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Fine-scale genetic structure and phenotypic divergence of a passerine bird population inhabiting a continuous Mediterranean woodland

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Genetic differentiation between populations inhabiting ecologically different habitats might appear because of limited dispersal and gene flow, which may lead to patterns of phenotypic divergence and local adaptation. In this study, we use dispersal, genotypic (24 microsatellite loci) and phenotypic (body size and clutch size) data to analyse patterns of genetic structuring and phenotypic divergence in a blue tit (*Cyanistes caeruleus*) population inhabiting a continuous and heterogeneous woodland along a valley. The two slopes of the valley differ in their forest formations and environmental conditions. Findings showed that most blue tits reproduced within their natal slope. Accordingly, microsatellite analyses revealed that populations of blue tits established in the two slopes show subtle genetic differentiation. The two genetic populations diverged in clutch size, exceeding the level of differentiation expected based on genetic drift, hence suggesting divergent selection (or other processes promoting divergence) on this life-history trait. Our findings reveal that restricted dispersal and spatial

1. Introduction

Dispersal is a key life-history trait playing a crucial role in determining the levels of gene flow among populations and thus adaptation to local environments [1]. Limited or restricted gene flow due to geographical barriers is regarded as the main cause of genetic divergence [2]. Indeed, genetic differentiation between populations typically increases with geographical distance [3–5]. Yet, genetic structuring can emerge at small scales because dispersal, and consequently gene flow, can be non-random [6] or because landscape configuration and local ecological conditions limit gene flow [7–9]. Limited gene flow is expected to enhance the capacity of populations to become adapted to local environmental conditions, whenever gene flow introduces foreign alleles that are less locally adapted [10,11].

Whenever individuals disperse from their natal territory, they must cope with novel environmental conditions to which they are not well adapted [12–14]. Accordingly, it is expected that selection against immigrant genes would reduce gene flow among populations, especially among contrasting environments, thus leading to genetic differentiation between populations, phenotypic divergence and local adaptation [14–19]. Population fragmentation because of restricted gene flow can favour the maintenance of local adaptations when genetic diversity is high enough in local populations [20,21]. Local adaptation could be constrained by immigrant genes from marginal and poor-quality habitats [22,23]. On the other hand, genetic differentiation may reduce the local genetic diversity and impact local adaptation if the effective population size is small [24]. In this way, dispersal can benefit populations by reducing close inbreeding as well as the consequent loss of genetic diversity [18,25,26].

Some studies show that non-random dispersal and genetic population differentiation can occur between neighbouring populations of highly mobile animals, such as birds [8,18,19,22,27–30]. Population genetic structuring has been shown to be accompanied by phenotypic differentiation in reproductive strategies, behaviour and morphology [14,15,17,31–35]. However, correlational studies are insufficient to make conclusions about patterns of local adaptation (36,37; but refer to [33]). There are logistic concerns whenever studying the adaptive significance of phenotypic divergence in birds (e.g. difficulty in carrying out common garden or reciprocal transplant experiments [38]). One way to study local adaptation is to measure both the neutral genetic divergence and the quantitative phenotypic variation among populations and compare them using the P_{ST}–F_{ST} method, which allows one to infer the role of selection and genetic drift in shaping the phenotypic variation among populations [39–41]. The P_{ST}–F_{ST} comparison quantifies the phenotypic differentiation (P_{ST}) in relation to the level of divergence expected according to genetic drift alone (F_{ST}). Previous studies using this approach found evidence of local adaptation in avian coloration, morphometry, behaviour and reproductive parameters [18,33,42–47]. Nonetheless, results derived from P_{ST}–F_{ST} comparisons should be interpreted with caution, especially if the genetic markers used have high mutation rates [48].

In the present study, we analysed the natal dispersal, the genetic differentiation and the phenotypic divergence in a population of blue tits (*Cyanistes caeruleus*) inhabiting a continuous Mediterranean woodland located along a valley. The two slopes of the valley differ in forest formations and environmental conditions. Despite their potential for dispersal, blue tits only disperse over short distances, typically less than 1 km from their natal territory [18,30,49–51]. This restricted dispersion reduces gene flow between nearby blue tit populations and enhances genetic differentiation [8,19,30], which can lead to local adaptation [15,18,19]. Nonetheless, studies reporting a genetic structure among blue tit populations usually compared distant geographical populations or habitat patches in a mosaic landscape (1–28 km). The exceptions examining these processes in a continuous space in birds are the studies by Garant *et al.* [17] and Garroway *et al.* [52] that documented local adaptation and spatial genetic structure in the great tit (*Parus major*). Here, we show that limited dispersal can lead to genetic differentiation in a blue tit population inside a continuous woodland formed by different forest formations and without exhibiting any geographical barrier. Moreover, we found evidence of local adaptation in clutch size, since there was a divergence in this trait exceeding the level of divergence expected according to genetic drift alone.

2. Material and methods

2.1. Study area and sampling

Fieldwork was carried out during 2017, 2018 and 2019 in a woodland located at 1700–1800 m in the Sierra Nevada National Park (southeast Spain, 36°57′ N, 3°24′ W). This woodland is located along a valley. The east-facing slope of the valley is composed of two contiguous forest formations of Holm oaks (*Quercus ilex*) and Pyrenean oaks (*Q. pyrenaica*), while the west-facing slope is composed of Scots pines (*Pinus sylvestris*) and Pyrenean oaks (figure 1). Consequently, the two slopes differed in their environment (electronic supplementary material, appendix S1). The Pyrenean oak forests from the east-facing and west-facing slopes are henceforth referred to as dry and humid Pyrenean oak forests (figure 1).

Blue tits bred in nest boxes that were checked regularly to determine breeding parameters [53]. Birds were sexed and banded with aluminium rings for further identification. Birds were weighed to the nearest 0.1 g and their tarsus length was measured to the nearest 0.01 mm. We collected blood samples that were preserved in 1.5 ml tubes in absolute ethanol and stored at −20°C. In total, from 2017 to 2019, we placed 180 nest boxes on the east-facing slope (Holm oak forest: 40; dry Pyrenean oak forest: 140) and 210 nest boxes on the west-facing slope (Scots pine forest: 70; humid Pyrenean oak forest: 140). The occupation rate by blue tits was 62.5% in the Holm oak forest, 75.8% in the dry Pyrenean oak forest, 33.8% in the Scots pine forest and 74.4% in the humid Pyrenean oak forest. Capture–mark–recapture data from 2016 to 2019 were used to examine natal dispersal from their natal to their respective first-breeding nest box between the four forest formations and the two slopes of the valley. The number of ringed nestlings per nest box in each forest was: 214 in the Holm oak forest; 565 in the dry Pyrenean oak forest; 137 in the Scots pine forest; and 615 in the humid Pyrenean oak forest [53].

2.2. Microsatellite genotyping

We genotyped 171 blue tits (east-facing slope: 78, west-facing slope: 93). Ten microlitres of blood per blue tit was used for DNA extraction using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions except for the proteinase K digestion time, which was increased to 1 h, and the final elution volume, which was reduced to 50 μl. Twenty-seven polymorphic microsatellite markers were tested. Microsatellite genotyping is described in electronic supplementary material, appendix S2. According to microsatellite marker screening, three loci were discarded (electronic supplementary material, appendix S2), the remaining 24 loci being neutral to selection and used for subsequent genetic analyses.

Multi-locus genotypes were used to estimate the genetic diversity of the blue tit populations of the two slopes of the valley (i.e. the east-facing and the west-facing) and for the overall population. Estimates included the number of alleles (K), and the observed and expected heterozygosity (H_O and H_E, respectively), calculated in ARLEQUIN 3.5 [54]. Genetic diversity estimates (K, H_O, H_E) were also calculated for all loci, including those that were not finally used in subsequent genetic analyses (electronic supplementary material, appendix S2).

We analysed patterns of spatial genetic structure using the model-based Bayesian Markov chain–Monte Carlo clustering method implemented in the software STRUCTURE 2.3.4 [55–57], which assigns individuals to inferred populations based on their multi-locus genotypes. We ran STRUCTURE assuming correlated allele frequencies, admixture and sampling locations (the two slopes of the valley) as priors. We conducted five independent runs for each value of cluster K (1–10), with 1 000 000 Markov chain–Monte Carlo (MCMC) iterations followed by a burn-in of 100 000 steps. The optimal number of genetic clusters was estimated using the log probabilities [$\Pr(X|K)$] and Evanno's ΔK method [58], implemented in STRUCTURE HARVESTER [59]. Optimal alignments of replicates of the same K and graphical representation of selected clusters were performed using the software CLUMPAK [60]. Since patterns of genetic structure and gene flow in the blue tit may be affected by male-biased philopatry [18,30], analyses of spatial genetic structure were performed both for all sampled individuals pooled together and considering male and female genotypes separately.

The inbreeding and outbreeding coefficients for each population were defined previously through the results of genetic population structuring conducted in STRUCTURE 2.3.4 [55–57]. The extent of geographical structuring of the genetic variation between individuals from the two slopes of the valley was evaluated based on F_{ST} statistics, using the analysis of molecular variance (AMOVA) [61],
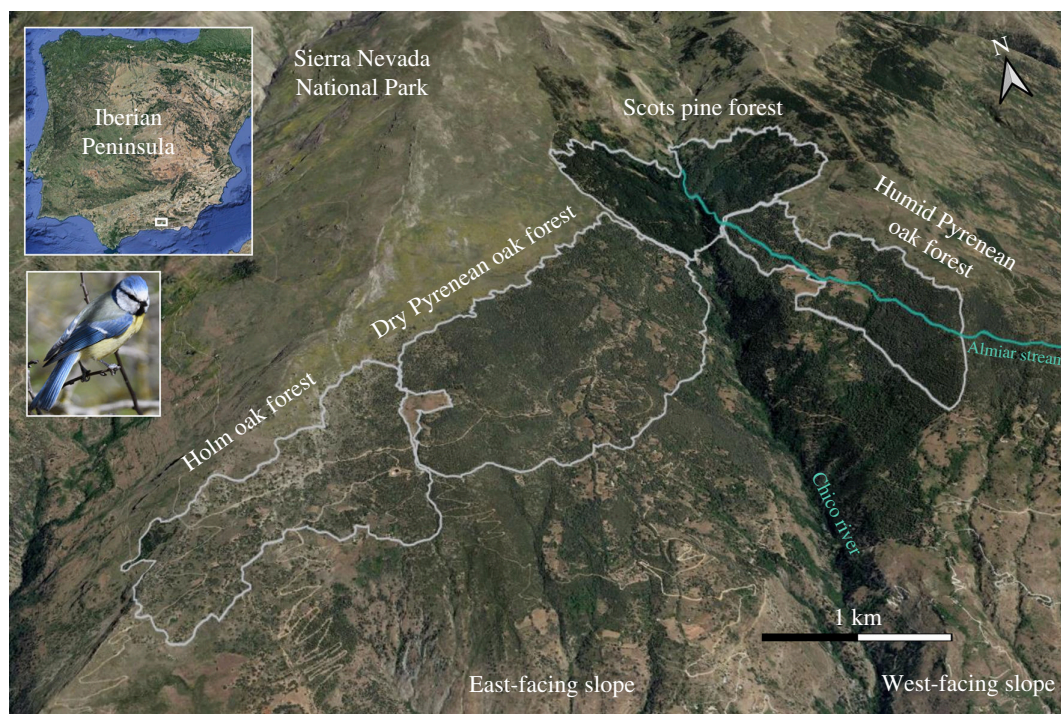
Figure 1. Map of the study area showing the valley where blue tits (*C. caeruleus*) reproduced. Note the difference in forest density and the presence of a stream crossing the west-facing slope. Map created using the software QGIS 3.10.5 connected to Google Earth.

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implemented in ARLEQUIN 3.5 [54]. The significance of variance components and F-statistics (F_{ST}, F_{IS} and F_{IT}) were assessed by 10 000 permutations. As above, F-statistics were also calculated considering male and female genotypes separately.

2.3. Phenotypic population differentiation

Previous research found that females adjusted their clutch size to the slope of the valley where they bred [53]. Here, we used the F_{ST}–Q_{ST} (P_{ST}) approach to examine the relative importance of genetic drift and natural selection in explaining the variation in such life-history (clutch size) and morphological (body mass and tarsus length) traits between the two slopes of the valley. F_{ST} estimates the extent of population genetic differentiation, while Q_{ST} estimates the differentiation of quantitative genetic traits [62]. However, Q_{ST} uses purely additive genetic variance, and its estimation requires rearing individuals from different populations in a common environment [33], which was unfeasible for this study. For this reason, we used P_{ST}, analogous to Q_{ST}, to quantify the between populations variance in quantitative phenotypic traits [39,63]. We calculated the phenotypic differentiation (P_{ST}) for each quantitative trait: clutch size, body mass and tarsus length (electronic supplementary material, appendix S3).

The F_{ST}–P_{ST} comparisons allow the detection of local adaptation by testing the relationship between neutral and quantitative genetic variation among populations. There are three possible outcomes from the F_{ST}–P_{ST} comparisons [64,65]. If P_{ST} is higher than F_{ST}, then the degree of divergence in phenotypic quantitative traits exceeds that achievable by genetic drift alone (measured through neutral markers), and consequently, natural selection favours different phenotypes in every different population (diversifying selection). If P_{ST} equals F_{ST}, then the relative effects of natural selection and genetic drift are indistinguishable, hence natural selection is not implied in population differentiation. Lastly, if P_{ST} is lower than F_{ST}, then natural selection is favouring the same phenotype in different populations, so phenotypic differentiation is less than expected on the basis of neutral divergence [65]; see electronic supplementary material, appendix S3, for more details.

3. Results

3.1. Natal dispersal

We recaptured 11 blue tits that were ringed as nestlings, seven individuals at the east-facing slope of the valley and four at the west-facing slope. Only one blue tit (1 out of 11; 9.09%) dispersed between slopes, being born in the west-facing slope (humid Pyrenean oak forest) and reproducing for the first time in the east-facing slope (dry Pyrenean oak forest).

3.2. Genetic population differentiation and structure

Between 2 and 40 alleles were detected per locus (mean = 14), with a mean H_O of 0.58 (range = 0.05–0.87) and a mean H_E of 0.73 (range = 0.45–0.95) (electronic supplementary material, table S1). The mean H_O of the populations of the east-facing and west-facing slopes were 0.58 and 0.54, respectively, and their mean H_E were 0.73 and 0.70, respectively.

The geographical structuring inferred from the microsatellite markers between the populations of the two slopes of the valley revealed that most of the variation lies within individuals (table 1). The fixation index F_{ST} among populations indicated a significant genetic divergence between both slopes (F_{ST} = 0.016, $p < 0.001$, 1.57% of the variance; table 1). In accordance, the F_{IS} estimate significantly differed from zero (average F-statistic over all loci, F_{IS} = 0.220, $p < 0.001$, 18.94% of the variance; table 1), suggesting outbreeding within both genetic populations. Also, F_{IS} estimates of each genetic population were statistically significantly different from zero (east-facing slope = 0.176, west-facing slope = 0.207; in both cases, $p < 0.001$). Lastly, the F_{IT} estimate indicated that most of the variation lies within individuals (average F-statistic over all loci, F_{IT} = 0.231, $p < 0.001$, 79.5% of the variance; table 1). When considering male and female genotypes separately, the same pattern emerged (F_{ST} for males = 0.018, $p < 0.001$; F_{ST} for females = 0.015, $p < 0.001$; F_{IS} for males = 0.203, $p < 0.001$; F_{IS} for females = 0.188, $p < 0.001$; F_{IT} for males = 0.218, $p < 0.001$; F_{IT} for females = 0.200, $p < 0.001$; table 1).

Structure analyses considering all individuals revealed a maximum ΔK and $Pr(X|K)$ for K = 2, indicating the presence of two genetic clusters across the woodland (figure 2a). Genetic differentiation occurred mainly between the populations of the east-facing and west-facing slopes of the valley (figure 2a). Analyses considering male and female genotypes separately also revealed a maximum ΔK and $Pr(X|K)$ for K = 2 (figure 2b,c, respectively), with a similar pattern of genetic structure to that observed in the analysis considering all the sampled individuals. Overall, genetic population differentiation and structure analyses indicated the presence of subtle genetic differentiation within the woodland.

3.3. Phenotypic population differentiation

When considering the null assumption where $g/h^2 = 1$ (i.e. when the proportion of variance between populations due to additive genetic effects equals the proportion of variance due to genetic effects within populations), only the P_{ST} estimate for clutch size (0.052) was higher than the global pairwise F_{ST} (body mass: 0.013, tarsus length: 0.005). Hence, comparisons of quantitative trait differentiation (P_{ST}) with its expectation under neutrality (F_{ST}) revealed evidence of divergent selection for clutch size ($P_{ST} > F_{ST}$). The P_{ST} values for clutch size were higher than the global F_{ST} when altering the assumptions about heritability and the values of additive genetic proportion. On the other hand, the P_{ST} values for tarsus length never exceeded the global F_{ST}, while the P_{ST} values for body mass were higher than the global F_{ST} only at medium to high g values ($g > 0.4$) when heritability was relatively low (electronic supplementary material, appendix S3). Therefore, the results from F_{ST}–P_{ST} comparisons suggest that clutch size was under diversifying selection.

4. Discussion

Our findings reveal that, despite having a high dispersal capacity, blue tits in our study sites showed genetic and phenotypic differentiation within a continuous population. This pattern was mainly driven by non-random dispersal—as suggested by both the genetic and the capture–mark–recapture data—associated with environmental heterogeneity within the woodland. Previous studies already showed that capture–mark–recapture data provide accurate information on recent levels of gene flow

Table 1. Hierarchical AMOVAs for the two genetic blue tit (*C. caeruleus*) populations analysed by microsatellite marker data, considering all sampled individuals and male and female genotypes separately.

source of variation	degrees of freedom	sum of squares	variance components	percentage of variation
all individuals				
among populations	1	29.426	0.120	1.57
among individuals within populations	169	1522.840	1.454	18.94
within individuals	171	1043.500	6.102	79.49
total	341	2595.766	7.677	100
male genotypes				
among populations	1	20.814	0.148	1.88
among individuals within populations	76	705.257	1.566	19.92
within individuals	78	479.500	6.147	78.20
total	155	1205.571	7.862	100
female genotypes				
among populations	1	19.161	0.112	1.45
among individuals within populations	91	817.812	1.424	18.55
within individuals	93	571.00	6.139	80.00
total	185	1407.973	7.675	100

among bird populations [16,18,30,66–68]; but see [69]. Widespread highly mobile species are likely to experience several environmental conditions and geographical barriers, and thus, they are expected to experience genetic population structure at large geographical scales [70–73], often following an isolation-by-distance pattern (e.g. [3,72,74]). However, genetic structure can be observed at small spatial scales depending on the landscape composition and environmental conditions [8], which can restrict dispersal and gene flow among populations when organisms match habitat selection to natal conditions [75]. Hence, our results show that genetic structure (and probably local adaptation) can occur even among smaller population patches than previously thought (but see [17]), since we showed subtle genetic structuring associated with environmental heterogeneity even within the same woodland and for a species with a potential high dispersal capacity.

The blue tits inhabiting the continuous woodland displayed a limited dispersal, with all individuals, except for one, reproducing within their natal slope of the valley. The strong philopatry of blue tits can explain the genetic differentiation among populations on the two slopes of the valley covered by the woodland, although we acknowledge that the reduced recapture rate precludes us from examining accurate gene flow estimates and other dispersal dynamics. Blue tits often breed with related partners and females show less promiscuity with genetically distant males ([49]; see also [76]), contributing to the genetic population structure and potentially leading to local adaptation [77]. Our results were similar to other studies that examined patterns of genetic structure between neighbouring and fragmented populations of tits (Paridae) (table 2) and other passerine birds [27,32,88]. However, we found a level of genetic differentiation higher than expected given the extremely short distance that separates the two slopes of the valley (see table 2 for geographical and genetic distances in other blue tit populations), and the fact that the two slopes of the valley are connected through a pine forest formation and, thus, form a continuum (figure 1).

Despite the statistically significant genetic differentiation of the two subpopulations, blue tits still showed some degree of outbreeding within both slopes of the valley, suggesting the existence of some gene flow, an expected finding if one considers the short geographical distance that separates the two blue tit subpopulations [30]. This scenario, *a priori*, could prevent genetic and phenotypic divergence as gene flow might introduce maladaptive alleles into the locally adapted populations [10,11]. Nonetheless, divergent selection can maintain local adaptation and adaptive divergence
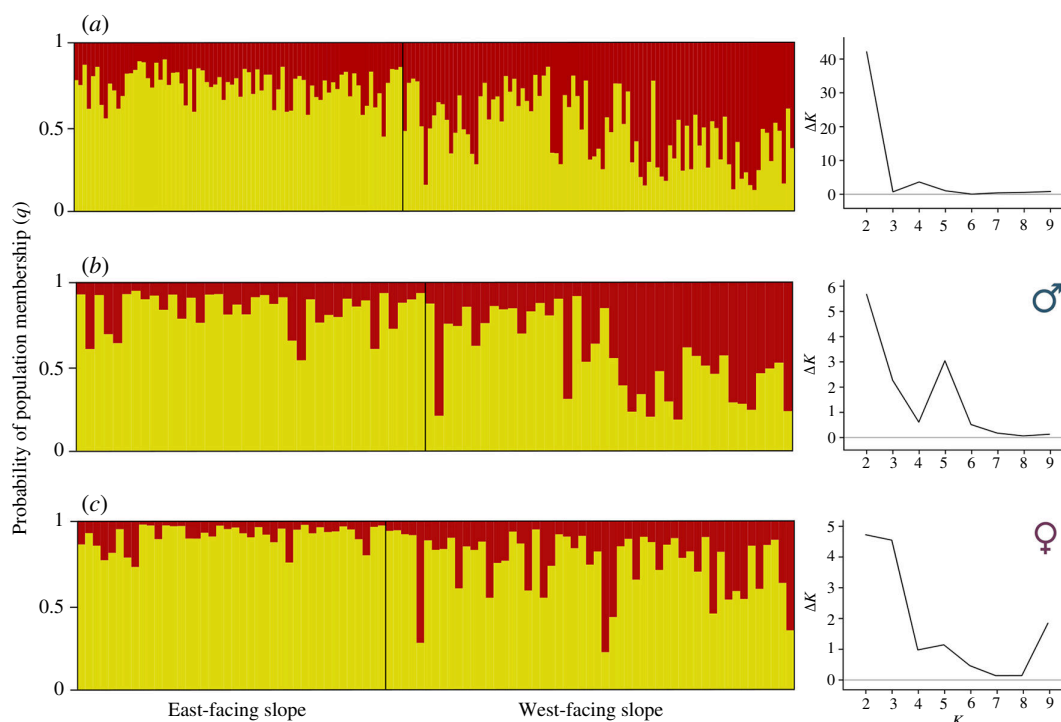
Figure 2. Genetic assignment based on the Bayesian clustering analysis implemented in STRUCTURE software, considering (a) all individuals, (b) males only and (c) females only. Each individual is represented by a vertical line, which is partitioned into two coloured segments each representing the individual's probability of pertaining to one of the two populations (q) that the genetic clustering gave. Right: Bayesian clustering shows the most probable number of genetic clusters according to ΔK values.

despite the homogenizing effects of gene flow [89–91], especially when environmental heterogeneity generates spatially contrasting selection pressures [6,43]. This would occur if local populations retain high genetic diversity [20,21]. The blue tits inhabiting the continuous woodland studied here exhibited moderate to high levels of genetic diversity (heterozygosity) both at population and loci levels (see §3 and electronic supplementary material, appendix S2), and gene flow seems to maintain such genetic diversity in each slope of the valley. Several studies have revealed significant associations between individual genetic quality and several fitness-related traits in blue tits, such as the probability of infection by parasites [92], carotenoid-based feather coloration [78,93], egg quality [93], clutch size [94] and the probability of local recruitment [82,94]. Therefore, it seems that reproductive success is not constrained by potential genetic inbreeding within each subpopulation, while the phenotypic divergence between both subpopulations still seems to be maintained by diversifying selection.

We found some evidence that blue tits were locally adapted to the conditions of the slope they inhabit, as clutch size was differentiated between both slopes of the valley to a greater extent than expected if we consider genetic drift alone (i.e. $P_{ST} > F_{ST}$), suggesting that selection favours different clutch sizes in the two slopes of the valley. These findings are congruent with a previous study in which we found that females adjust their clutch size to the rearing conditions in the slope where they bred, thus maintaining the breeding success constant throughout the woodland [53]. Concretely, females from the east-facing slope lay on average one egg less than females from the humid Pyrenean oak forest on the west-facing slope [53]. The heritability of clutch size is high in the blue tit [18] and moderately high in other passerine species [95,96], indicating a substantial additive genetic variance for this life-history trait that, hence, provides considerable options and responses for selection to act on. However, studies reporting phenotypic differentiation in avian traits, including the clutch size, exceeding the level of genetic neutral divergence rely on large scales across the species breeding ranges ([42–47]; but see [18]). In fact, few studies have found evidence of local adaptation in bird populations at such small scales (table 2). Garant et al. [17] showed that non-random gene flow and divergent selection generate genetic differentiation in great tit nestling body mass in a continuous woodland. Similarly, Garroway et al. [52] found evidence of fine-scale genetic structure associated with malaria infection risk and local conspecific density. Postma and van Noordwijk [14] and Postma et al. [85,97] showed that non-random gene flow is the main cause for the genetic differences in the clutch size

Table 2. Summary of studies reporting the geographical and the genetic distance among natural populations of tits (Paridae), namely, blue tits (*C. caeruleus*), great tits (*P. major*) and black-capped chickadees (*P. atricapillus*). The table summarizes the studies examining genetic structure among populations at medium to low geographical distances (continental large-scale studies are excluded).

species	geographical distance (km)[a,b]	genetic distance (F_{ST} values)[a,c]	habitat types	genetic marker	reference
blue tit (*C. caeruleus*)	0 km	**0.016**	continuous woodland	microsatellites	This study
	<2 km	0.006	mosaic of forest patches	microsatellites	Ferrer *et al.* [78]
	1–28 km	0.000–0.017	mosaic of forest patches	microsatellites	Ferrer *et al.* [8]
	2–90 km	0.000–**0.010**	mosaic of forest patches	minisatellites	Verheyen *et al.* [79]
	5–440 km	**0.008–0.087**	mosaic of forest patches	SNPs	Perrier *et al.* [73]
	5–440 km	0.009–**0.054**	mosaic of forest patches	SNPs	Szulkin *et al.* [80]
	5–493 km	0.001–0.049	mosaic of forest patches	microsatellites	Porlier *et al.* [19]
	5–9 km	**0.028–0.063**[d]	urban parks and forest patch	microsatellites	Senar & Björklund [81]
	7 km	**0.005**	mosaic of forest patches	microsatellites	Ferrer *et al.* [82]
	7 km	**0.033**	mosaic of forest patches	microsatellites	García-Navas *et al.* [18]
	10–50 km	0.002–0.004	urban parks and forest patches	microsatellites	Markowski *et al.* [83]
	20 km	**0.003**	mosaic of forest patches	microsatellites	Ortego *et al.* [30]
	25 km	−0.003 and **0.012**[e]	mosaic of forest patches	microsatellites	Blondel *et al.* [84]
	25 km	**0.004**	mosaic of forest patches	SNPs	Dubuc-Messier *et al.* [33]
	Not available	**0.007–0.021**	mosaic of forest patches	microsatellites	Blondel *et al.* [22]
great tit (*P. major*)	500 m–3 km	0.000–**0.190**	urban parks and forest patch	microsatellites	Björklund *et al.* [28]
	5–150 km	0.003–**0.011**	mosaic of forest patches	microsatellites	Postma *et al.* [85]
	7 km	0.006	mosaic of forest patches	microsatellites	García-Navas *et al.* [86]
	10–50 km	0.003–**0.010**	urban parks and forest patches	microsatellites	Markowski *et al.* [83]
	500 km	**0.010**	mosaic of forest patches	SNPs	van Bers *et al.* [87]

(*Continued.*)

Table 2. (Continued.)

species	geographical distance (km)[a,b]	genetic distance (F_{ST} values)[a,c]	habitat types	genetic marker	reference
black-capped chickadee (*P. atricapillus*)	26–1500 km	**0.009**–0.316	not reported	microsatellites	Adams *et al.* [71]

[a]When more than two populations were compared, the table shows a range of values (minimum to maximum geographical distance, and minimum to maximum F_{ST} values).

[b]When geographical distances among populations were not reported in the original study, Euclidean distances were estimated based on coordinates and map information.

[c]Statistically significant pairwise F_{ST} values are marked in bold.

[d]The study reported G_{ST} values instead of F_{ST} values.

[e]The study reported the F_{ST} values in two separate years.

between two island great tit populations only 1.3 km apart. Regarding blue tits, García-Navas *et al.* [18] found that the divergence in clutch size and morphological traits exceeded the neutral genetic differentiation in two blue tit populations separated by 7 km. Charmantier *et al.* [98] and Blondel *et al.* [22] showed evidence of local adaptation for several traits occurring in blue tit populations inhabiting forest patches separated by a few kilometres. Finally, Camacho *et al.* [31] documented a genetic and phenotypic divergence for tarsus length at a short spatial distance (1.1 km) in two pied flycatcher (*Ficedula hypoleuca*) populations. Our findings and the aforementioned studies reveal that phenotypic divergence can occur over surprisingly small spatial scales even for birds, with high potential for dispersal. However, to our knowledge, only the present study and those by Garant *et al.* [17] and Garroway *et al.* [52] provide evidence for such differentiation in phenotypic traits (clutch size, body mass and resistance to *Plasmodium* parasites, respectively) over continuous systems. Still, the results derived from the P_{ST}–F_{ST} comparison should be interpreted with caution since the analyses were run using microsatellites, genetic markers which have typically high mutation rates and hence can bias the F_{ST} estimate [48]. Other processes apart from divergent selection, such as habitat match selection to natal conditions [75], may operate to produce the observed clutch size differentiation. Additional experiments would be needed to test these hypotheses.

Environmental heterogeneity within the woodland, together with limited dispersal, seems to be behind the evolutionary process disentangled in this study. The study area's topography results in a microscale geographic heterogeneity similar to the evolution canyon models described in [99,100]. In these systems, the opposite and closely neighbouring slopes of a valley display marked climatic and biotic contrasts which determine the ecological and evolutionary processes developing on each slope. The east-facing slope of the woodland studied here receives more solar radiation, has a higher temperature and lower humidity, less tree cover, lower nest infestation by nest-dwelling ectoparasites and a lower caterpillar abundance during the spring compared with the west-facing slope ([53,101,102]; electronic supplementary material, appendix S1). In evolution canyon models, the microclimatic inter-slope differences determine the level of gene flow among slopes and produce adaptive divergence [103,104]. Although the microclimatic and biotic contrasts from our study area are not as divergent as those from evolution canyons described by Nevo [99] in Israel—in which the two slopes unfold xeric, savannoid and shade, forested ecosystems—they seem to be enough to allow a genetic and phenotypic divergence within the blue tit population, suggesting that small variation in evolutionary canyons is sufficient to generate divergent evolution. A similar example is found in populations of citril finches (*Carduelis* (before *Serinus*) *citrinella*) in the Pyrenees, where birds from the north-facing slope are genetically and morphologically distinct from birds from the south-facing slope, which is drier and sunnier and has a lower abundance of pines [35,88]. In our study system, the inter-slope variation in clutch size [53] and the selection favouring such different clutch sizes between the two slopes of the valley suggest that blue tits face distinct selective pressures in each slope during their reproductive period. An important factor constraining all stages of avian reproduction is food availability [105]. Caterpillars are the main food source for blue tits during the spring [106–108], and we found that their abundance is lower in the east-facing slope [101,102]. Thus, food availability could be one of the most probable factors of selection underlying the phenotypic divergence observed between the

two blue tit subpopulations and may explain why the clutch size is lower in the east-facing slope than in the west-facing slope of the valley.

5. Conclusions

We found genetic and phenotypic divergence among subpopulations of blue tits at surprisingly small scales (inside a continuous woodland). The topography and microclimatic geographic heterogeneity of our study area (a Mediterranean woodland located in a valley with two slopes) create different selective pressures when blue tits reproduce. The east-facing slope of the valley has a drier environment, with less tree cover and food availability, while the west-facing slope of the same valley exhibits a more humid environment with more tree cover and diversity, as well as higher caterpillar abundance. Consequently, blue tits adjust their reproductive effort to the slope where they breed by showing different clutch sizes [53]. Here, we provide evidence that an adaptive process seems to underlie such between-slope phenotypic variation. Concretely, blue tits from the opposite slopes are subtly genetically differentiated into two subpopulations, and selection (or other processes promoting divergence) seems to favour different clutch sizes between these subpopulations. This evolutionary scenario may seem surprising given that all the forestry formations of the woodland that cover the studied valley are connected, thus forming a continuum of habitats, and given that blue tits have a high dispersal potential.

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