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The role of historical biogeography in shaping colour morph diversity in the common wall lizard

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Abstract

The maintenance of polymorphisms often depends on multiple selective forces, but less is known on the role of stochastic or historical processes in maintaining variation. The common wall lizard (*Podarcis muralis*) is a colour polymorphic species in which local colour morph frequencies are thought to be modulated by natural and sexual selection. Here, we used genome-wide single-nucleotide polymorphism data to investigate the relationships between morph composition and population biogeography at a regional scale, by comparing morph composition with patterns of genetic variation of 54 populations sampled across the Pyrenees. We found that genetic divergence was explained by geographic distance but not by environmental features. Differences in morph composition were associated with genetic and environmental differentiation, as well as differences in sex ratio. Thus, variation in colour morph frequencies could have arisen via historical events and/or differences in the permeability to gene flow, possibly shaped by the complex topography and environment. In agreement with this hypothesis, colour morph diversity was positively correlated with genetic diversity and rates of gene flow and inversely correlated with the likelihood of the occurrence of bottlenecks. Concurrently, we did not find conclusive evidence for selection in the two colour loci. As an illustration of these effects, we observed that populations with higher proportions of the rarer yellow and yellow-orange morphs had higher genetic diversity. Our results suggest that processes involving a decay in overall genetic diversity, such as reduced gene flow and/or bottleneck events have an important role in shaping population-specific morph composition via non-selective processes.

KEYWORDS
biogeography, colour polymorphism, gene flow, genetic diversity, *Podarcis muralis*, selection

Pedro Andrade and Catarina Pinho shared joint senior authorship.

1 | INTRODUCTION

The maintenance of colour polymorphisms, that is, the coexistence of different genetically-determined colour phenotypes in sympatry, can be explained by several evolutionary processes (Ford, 1945; Gray & McKinnon, 2007). Evidence suggests that several forms of balancing selection can drive the coexistence of multiple colour morphs over time (e.g. Gillespie & Oxford, 1998; Kim et al., 2019). Colour morphs may be maintained through negative frequency-dependent selection, where the least common morph is favoured (Gray & McKinnon, 2007; Roulin, 2004; Wellenreuther et al., 2014). Colour morphs can also be maintained when individuals that are heterozygous at colour loci attain greater fitness (i.e. heterosis; Gray & McKinnon, 2007; Roulin, 2004; Roulin & Bize, 2007; Wellenreuther et al., 2014). Under such a scenario, colour disassortative mating can facilitate the persistence of multiple colour morphs (Gray & McKinnon, 2007; Wellenreuther et al., 2014; but see Jiang et al., 2013).

Contrarily, if colour morphs are not affected by any form of balancing selection they may be lost or become fixed in a population due to drift (Gray & McKinnon, 2007; Roulin, 2004). Moreover, high levels of assortative mating, mediated by non-random mate choice and/or spatial segregation can also result in the loss of polymorphism (Gray & McKinnon, 2007; Maan et al., 2006). Previous studies have suggested that the loss of a colour morph can promote accelerated speciation (i.e. morphic speciation; Corl et al., 2010). In turn, some studies have found greater rates of speciation associated with colour polymorphic species, including birds (Hugall & Stuart-Fox, 2012) and lacertids lizards (Brock et al., 2021). Even when colour morphs are not lost, colour morph frequencies are expected to change over time due to a multitude of evolutionary forces, such as balancing selection, gene flow, drift, and stochastic events (reviewed in McLean & Stuart-Fox, 2014). Many studies have focused on explaining why different morphs occur and how they persist in natural populations, but it is equally important to understand the different factors that can lead to specific morphs becoming rare or even disappearing altogether, in this way shaping the evolutionary trajectory of a polymorphic system.

Lizards have repeatedly evolved colour polymorphisms (Stuart-Fox et al., 2020), making them excellent candidates to test how different evolutionary processes interact to explain morph diversity. The common wall lizard (*Podarcis muralis*) has been extensively studied to understand the factors that promote the evolution and maintenance of colour polymorphisms. In this species, five ventral colour morphs have been described: white, orange, and yellow pure morphs, and white-orange and yellow-orange mosaic morphs (Pérez i de Lanuza et al., 2013). Colours involved in this polymorphism are discriminated by lizards (Pérez i de Lanuza, Abalos, et al., 2018), and colour variation is largely explained by two loci (*SPR* controls orange pigmentation and *BCO2* controls yellow pigmentation; Andrade et al., 2019). Both natural and sexual selection have been invoked to explain the maintenance of this colour polymorphism. On one hand, temperature seasonality and precipitation (i.e. environmental-dependent selection) as well as microhabitat preferences have been shown to modulate local colour morph frequencies (Pérez i de Lanuza & Carretero, 2018; Pérez i de Lanuza, Sillero, et al., 2018). On the other hand, colour morph richness (i.e. number of morphs) positively correlates with male-biased sex ratios (Pérez i de Lanuza et al., 2017), suggesting that the intensity of sexual selection could promote the maintenance of the polymorphism. In addition, high frequencies of positive assortative pairing by colour morph have been recorded in this species (Pérez i de Lanuza et al., 2013, 2016), although this effect is likely not strong enough to result in within-population genetic divergence by colour morph (Aguilar, Andrade, Afonso, et al., 2022). Other studies have, however, suggested mixed evidence for the role of sexual selection in maintaining the polymorphism (Abalos et al., 2016, 2020). In general, these studies have looked at how population-level processes affect the evolutionary dynamics of this polymorphic system. However, morph variation (and genetic diversity as a whole) is highly contingent on processes that act at larger spatial and temporal scales (Aavik et al., 2020; Ferreira et al., 2023; Salvi et al., 2013). For example, in the tawny dragon lizard (*Ctenophorus decresii*) differences in colour morph frequencies between populations can be associated with divergence driven by Pleistocene glacial refugia (McLean et al., 2014). Understanding the factors that are responsible for between-population variation in morph frequencies is thus important to paint a complete picture of the evolutionary phenomena that impact polymorphic systems.

In this study, we generated a large dataset of genome-wide markers to investigate patterns of genetic divergence and diversity in populations of *P. muralis* from the Pyrenees, a region where both biotic (e.g. sex ratio) and abiotic factors (environmental features and microhabitat composition) have been implicated in shaping colour morph composition (Pérez i de Lanuza et al., 2016, 2017; Pérez i de Lanuza & Carretero, 2018; Pérez i de Lanuza, Sillero, et al., 2018). With this information, we investigated how levels and patterns of genetic variation correlate with colour morph composition across populations to understand if, and how, the evolutionary history of these populations may play a role in shaping morph evolution at a regional scale, in particular looking at the roles of gene flow, recent bottlenecks and selection.

2 | MATERIALS AND METHODS

2.1 | Fieldwork and data collection

Podarcis muralis lizards were collected from 55 populations (= localities) in the central and eastern Pyrenees from 2018 to 2020 (Figure 1; Table S1). From each population, we aimed to sample at least 50 adult individuals (snout-to-vent length > 55 mm) to obtain accurate estimates of ventral colour morph frequencies (Pérez i de Lanuza et al., 2017). We also collected a small tissue sample (i.e. tail tip) from each individual for genetic analyses, and stored them in ethanol.

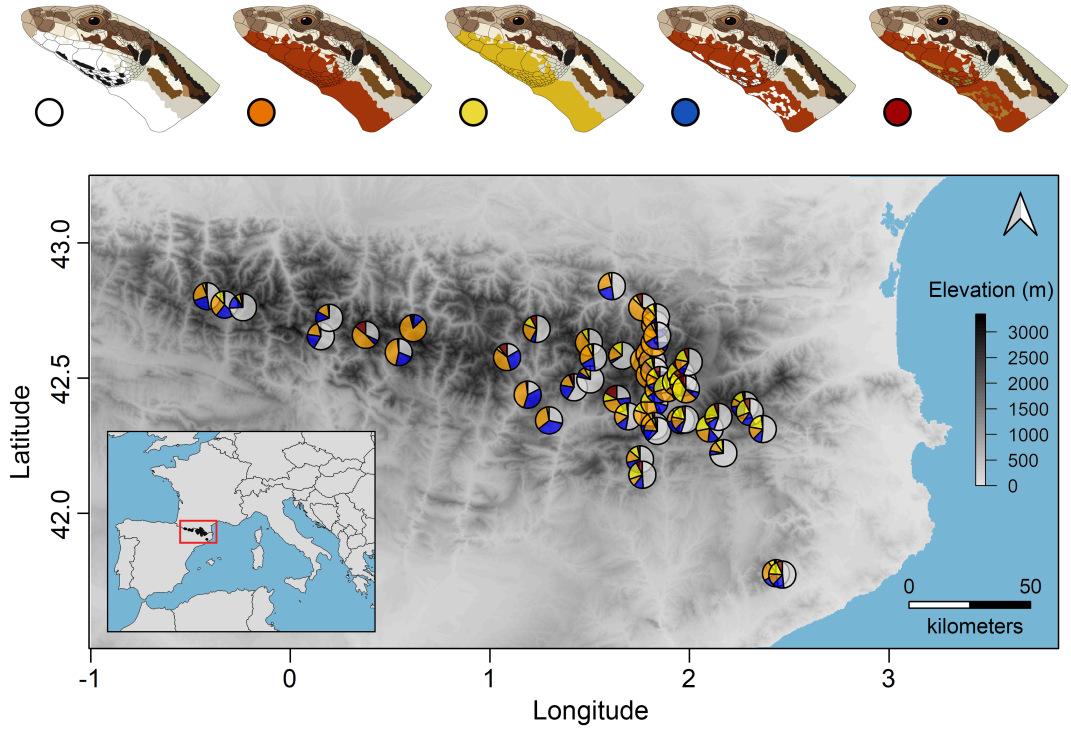

FIGURE 1 Distribution of the sampled populations across the Pyrenees along with colour morph frequencies at each of them.

2.2 | Double-digest RAD sequencing

For one of the populations, namely Angostrina, samples were already sequenced for a previous study (Aguilar, Andrade, Afonso, et al., 2022) and thus, we randomly selected 10 individuals from this sample. For the other 54 populations, we randomly selected 10 tails per population to extract DNA using a commercial column-based extraction kit (EasySpin; Citomed). Then, following Peterson et al. (2012), we constructed double-digest restriction site-associated DNA (ddRAD) sequencing libraries. First, we double-digested 0.15 μg of genomic DNA using MspI and EcoRI-HF restriction enzymes (New England Biolabs; Ipswich). Then, we dual-indexed each sample individually and pooled them, prior to size selection (~300–700 bp) using AMPure beads (Beckman; USA). We enriched the final library via 12 PCR cycles using a Q5 High-Fidelity DNA Polymerase (New England Biolabs; Ipswich). The final library was paired-end sequenced in four lanes of an Illumina NovaSeq 6000 instrument (Novogene; China) to a length of 150 bp.

2.3 | SNP catalogue and filtering

We began by evaluating the quality of raw reads in FastQC version 0.11.8 (Andrews, 2018). Subsequently, we removed reads with an average Phred score lower than 20, uncalled bases and degenerated cut sites and/or barcodes and demultiplexed reads by individual, using Stacks version 2.62 (Catchen et al., 2013). We aligned the demultiplexed reads to the *P. muralis* genome (Andrade et al., 2019) using BWA-MEM version 0.7.17 (Li & Durbin, 2009). Then, we built a SNP catalogue and removed positions with heterozygosity greater than 0.7 to limit the impact of paralogous and/or repetitive loci (Paris et al., 2017; Rochette & Catchen, 2017) using Stacks (Catchen et al., 2013). Using VCFtools version 0.1.16 (Danecek et al., 2011) we applied the following filtering steps: (i) minimum coverage of 5 and minimum mean coverage of 15 per locus; (ii) minimum allele frequency of 0.05; (iii) missing data per individual below 90%, 70% and 50%; (iv) non-missing data per locus above 50%, 60%, 70% and 95%. Filtering steps (iii) and (iv) were interspersed to reduce data loss (O'Leary et al., 2018). To further exclude paralogous and tightly linked SNPs (e.g. Aguilar, Andrade, Afonso, et al., 2022; Caeiro-Dias et al., 2021), we identified and removed RADtags with more than eight SNPs, and then, selected one random SNP in each of the RADtags exhibiting eight or less SNPs, using a custom R script (R Core Team, 2022). The final SNP catalogue was built by specifying the list of SNPs to be kept using VCFtools (Danecek et al., 2011). We also excluded SNPs showing a $r^2 > .2$ to account for linkage disequilibrium using PLINK version 1.9 (Purcell et al., 2007). Finally, we removed populations for which less than 5 samples were retained and repeated the SNP calling and filtering steps without those populations (i.e. one population, Fontviva, was removed and all the analyses detailed below were conducted on the remaining 54 populations).

2.4 | Population genetics

We inspected the population structure following two main approaches. First, we carried out a principal component analysis (PCA) using PLINK (Purcell et al., 2007). We retained the first three

principal components (PC) and tested whether populations differed based on their PC scores using a MANOVA; we also tested the effect of latitude, longitude and their interaction on the PC scores via multivariate regression. Second, we ran ADMIXTURE version 1.3.0 (Alexander et al., 2009) with 20 replicates for $K = 1$–55 (number of populations + 1) and calculated 20-fold cross-validation error to estimate the most suitable value of K. To further characterize these populations, we calculated two complementary measures of genetic diversity, namely, pi (π) and Wu and Watterson's theta (θ_w) and tested whether populations differed in these summary statistics using a Kruskal-Wallis test. We calculated π as described in Clark et al. (2022), whereas θ_w was calculated using the 'popgenstuff' R package.

2.5 | Genetic variation and the physical environment

To evaluate the role of the physical environment in determining overall patterns of genetic variation, we assessed whether genetic differences between populations were associated with geographic distances (isolation by distance, IBD) and/or environmental differences (isolation by environment, IBE). To do so, we first obtained a matrix of genetic differentiation values (fixation index, F_{ST}) between populations using the 'StAMPP' R package version 1.6.3 (Pembleton et al., 2013). Then, we obtained a topographic distance matrix using the 'topoDistance' R package version 1.0.1, which calculates topographic least cost paths using horizontal and vertical distances as input, thus providing more reliable distances (Wang, 2020). For each of the populations, we recorded the 19 bioclimatic variables from the Worldclim 2.0 dataset (www.worldclim.org) at 30 arcsec resolution (~1 km^2); this set of variables describes the mean values and seasonality of temperature and precipitation as well as its extremes (Fick & Hijmans, 2017). We then summarized these variables via PCA, which resulted in three principal components following the Kaiser-Guttman criterion (Guttman, 1954). Using these PC, which explained 91.98% of the total variance (see Table S2), we calculated pairwise Euclidean distances between populations to generate a matrix of environmental differences. To formally test whether genetic differentiation could be driven by topographic distance and/or environmental features (and their contribution), we fitted a multiple matrix regression with randomization (MMRR; Wang, 2013) with the F_{ST} matrix as the dependent variable, and topographic and environmental matrices as independent variables.

2.6 | Co-variation between genetic background and colour morph composition

To describe the colour morph composition, we explored how colour morph frequencies varied across populations by testing whether each morph showed differences in variance through a Levene test. Then, we calculated Shannon, richness and evenness indices as described in Pérez i de Lanuza et al. (2017). Using these data, we evaluated which features can shape morph composition in the Pyrenees by fitting MMRRs with morph diversity indices as dependent variables and the following variables as predictors: (i) F_{ST}, to evaluate the possible role of historical divergence (e.g. Pérez i de Lanuza et al., 2019) or gene flow; (ii) topographic distance; (iii) environmental distance, as climate has been shown to impact local morph composition in the Pyrenees (Pérez i de Lanuza, Sillero, et al., 2018); and (iv) sex ratio, calculated as $\frac{\text{no males} - \text{no females}}{\frac{N}{2}}$. We fitted MMRRs for all individuals together and for males and females separately.

In addition, we tested for correlations between genetic diversity (π and θ_w) and morph composition at the population level. To formally test these assumptions, we calculated Spearman's correlations between morph composition indices (i.e. Shannon diversity index, evenness, and richness) and genetic diversity indices (π and θ_w). Further, we calculated Spearman's correlations between the proportion of each colour morph and the genetic diversity indices. The aforementioned analyses were carried out considering all individuals of a population together, and separately by sex. We accounted for multiple testing by correcting p-values following Benjamini and Hochberg (1995).

Finally, we tried to assess whether patterns of genetic diversity could be associated with differences in gene flow and recent bottlenecks. To do so, we first calculated the levels of gene flow across the study area using the estimated effective migration surface (EEMS) software (Petkova et al., 2016). We ran EEMS on the dissimilarity matrix calculated using the bed2diffs module, with 5 million MCMC iterations, 1 million burn-in iterations, and a deme density of 750. MCMC chain convergence was evaluated through the output from the 'rEEMSplots' R package version 0.0.1 (Petkova et al., 2016). Then, we extracted the estimates of gene flow at each of the populations and tested whether they were correlated with colour morph composition indices. To evaluate if populations suffered recent important reductions in genetic diversity, we used the software BOTTLENECK version 1.2.02 (Cornuet & Luikart, 1996) under the IAM model, using 100 SNPs randomly selected from our dataset. Then, we tested if populations in which a bottleneck event was detected ($p < .05$) differed from those in which it was not ($p > .05$) in their morph composition indices through a Kruskal–Wallis test. We accounted for multiple testing by correcting p-values following Benjamini and Hochberg (1995).

2.7 | Selection on colour morphs

To test whether colour loci were under selection, we first calculated F_{ST} between populations for our panel of SNPs to be used as a 'null distribution' (e.g. Curlis et al., 2021; Runemark et al., 2010), using the R package 'hierfstat' version 0.11.5 (Goudet, 2005). Then, guided by the available information on the genetic basis of colouration in *P. muralis*—the alleles that explain the deposition

of orange and yellow pigments are recessive towards the non-coloured alleles at the *SPR* and *BCO2* loci, and orange is epistatic over yellow (Aguilar, Andrade, & Pérez i de Lanuza, 2022; Andrade et al., 2019)—we estimated the allelic frequencies of the orange and yellow loci. We did this employing the colour morph frequencies recorded in the field (see Table S1), assuming these two loci were in Hardy–Weinberg equilibrium (e.g. Liu et al., 2022). We first estimated the allelic frequencies at the orange locus, where the frequency of the recessive allele ('*q*', hereafter, '*o*' for the orange locus) was estimated based on the proportions of the morphs that express the orange colouration:

$$'o^2' = \text{prop. orange} + \text{prop. white} - \text{orange} + \text{prop. yellow} - \text{orange};$$

thus, '*o*' = $\sqrt{'o^2'}$ and '*O*' = 1 – '*o*'.

For the yellow locus, however, we cannot calculate the allelic frequencies as above, because orange colouration is epistatic over yellow (Aguilar, Andrade, & Pérez i de Lanuza, 2022; Andrade et al., 2019). Thus, a portion of the orange morph individuals would also carry one or two copies of the recessive allele at the yellow locus. For this reason, we calculated the frequency of the yellow recessive allele ('*q*', i.e. '*y*' for the yellow locus) considering the morphs that show the yellow colouration plus a portion of the orange individuals based on the ratio of the homozygous recessive genotype at the yellow locus ('yy-ratio'). This ratio was calculated for each population by employing all morphs but the orange (for which the presence of yellow colouration is not evident) as:

$$'\text{yy} - \text{ratio}' = \frac{\text{prop. yellow} + \text{prop. yellow} - \text{orange}}{\text{prop. white} + \text{prop. yellow} + \text{prop. white} - \text{orange} + \text{prop. yellow} - \text{orange}}$$

Finally, the proportion of the recessive yellow allele ('y') was calculated as:

$$'y^2' = \text{prop. yellow} + \text{prop. yellow} - \text{orange} + (\text{prop. orange} \times '\text{yy} - \text{ratio}');$$

thus, '*y*' = $\sqrt{'y^2'}$ and '*Y*' = 1 – '*y*'.

As the 'yy-ratio' might be dependent on the phenotyping (i.e. whether to classify an individual as orange or as yellow-orange) we repeated the simulations increasing or decreasing the value of this ratio by 0.05, 0.1, 0.15 and 0.2 (when this ratio resulted in negative values they were substituted by 0). However, our results remained qualitatively unchanged after such modifications to this parameter.

Then, using the estimated allelic frequencies, we simulated colour loci information for as many individuals per population as those that passed the filtering steps for our ddRAD-seq dataset (i.e. 486 individuals belonging to 54 populations). We replicated the simulation of the genotypes of the 486 individuals 999 times, which allowed us to obtain an average F_{ST} value and the corresponding standard deviation for each colour locus. Finally, we tested whether these loci were under selection by comparing random F_{ST} values from the 'null' distribution with the mean F_{ST} estimated for the colour loci, running 999 permutations, allowing us to obtain a *p*-value. We would expect that loci under diversifying selection would show greater differentiation than neutral markers, whereas the opposite scenario would be expected for loci under balancing selection (Cox & Davis Rabosky, 2013). Finally, as low frequency variants can be skewed towards lower F_{ST} values, we tested the correlation between minor allele frequency (MAF) and F_{ST} for the SNP panel. Due to the positive significant correlation between these two variables ($r_S = .422$, $p < .001$), we repeated this analysis by comparing the simulated colour loci to putatively neutral SNPs of similar allelic frequencies (i.e. dividing the SNP panel by the minor allele frequency in bins of 0.1).

3 | RESULTS

3.1 | Population genetics of *Podarcis muralis* in the Pyrenees

Our variant call identified 14,896 SNPs for 486 individuals from the 54 populations (9 ± 1.1 samples/population, ranging from 6 to 10 samples). The coverage for this panel was approximately 23.4*X* ± 11.1 per individual and 23.5*X* ± 3.1 per locus. We began our genetic analyses by testing for the existence of population structure with a PCA. The first three principal components of the PCA explained 62.8% of the total variance in the dataset and suggested some degree of genetic structure (Figure S1). Genetic differentiation was significant when we compared the 54 populations for these three components (MANOVA: Wilk's lambda = .002; $p < .001$). The interaction between latitude and longitude correlated with the three components (Multivariate regression: Pillai's trace = .127; $p < .001$), suggesting that geography plays an important role in defining population structure of *P. muralis* in the Pyrenees. These results were corroborated by the ADMIXTURE analysis. Variability in the ancestry coefficients seemed to be, at least in part, explained by both latitude and longitude, throughout several values of K (Figure 2a–c). For this analysis, the best-supported number of ancestral populations corresponded to $K = 9$ (see Figure S2 for the cross-validation errors), which suggests a strong substructure in this region. This is reflected in the high values of average genetic differentiation between populations ($F_{ST} = 0.154 \pm 0.095$). Overall genetic diversity differed between populations both for π (Kruskal–Wallis: $X^2 = 387.68$, $p < .001$, mean ± SD = 0.182 ± 0.041) and θ_w (Kruskal–Wallis: $X^2 = 382.22$, $p < .001$, mean ± SD = 0.185 ± 0.043).

To understand if the physical environment is associated with these patterns of genetic variation, we assessed if there was any relationship between overall genetic differentiation and different measures of environmental variation. In line with the population structure results, the MMRR indicated that genetic divergence between populations was influenced by topographic distance ($\beta = .492$, $p = .001$; Figure 2d). However, environmental differences per se

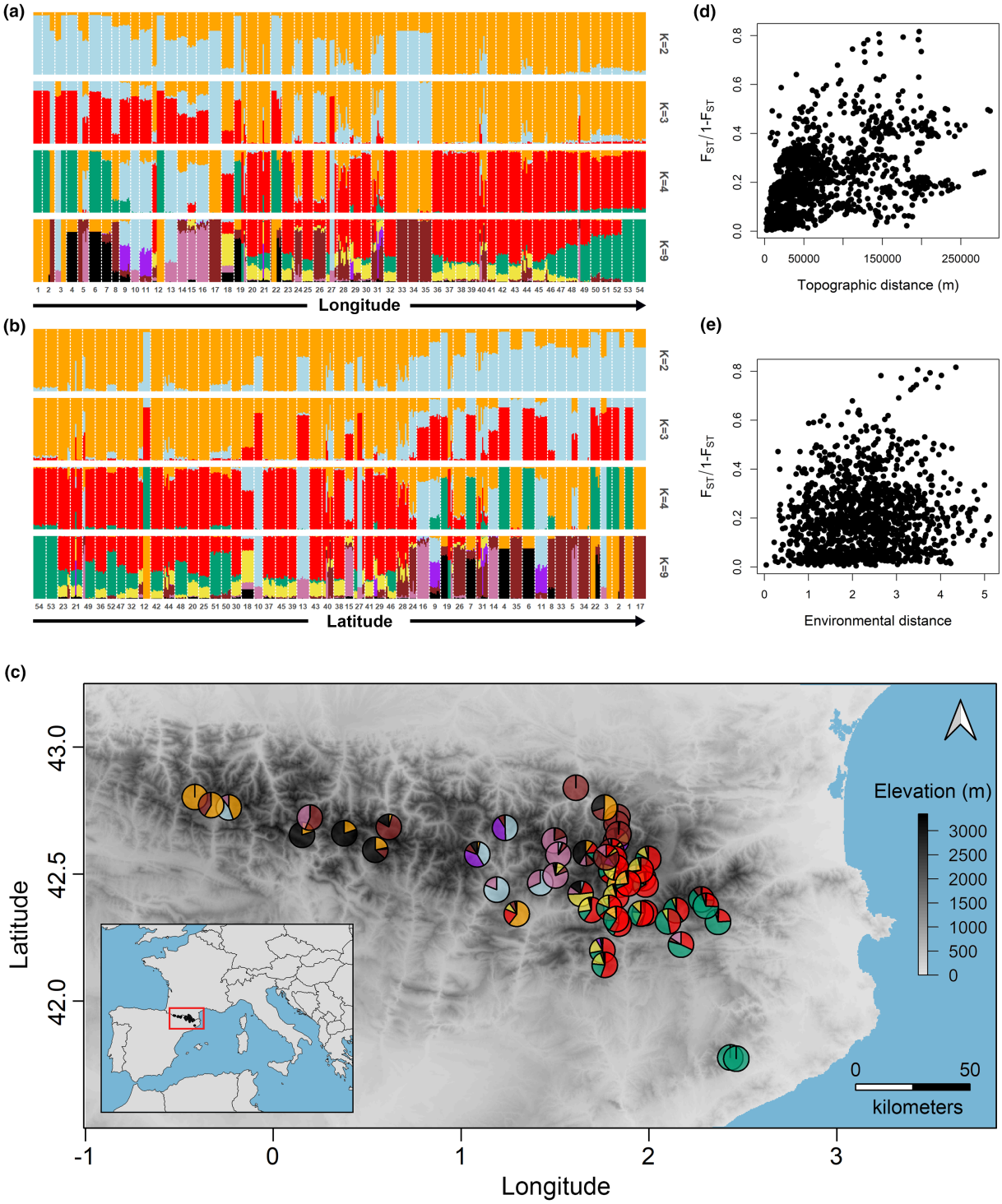

FIGURE 2 Genetic differentiation between Pyrenean populations of *Podarcis muralis* based on a panel of 14,896 SNPs. ADMIXTURE results for $K=2$–4 and 9 (most likely value of K), panels were ordered by longitude (a) and latitude (b). Different populations in the plots are separated by dashed vertical white lines and identified by a population number (for correspondence, see Table S1). (c) Projection of the most likely value of K ($K=9$) in the map; colours in each population indicate the average assignment to a cluster across samples. (d) Relationship between genetic distances, estimated as pairwise F_{ST} and topographic distances. (e) Relationship between genetic distances, estimated as pairwise F_{ST} and environmental distances.

did not seem to explain genetic differences ($\beta = -.009$, $p = .879$; Figure 2e).

3.2 | Colour morph composition across populations is associated with patterns of genetic variation

To understand how colour morph diversity is partitioned at a large geographical scale, we started by calculating the relative frequency of wall lizard morphs across the 54 sampled populations distributed across the Pyrenees. Similar to previous studies (Pérez i de Lanuza et al., 2017), we found that morph composition differs across populations (Levene test; $F = 16.419$, $p < .001$). For example, the frequency of the most common morph overall (white) varied between 1.33% and 78.43%, whereas the least common morph (yellow-orange) varied between 0% and 17.19%. The largest range was observed for the orange morph, which varied between 1.85% and 82.67% of the individuals across populations.

These differences in morph composition were significantly associated with genetic divergence (Table 1). Specifically, differences in morph composition estimated as distances of the Shannon diversity index were associated with genetic and environmental differences, but only for males. Similarly, the number of morphs (i.e. richness) was explained by genetic differences for the whole sample, whereas for males, differences in colour morph richness were associated with genetic differentiation and differences in sex ratios. Last, evenness estimates in males were also associated with environmental differences (Table 1).

We also found that levels of genetic diversity within populations correlated with indices of morph diversity and composition. Specifically, populations with higher genetic diversity showed greater colour morph diversity (Table 2). Levels of genetic diversity also tended to vary depending on the proportion of the different morphs. For example, for the overall sample and males, there was a negative correlation between the frequency of orange individuals and genetic diversity (Table 3). In a similar fashion, the proportion of white-orange females negatively correlated with genetic diversity. Whereas for the yellow and yellow-orange morphs, we found a positive correlation with genetic diversity.

The correlation between lower genetic diversity and lower morph diversity could be explained by historical colonization events of this highly heterogeneous landscape, if these play a role in determining the availability of alleles at the two causal loci that control for the polymorphism (Andrade et al., 2019). To test this, we estimated patterns of gene flow and the probability of the occurrence of recent bottlenecks for the 54 populations. Results from EEMS suggested that the study region is characterized by a complex suite of corridors and barriers to gene flow (Figure 3). When we look specifically at effective migration rate estimates for each population, we observe that populations in which gene flow was lower also tended to show less diversity in colour morph composition (Table 4). Similarly, populations in which we found evidence for bottleneck events ($p < .05$ under the IAM model) also showed lower values of richness and evenness (i.e. lower colour morph diversity), but this association was only significant for males (Table 5).

TABLE 1 MMRR results on the association between the predictors and indices of morph composition in *Podarcis muralis* populations.

		Predictors			
	Morph composition	F_{ST}	Environ.	Geography	Sex ratio
Overall	Shannon	$\beta = .004$	$\beta = -.097$	$\beta = .177$	$\beta = -.094$
		$p = .969$	$p = .184$	$p = .072$	$p = .242$
Males	Shannon	$\beta = \mathbf{.255}$	$\beta = \mathbf{-.166}$	$\beta = .049$	$\beta = .091$
		$p = \mathbf{.008}$	$p = \mathbf{.024}$	$p = .608$	$p = .216$
Females	Shannon	$\beta = -.132$	$\beta = -.014$	$\beta = .144$	$\beta = -.094$
		$p = .142$	$p = .843$	$p = .099$	$p = .200$
Overall	Richness	$\beta = \mathbf{.292}$	$\beta = -.129$	$\beta = .027$	$\beta = -.014$
		$p = \mathbf{.002}$	$p = .060$	$p = .764$	$p = .875$
Males	Richness	$\beta = \mathbf{.273}$	$\beta = -.073$	$\beta = .087$	$\beta = \mathbf{.187}$
		$p = \mathbf{.003}$	$p = .246$	$p = .267$	$p = \mathbf{.016}$
Females	Richness	$\beta = .070$	$\beta = -.129$	$\beta = .093$	$\beta = .033$
		$p = .416$	$p = .067$	$p = .308$	$p = .744$
Overall	Evenness	$\beta = -.076$	$\beta = -.053$	$\beta = .181$	$\beta = -.107$
		$p = .482$	$p = .508$	$p = .105$	$p = .216$
Males	Evenness	$\beta = .193$	$\beta = \mathbf{-.165}$	$\beta = .072$	$\beta = .090$
		$p = .074$	$p = \mathbf{.044}$	$p = .538$	$p = .312$
Females	Evenness	$\beta = -.120$	$\beta = .083$	$\beta = .131$	$\beta = -.072$
		$p = .241$	$p = .260$	$p = .187$	$p = .396$

Note: β and p-values were highlighted in bold when p-values were significant ($p < .05$).

TABLE 2 Results of Spearman's correlations between genetic diversity and morph composition indices of *Podarcis muralis* populations.

	Genetic diversity	Morph composition indices		
		Shannon	Richness	Evenness
Overall	π	r_S=**.484**	r_S=**.443**	r_S=**.310**
		p<**.001**	p<**.001**	p=**.023**
		p_{adj}<**.001**	p_{adj}=**.002**	p_{adj}=**.032**
Males	π	r_S=**.661**	r_S=**.600**	r_S=**.373**
		p<**.001**	p<**.001**	p=**.006**
		p_{adj}<**.001**	p_{adj}<**.001**	p_{adj}=**.011**
Females	π	r_S=**.335**	r_S=**.356**	r_S=.136
		p=**.014**	p=**.008**	p=.325
		p_{adj}=**.022**	p_{adj}=**.015**	p_{adj}=.344
Overall	θ_w	r_S=**.383**	r_S=**.400**	r_S=.220
		p=**.005**	p=**.003**	p=.110
		p_{adj}=**.010**	p_{adj}=**.007**	p_{adj}=.132
Males	θ_w	r_S=**.583**	r_S=**.544**	r_S=**.309**
		p<**.001**	p<**.001**	p=**.023**
		p_{adj}<**.001**	p_{adj}<**.001**	p_{adj}=**.032**
Females	θ_w	r_S=.216	r_S=**.295**	r_S=.013
		p=.117	p=**.031**	p=.925
		p_{adj}=.132	p_{adj}=**.039**	p_{adj}=.925

Note: r_S and p-values were highlighted in bold when p-values were significant after adjusting for multiple testing (p_{adj}<.05).

3.3 | Selection on colour morphs

To complement our population-level approach, we also checked for evidence of balancing selection. Allelic frequencies estimated from phenotypic frequencies were equal to 0.662±0.131 for the '*o*' allele (orange) and 0.426±0.146 for the '*y*' allele (yellow). Both colour loci showed lower mean F_{ST} values than the average of neutral SNPs (0.136±0.08), but differentiation was not significantly higher or lower than the panel of putatively neutral loci (orange locus: F_{ST}=0.075±0.019, p=.213; yellow locus: F_{ST}=0.085±0.021, p=.289; Figure 4a,b), arguing against an effect of selection. Since F_{ST} values were correlated with the minor allele frequency for the corresponding loci, we repeated this comparison looking at the respective classes of minor allele frequency (0.3–0.4 for the orange locus and 0.4–0.5 for the yellow locus; Figure 4c), but again none of the colour loci showed significantly lower F_{ST} (orange: p=.069; yellow: p=.077). However, it is important to note that both loci sit within the lower 10th percentile of the empirical distribution of values, in their respective allele frequency class. Therefore, these results should be interpreted with caution since this may argue against a strictly neutral scenario.

4 | DISCUSSION

Although the factors that promote the rise and persistence of colour polymorphisms in natural populations have been extensively studied in several taxa, the factors that explain how these co-occurring

TABLE 3 Results of Spearman's correlations between genetic diversity and the proportion of each colour morph (W=white, O=orange, Y=yellow, WO=white-orange and YO=yellow-orange).

	Nucleotide diversity	Morph diversity indices				
		W	*O*	*Y*	WO	YO
Overall	π	r_S=.047	r_S=**−.389**	r_S=**.626**	r_S=−.196	r_S=**.413**
		p=.735	p=**.004**	p<**.001**	p=.155	p=**.002**
		p_{adj}=.787	p_{adj}=**.010**	p_{adj}<**.001**	p_{adj}=.208	p_{adj}=**.006**
Males	π	r_S=−.022	r_S=**−.413**	r_S=**.586**	r_S=.253	r_S=**.465**
		p=.874	p=**.002**	p<**.001**	p=.065	p<**.001**
		p_{adj}=.904	p_{adj}=**.006**	p_{adj}<**.001**	p_{adj}=.093	p_{adj}=**.002**
Females	π	r_S=.124	r_S=−.287	r_S=**.526**	r_S=**−.311**	r_S=.258
		p=.370	p=.035	p<**.001**	p=**.022**	p=.060
		p_{adj}=.427	p_{adj}=.062	p_{adj}<**.001**	p_{adj}=**.041**	p_{adj}=.091
Overall	θ_w	r_S=.087	r_S=**−.367**	r_S=**.565**	r_S=−.259	r_S=**.340**
		p=.531	p=**.006**	p<**.001**	p=.058	p=**.012**
		p_{adj}=.590	p_{adj}=**.015**	p_{adj}<**.001**	p_{adj}=.091	p_{adj}=**.024**
Males	θ_w	r_S=.010	r_S=**−.376**	r_S=**.577**	r_S=.194	r_S=**.415**
		p=.944	p=**.005**	p<**.001**	p=.159	p=**.002**
		p_{adj}=.944	p_{adj}=**.013**	p_{adj}<**.001**	p_{adj}=.208	p_{adj}=**.006**
Females	θ_w	r_S=.184	r_S=−.257	r_S=**.421**	r_S=**−.353**	r_S=.133
		p=.182	p=.061	p=**.002**	p=**.009**	p=.339
		p_{adj}=.228	p_{adj}=.091	p_{adj}=**.006**	p_{adj}=**.019**	p_{adj}=.407

Note: r_S and p-values were highlighted in bold when p-values were significant after adjusting for multiple testing (p_{adj}<.05).

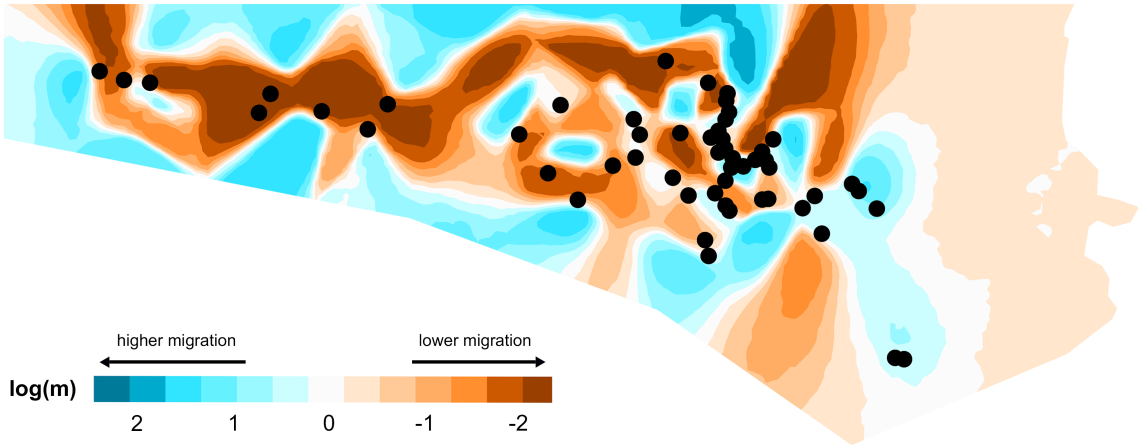

FIGURE 3 Estimated effective migration surface (EEMS) in the Pyrenees. Levels of gene flow above and below average are indicated in blue and brown respectively. Black dots correspond to sampled populations.

TABLE 4 Results of Spearman's correlations between migration estimates and morph composition indices for the overall sample, as well as for males and females separately.

		Morph composition indices		
	Migration	Shannon	Richness	Evenness
Overall	Log(m)	r_S = **.361**	r_S = **.329**	r_S = .225
		p = **.007**	p = **.015**	p = .101
		p_{adj} = **.030**	p_{adj} = **.034**	p_{adj} = .114
Males	Log(m)	r_S = **.428**	r_S = **.348**	r_S = **.295**
		p = **.001**	p = **.010**	p = **.031**
		p_{adj} = **.013**	p_{adj} = **.030**	p_{adj} = **.046**
Females	Log(m)	r_S = **.314**	r_S = **.286**	r_S = .184
		p = **.021**	p = **.036**	p = .183
		p_{adj} = **.038**	p_{adj} = **.046**	p_{adj} = .183

Note: r_S and p-values were highlighted in bold when p-values were significant after adjusting for multiple testing ($p_{adj} < .05$).

TABLE 5 Results of Kruskal–Wallis tests to infer the effect of recent bottlenecks in *Podarcis muralis* morph composition.

		Morph composition indices		
	Bottleneck	Shannon	Richness	Evenness
Overall	IAM	X^2 = 3.752	X^2 = 2.203	X^2 = 1.611
		p = .053	p = .138	p = .204
		p_{adj} = .158	p_{adj} = .248	p_{adj} = .263
		df = 1	df = 1	df = 1
Males	IAM	X^2 = **11.306**	X^2 = 2.950	X^2 = **15.711**
		p < **.001**	p = .086	p < **.001**
		p_{adj} = **.003**	p_{adj} = .193	p_{adj} < **.001**
		df = **1**	df = 1	df = **1**
Females	IAM	X^2 = 0.508	X^2 = 1.835	X^2 = 0.607
		p = .476	p = .176	p = .436
		p_{adj} = .476	p_{adj} = .263	p_{adj} = .476
		df = 1	df = 1	df = 1

Note: X^2 and p-values were highlighted in bold when p-values were significant after adjusting for multiple testing ($p_{adj} < .05$).

phenotypes vary across space and time between populations are still poorly understood. This is particularly true for *Podarcis muralis*, for which it has been particularly difficult to isolate the factors that control the evolutionary dynamics of ventral colour polymorphism (Abalos et al., 2020; Calsbeek, Hasselquist, et al., 2010; Pérez i de Lanuza, Abalos, et al., 2018; Pérez i de Lanuza et al., 2013; Pérez i de Lanuza, Sillero, et al., 2018; Sacchi et al., 2017). Previous studies suggest that a complex set of biological and environmental factors are likely to interact in leading to the prevalence of the different morphs across populations. Our findings build on this complex scenario, by showing how large-scale patterns of population divergence and colonization correlate with the presence of different morphs in distinct populations.

The Pyrenean populations of *P. muralis* show a pattern of relatively high genetic structure, and this differentiation is mostly associated with topographic distances between populations. Although colour morph frequencies often vary geographically (e.g. McLean & Stuart-Fox, 2014), differences in colour morph composition in *P. muralis* populations were associated with their genetic background rather than topographic distances. There are several processes that could promote genetic and phenotypic (i.e. colour morphs) differences concomitantly. First, historical divergence, which is associated with differences in colour morph composition between different lineages of this species (see figure S1 from Andrade et al., 2019) as well as in other polymorphic lizards (e.g. Corl et al., 2010; McLean et al., 2014). Second, the rise of barriers and corridors of gene flow between populations, which may also interact with stochastic and historical events, could foster the association between genetic and phenotypic differences (Eckert et al., 2008; McLean & Stuart-Fox, 2014). For instance, populations of the barn owl (*Tyto alba*) from the British Isles show remarkably high frequencies of the white morph as a result of a founder effect and subsequently reduced gene flow (Machado et al., 2022). Estimates of gene

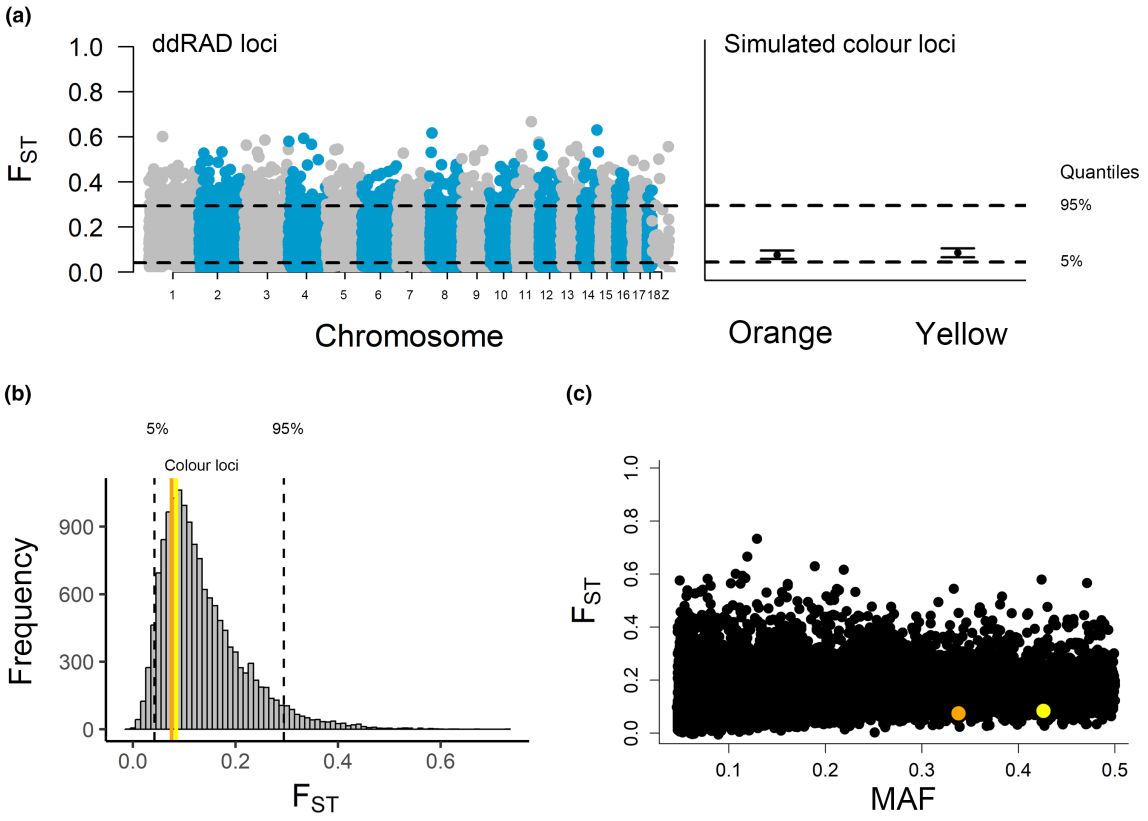

FIGURE 4 Test for selection on the simulated colour loci. (a) Values of genetic differentiation (fixation index, F_{ST}) for the panel of neutral SNPs, ordered by chromosome and position (on the left), and mean and standard deviation for the simulated F_{ST} values for the orange and yellow loci (on the right). (b) Position of the simulated colour loci F_{ST} values when compared to the F_{ST} values of the SNP panel. Quantiles (5% and 95%) are represented as dashed lines, and orange and yellow colour loci are indicated as orange and yellow vertical lines. (c) Correlation between F_{ST} and minor allele frequency (MAF) for the SNP panel used as the 'null distribution' to test for selection in the simulated orange and yellow colour loci (represented in orange and yellow, respectively).

flow in our study area support the latter scenario, showing a rather complex combination of high and low levels of gene flow, perhaps facilitated by the complex orography of the Pyrenees. This association between genetic divergence and differences in morph composition was not detected when considering only females. This result could be explained because *P. muralis* males are probably under stronger intrasexual selection (Pérez i de Lanuza et al., 2017), the main source of sexual selection acting on territorial lizards (Olsson & Madsen, 1995; Sullivan & Kwiatkowski, 2007; Tokarz, 1995). The relevance of male intra-sexual selection on colour morph evolution was suggested because male local morph diversity is increased in male-biased populations in which high levels of male competition are expected (Pérez i de Lanuza et al., 2017). Interestingly, the present study, which involves an independent and larger sample, confirms this pattern. Similarly, we found an association between the environment and colour morph composition as previously suggested by Pérez i de Lanuza, Abalos, et al. (2018) and Pérez i de Lanuza, Sillero, et al., (2018).

Genetic diversity was consistently associated with morph composition and diversity indices, specifically in the sense that more genetically diverse populations also had higher colour morph diversity. We speculate that processes involving a decay in overall genetic diversity, namely, historical bottlenecks or founder effects (particularly associated with reduced connectivity in the heterogenous terrain of the Pyrenees) are likely to lead to the reduction of the frequency of specific colour morphs (Calsbeek, Bonvini, et al., 2010; McLean et al., 2014). However, it should be noted that, since the alleles that are associated with orange and yellow pigmentation have a recessive mode of inheritance, it is possible that some populations still retain this variation at very low levels. Furthermore, the presence of the yellow carotenoid-based colouration can also be masked by orange pterins in the skin (Aguilar, Andrade, & Pérez i de Lanuza, 2022; Andrade et al., 2019), which could lead to underestimating the frequency of the 'y' allele. Supporting this, all populations included at least a few yellow and/or yellow-orange individuals, thus evidencing the persistence of the 'y' allele, even if at low frequencies. In any case, we observed that in populations in which the proportion of yellow and yellow-orange individuals was greater, so was the genetic diversity (overall sample and in males). On the contrary, greater frequencies of orange (overall and in males) and white-orange (females) were associated with lower genetic diversity. Such sex-specific trends for the orange colouration are likely derived from differences in the expression of orange colouration between sexes, since the white-orange mosaic colouration is more frequent in females than in

males (Pérez i de Lanuza et al., 2013). Thus, results from this study suggest that colour morph frequencies might serve as a rough proxy of genetic diversity. Nonetheless, whether these results can be extrapolated to the whole distribution of *P. muralis* or to other species should be addressed in future studies. For instance, it would be interesting to test if these findings could be replicated in other lineages, especially in those in which orange colouration is less frequent than yellow (Sacchi et al., 2007; also see figure S1 from Andrade et al., 2019).

In tandem with evidence for a role of historical processes in shaping colour variation across populations, we found no significant differentiation in either the orange or the yellow colour loci when compared to the genome-wide average; however, both loci had extremely low values of F_{ST}, which suggests that selection should not be completely ruled out. Therefore, these results also support an important role for genetic drift in generating differences in colour morph frequencies between populations. Our results are in line with those obtained by Runemark et al. (2010) in *Podarcis gaigeae*, in which they estimated the F_{ST} of a putative codominant triallelic locus responsible for the colour polymorphism in this species. They found that the F_{ST} of this putative locus did not significantly differ from the null F_{ST} distribution estimated from microsatellite data (Runemark et al., 2010). Another plausible scenario would be that colour morphs in *P. muralis* are maintained due to the elevated population densities this species shows in the Pyrenees (e.g. Aguilar, Andrade, Afonso, et al., 2022). Nonetheless, available evidence suggests that colour morph frequencies and the forces operating on them can greatly differ both within- (Pérez i de Lanuza et al., 2019; Sacchi et al., 2007) and between-species in the genus *Podarcis* (e.g. Brock et al., 2022), thus, highlighting the need to obtain a more comprehensive view of how colour polymorphisms are maintained in lacertids.

AUTHOR CONTRIBUTIONS

P. Aguilar, G.P.L., M.C., P. Andrade and C.P. contributed to conceptualization; P. Aguilar and G.P.L. contributed to data curation; P. Aguilar contributed to formal analysis; P. Aguilar contributed to investigation; G.P.L., M.C., P. Andrade and C.P. contributed to resources; G.P.L., M.C., P. Andrade and C.P. contributed to supervision; P. Aguilar contributed to visualization; P. Aguilar contributed to writing—original draft; P. Aguilar, G.P.L., M.C., P. Andrade and C.P. contributed to writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors do not have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Demultiplexed short-read RAD-sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) (Accession number: PRJNA1083934). Scripts and data required to carry out the following analyses have been deposited on Dryad (https://doi.org/10.5061/dryad.4xgxd25j0) and Zenodo (https://doi.org/10.5281/zenodo.10810050): (1) MMRR; (2) correlations between genetic diversity and morph composition; (3) possible influence of migration and recent bottleneck events on genetic diversity; and (4) test of selection on the colour loci.

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