively transcribed to RNA excluding the two most polymorphic markers (*Pca3* and *Pca8*).

Figure S6 Results of genetic assignments of blue tits based on the Bayesian method implemented in the program STRUCTURE considering $K = 2$–6 and the subset of markers not located in regions of the genome that are being actively transcribed to RNA excluding the two most polymorphic markers (*Pca3* and *Pca8*).

Table S1 Panel of 26 microsatellite loci used to genotype blue tits.

Table S2 Pairwise F_{ST} values estimated at all typed loci for the studied blue tit populations.

Table S3 Pairwise F_{ST}-values estimated at two subsets of loci that were classified by considering whether they are located or not in regions of the genome that are being actively transcribed to RNA.

Table S4 Multiple Matrix Regressions with Randomization (MMRR) for genetic differentiation (F_{ST}) estimated for the subset of markers located in regions of the genome that are being actively transcribed to RNA in relation with nine isolation-by-resistance (IBR) scenarios.

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