

The role of environment and core–margin effects on range-wide phenotypic variation in a montane grasshopper

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Abstract

The integration of genetic information with ecological and phenotypic data constitutes an effective approach to gain insight into the mechanisms determining interpopulation variability and the evolutionary processes underlying local adaptation and incipient speciation. Here, we use the Pyrenean Morales grasshopper (*Chorthippus saulcyi moralesi*) as study system to (i) analyse the relative role of genetic drift and selection in range-wide patterns of phenotypic differentiation and (ii) identify the potential selective agents (environment, elevation) responsible for variation. We also test the hypothesis that (iii) the development of dispersal-related traits is associated with different parameters related to population persistence/turnover, including habitat suitability stability over the last 120 000 years, distance to the species distribution core and population genetic variability. Our results indicate that selection shaped phenotypic differentiation across all the studied morphological traits (body size, forewing length and shape). Subsequent analyses revealed that among-population differentiation in forewing length was significantly explained by a temperature gradient, suggesting an adaptive response to thermoregulation or flight performance under contrasting temperature regimes. We found support for our hypothesis predicting a positive association between the distance to the species distribution core and the development of dispersal-related morphology, which suggests an increased dispersal capability in populations located at range edges that, in turn, exhibit lower levels of genetic variability. Overall, our results indicate that range-wide patterns of phenotypic variation are partially explained by adaptation in response to local environmental conditions and differences in habitat persistence between core and peripheral populations.

Introduction

Disentangling the relative contribution of natural selection and random genetic drift on phenotypic diversity is of great importance for understanding the mechanisms shaping intraspecific variation and incipient speciation processes (Leinonen *et al.*, 2006, 2008; Oneal & Knowles, 2013; Bertrand *et al.*, 2016). Phenotypic

divergence can arise as a consequence of purely stochastic processes such as bottlenecks or founder effects, which can lead to genetic drift in genomic regions involved in trait expression (Lande, 1976; Zhan *et al.*, 2005). However, numerous studies have found local adaptation as the main evolutionary force responsible for phenotypic differentiation in natural populations (Kekkonen *et al.*, 2012; Oneal & Knowles, 2013; Ortego *et al.*, 2015b; see reviews in Merilä & Crnokrak, 2001; Leinonen *et al.*, 2008), and considerable research has been devoted to identify the ecological conditions under which this phenomenon arises (Schluter, 2000; Nosil & Crespi, 2004; Räsänen & Hendry, 2008). Theoretical models have shown that local adaptation can occur even in the face of high gene flow when

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environmental heterogeneity results in spatially and temporally contrasting selection pressures (Merilä & Crnokrak, 2001; Räsänen & Hendry, 2008; see also Edelaar *et al.*, 2012; Edelaar & Bolnick, 2012 and references therein). Although several studies have empirically demonstrated that adaptive differentiation is possible in the presence of realized gene flow (García-Navas *et al.*, 2014; Egan *et al.*, 2015), it is more likely to occur when realized dispersal does not counteract the effects of local selection (Nosil & Crespi, 2004; Räsänen & Hendry, 2008). Though the different mechanisms potentially resulting in phenotypic divergence are not mutually exclusive and, in fact, both deterministic (local adaptation) and stochastic (random genetic drift) processes can act in concert when shaping phenotypic variation in natural populations (Fornel *et al.*, 2010).

Numerous organisms exhibit a considerable morphological variation across their distribution range, which suggests that local adaptation processes in response to spatially varying evolutionary pressures are at play (Tregenza *et al.*, 2000; Levy & Nufio, 2015). Accordingly, a broad plethora of selective agents has been identified as drivers of phenotypic divergence and life-history trait variation in natural populations, including environmental factors such as temperature (San Martín y Gómez & Van Dyck, 2012; Wojcieszek & Simmons, 2012; Laiolo & Obeso, 2015) and elevation (Berner *et al.*, 2004; Keller *et al.*, 2013; Laiolo *et al.*, 2013). Landscape dynamics and spatiotemporal changes in habitat suitability and fragmentation can also have a considerable impact on phenotypic variation (Thomas *et al.*, 2001; Hanski *et al.*, 2004; Dytham, 2009; Berggren *et al.*, 2012). In this sense, theoretical and empirical studies suggest that populations at expanding range margins (i.e. peripheral populations) or unstable habitats should experience selection towards phenotypes with a higher dispersal capability (Hughes *et al.*, 2007; Dytham, 2009; Hill *et al.*, 2011) due to increased local extinction rates and reduced mate availability associated with low habitat persistence and small population sizes (Denno *et al.*, 1991; Denno, 1994). Beyond selection, colonizers are also likely to constitute an unrepresentative sample of individuals from the origin population. On this regard, several studies have shown that dispersing individuals are larger, which may be due to the fact that individuals with such phenotype are more likely to be successful immigrants (Zera & Denno, 1997; Debeffe *et al.*, 2012; San Martín y Gómez & Van Dyck, 2012). This dispersive morph can get fixed quickly due to the strong genetic drift characterizing recently founded populations (O’Riain *et al.*, 1996; Hampe & Petit, 2005; Calabuig *et al.*, 2010; Fountain *et al.*, 2016). Most of the studies addressing this question have focused on recent range expansions (Hill *et al.*, 2011) or the persistence of habitats at short temporal scales (Heidinger *et al.*, 2010; Berggren *et al.*, 2012; see however Denno *et al.*, 1991), but the effects of long-term habitat stability (e.g. related

to Quaternary climate fluctuations and range shifts; Hewitt, 2000) on dispersal-related traits have received little attention.

In this study, we use the Pyrenean Morales grasshopper *Chorthippus saulcyi moralesi* (Uvarov 1954) as model system to investigate the relative role of genetic drift and local adaptation in phenotypic trait variation across range-wide distributed populations of the species (Spitze, 1993; Brommer, 2011; Leinonen *et al.*, 2013). The Pyrenean Morales grasshopper is a brachypterous and small body-sized (σ : 14.0–18.0 mm; ρ : 16.5–21.5 mm) gomphocerinae (Orthoptera: Acrididae) belonging to the *Chorthippus* group *binotatus* species complex (Default, 2011). This grasshopper is a narrow endemism whose patchy distribution is restricted to the central and eastern portion of the Pyrenees (Lucia-Pomares, 2002; Default, 2011). It inhabits different montane environments, from mesophilic shrubby habitats to subalpine grasslands (Lucia-Pomares, 2002) at altitudes above 1000 m (from 1100 up to 2400 m.a.s.l.; Default, 2011). The intricate orography of this area and the spatial configuration of the mountains that conform the Pyrenees are likely to be responsible for the strong spatial genetic structure observed in populations of this species (Noguerales *et al.*, 2016). Thus, both the abiotic/geographical framework and the early stage of population genetic differentiation represented in this species provide a well-suited scenario to investigate the evolutionary forces shaping phenotypic variation across an entire species distribution range (Storz, 2002; Hangartner *et al.*, 2012). Specifically, we first tested the null hypothesis that phenotypic differentiation arises from the effect of genetic drift, the main evolutionary force underlying the deep genetic structure observed among populations of the study species (Noguerales *et al.*, 2016). Second, we used distance-based redundancy analyses (dbRDAs; Legendre & Anderson, 1999) to analyse the association between phenotypic differentiation and environmental variation in order to test whether morphological trait variation is shaped by local adaptation in response to spatially varying selection pressures (Defaveri & Merilä, 2013). Finally, we tested the hypothesis that the development of dispersal-related traits is associated with different parameters related to population persistence and turnover, including habitat suitability stability over the last 120 kya, distance to the species distribution core and population genetic variability.

Materials and methods

Sampling and study area

Between 2012 and 2014, we collected 202 individuals of *C. saulcyi moralesi* from 11 populations dispersed across the Pyrenees (Spain, France and Andorra).

According to our own surveys and occurrence records available in the literature (Lucia-Pomares, 2002; Defaut, 2011), our sampling covered the entire distribution range of the taxon (~7000 km²; Fig. S1). Population codes and more information on sampling sites are presented in Table S1.

Genetic data and analysis

Genomic DNA from the muscle tissue of the hind femur was extracted using a salt extraction protocol (Aljanabi & Martinez, 1997). All individuals were genotyped at 18 polymorphic microsatellites markers whose characteristics and PCR cycling conditions are detailed in Basiita *et al.* (2016). We performed PCR amplifications and genotyping following the procedure described in Ortego *et al.* (2015a). We tested for deviations from Hardy–Weinberg equilibrium, linkage disequilibrium (LD) and the presence of null alleles as described in Nogueras *et al.* (2016). Two loci (*Cbin08* and *Cbin36*) were discarded from all downstream analyses because of HW disequilibrium in all populations and the presence of null alleles (Nogueras *et al.*, 2016). We did not find evidence for LD between any pair of loci in any sampling population after sequential Bonferroni corrections (Rice, 1989).

We quantified neutral genetic differentiation among populations calculating pairwise F_{ST} values and testing their significance with Fisher's exact tests after 10 000 permutations using ARLEQUIN (Excoffier & Lischer, 2010). We calculated global F_{ST} values over all populations and 95% confidence intervals (95% CI) by 10 000 bootstrapping replicates over loci using FSTAT (Goudet, 1995). Due to the high frequency of null alleles in Orthoptera, we applied the so-called ENA method to calculate global and pairwise F_{ST} values corrected for null alleles (F_{STNA}) using the software FREEANA (Chapuis & Estoup, 2007; e.g. Ortego *et al.*, 2015a). FREEANA was also used to obtain 95% CI by 10 000 bootstrapping replicates over loci.

Morphological data and phenotypic divergence

A total of 167 adult specimens (94 males and 73 females) were selected for linear and geometric morphometric analyses of body size and forewing length (FWL) and shape, allowing us to obtain measurements for about eight individuals (range: 5–9) of each sex per population. However, in one population (Creueta), no female could be captured and this locality was excluded from the subsequent analyses for this sex. Body size strongly correlates with life-history and fitness-related traits, and thus, it constitutes a key character and target of selection (Blanckenhorn, 2000; Whitman, 2008; Kanuch *et al.*, 2012). Forewings are strongly sclerotized structures involved in sound production and courtship rituals in Orthoptera (Petit *et al.*, 2006; Routtu *et al.*, 2007; Klingenberg *et al.*, 2010). FWL is considered a good proxy of dispersal ability (Thomas *et al.*, 2001; Simmons & Thomas, 2004; Heidinger *et al.*, 2010). Thus, this trait is expected to evolve under both natural and sexual selection (Routtu *et al.*, 2007; Klingenberg *et al.*, 2010).

We took linear measurements of left femur and left FWL to the nearest 0.1 mm using a stereoscopic microscope LEICA S8 APO (Leica Microsystems GmbH, Wetzlar, Germany) and the Leica LAS image analysis software v. 2.8.1. We used femur length (FL) as a proxy for body size because the total length of females varies substantially with the oviposition cycle (Hochkirch & Gröning, 2008). Femur length was strongly correlated with structural body length excluding the abdomen (i.e. head + thorax) ($r^2 = 0.96$, $P < 0.001$) as it has been reported for a large number of Orthoptera species (see, e.g. Ortego *et al.*, 2012; Laiolo *et al.*, 2013; Eweleit & Reinhold, 2014). We calculated an unbiased index of forewing size by expressing its length relative to FL. We took digital images of forewings and digitized 10 homologous landmarks using TpsDIG (Rohlf, 2015) in order to characterize the shape variation for this trait (Fig. 1). Once all specimens were digitized, they were

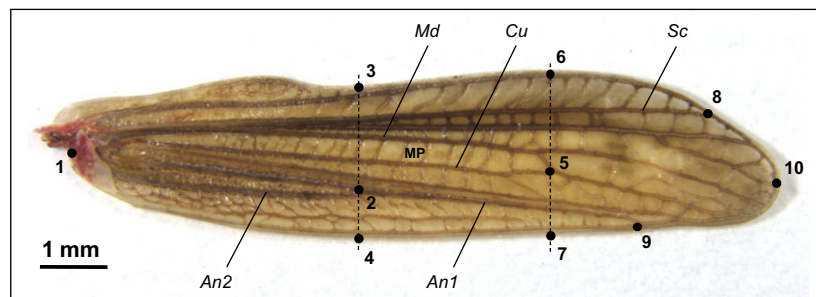


Fig. 1 Positions of the 10 landmarks used to characterize tegmina shape in the Pyrenean Morales grasshopper. The scale bar was used to standardize landmark distances to the same absolute scale across all images. We indicate the main traits (veins and plates) used to define the position of the landmarks: *Md*, media vein; *Cu*, cubitus vein; *Sc*, subcosta vein; *An1* and *An2*, anal 1 and anal 2 veins, respectively. *MP*, median plate, delimited by media and cubitus veins (see Bethoux & Nel, 2001; Petit *et al.*, 2006).

aligned and superimposed to a common coordinate system using a generalized Procrustes analyses (GPA; Rohlf & Slice, 1990). Next, morphometric variation in forewing shape was assessed separately for each sex by a relative warps (RW) analysis on the adjusted landmark coordinates using the default weighting factor ($\alpha = 0$). Given that the components of variance are not differentially weighted by their bending energy, this analysis is equivalent to a principal component (PC) analysis (Zelditch *et al.*, 2004). We retained the two first RW, which accounted for a high proportion of shape variation in both males (RW1: 57.51%; RW2: 19.60%) and females (RW1: 44.38%; RW2: 33.52%). RW scores (analogous to PC scores) were used in subsequent morphological analyses. GPA and RW analyses were conducted using TpsRELW (Rohlf, 2015). Forewing shape variation (FWS) was visually displayed using thin-plate spline diagrams using TpsPLIN (Rohlf, 2015). Finally, we conducted a canonical variates analyses (CVAs) to examine whether the differences among populations in forewing shape were significant. We calculated Mahalanobis distances (D_2) between populations and tested their significance by permutation tests with 10 000 replicates. CVA of forewing shape was conducted using MORPHOJ v.1.05d (Klingenberg, 2011). Differences between populations in FL, FWL and wing length relative to FL were analysed using one-way ANOVAS.

We studied the processes underlying morphological differentiation by comparing phenotypic and neutral genetic differentiation. Morphological differentiation was determined using P_{ST} statistics (Spitze, 1993; Brommer, 2011; Leinonen *et al.*, 2013). P_{ST} is used as a surrogate of Q_{ST} when estimating purely additive genetic variance and genotype–environment interactions is not straightforward. P_{ST} is statistically equivalent to F_{ST} for morphological characters (Brommer, 2011). If the phenotypic differentiation is solely attributable to random genetic drift, P_{ST} and neutral F_{ST} should exhibit a positive correlation, whereas if they are uncoupled, it would indicate that the phenotype is plastic or controlled by selection. In the latter scenario, P_{ST} could exhibit either smaller or higher values than neutral genetic differentiation that would suggest divergent and stabilizing selection, respectively (Merilä & Crnokrak, 2001; McKay & Latta, 2002; Leinonen *et al.*, 2006; Whitlock, 2008). Pairwise P_{ST} values were calculated as:

$$P_{ST} = \frac{(c/h^2)\sigma_{GB}^2}{(c/h^2)\sigma_{GB}^2 + 2\sigma_{GW}^2}, \quad (1)$$

where the scalar c indicates the proportion of the total variance that is presumed to be due to additive genetic effects across populations, h^2 is the assumed additive genetic proportion of differences between individuals within populations (narrow-sense ‘heritability’), and σ_{GB}^2 and σ_{GW}^2 are the between- and within-population variance components, respectively.

The variation in c and h^2 parameters affects the estimate of P_{ST} and, consequently, the reliability of the comparisons between P_{ST} and F_{ST} . We considered that the proportion of phenotypic variance that is due to additive genetic effects is the same within and among populations (i.e. $c = h^2$), a biologically realistic assumption (Brommer, 2011). The reported results were obtained using $h^2 = 0.5$, an estimate of heritability equal to the mean value previously calculated for several morphological traits in a close grasshopper species, *Chorthippus brunneus* (Butlin & Hewitt, 1986; see also Klingenberg *et al.*, 2010 for heritabilities of shape variables in Orthoptera forewings). However, we computed P_{ST} values by varying the c and h^2 parameters (c/h^2 ratio: 0.1–2.0) to check whether our conclusions hold even if the proportion of phenotypic variance across populations due to additive genetic effects (c) is much lower than heritability h^2 (sensitivity analysis; Wojcik *et al.*, 2006). We calculated pairwise P_{ST} values separately for females and males and for each morphological trait: FL, FWL, forewing length relative to femur length (RFWL) and the two retained RW components summarizing information on variation in forewing shape. In addition, we calculated a weighted P_{ST} index of FWS averaging P_{ST} values obtained for the two RW components and using its respective explained variances as offset. We estimated the 95% CI for each P_{ST} value by 10 000 bootstrap replicates using the BOOT package (Ripley, 2016) as implemented in R 3.2.3 (R Core Team, 2015).

We followed two complementary methods for comparing P_{ST} and F_{ST} according to the suggestions by Barley *et al.*, 2015 (see also Lehtonen *et al.*, 2009; Wojcieszek & Simmons, 2012). At first, we tested the association between neutral genetic (F_{ST}) and phenotypic differentiation (P_{ST}) using one-tailed Mantel tests, in order to assess the role of genetic drift or local adaptation in morphological divergence (Lehtonen *et al.*, 2009). A significant positive relationship would suggest that population phenotypic differentiation is mostly driven by neutral genetic drift. Conversely, the absence of association between genetic and phenotypic differentiation would suggest that morphological variation among populations is shaped by selective pressures favouring different phenotypes under certain ecological conditions (Lehtonen *et al.*, 2009; Ortego *et al.*, 2015b). Secondly, we compared 95% confidence intervals of global F_{ST} , F_{STNA} and P_{ST} for each trait and sex to determine whether their mean values were significantly different. Additionally, we used one-tailed Mantel tests to analyse the potential association between phenotypic differentiation (P_{ST}) and pairwise Euclidean geographical distances in order to test whether more isolated demes show a higher degree of morphological differentiation. All Mantel tests were conducted with 10 000 permutations using ZT software (Bonnet & Van de Peer, 2002).

Environmental effects on phenotypic differentiation (P_{ST})

In order to investigate the potential effects of elevation and climate on phenotypic differentiation, we characterized the environmental space of each population using a principal component analysis (PCA) applied to the 19 present-day bioclimatic variables from the WorldClim data set (Hijmans *et al.*, 2005). Subsequently, we applied a 'Kaiser varimax' rotation to maximize the differences in factor loadings on the principal components and make easier their interpretation (Norman & Streiner, 2000). After applying rotation, PC scores for the three PC axes retained for subsequent analyses remained uncorrelated (Pearson's correlations, PC1 and PC2: $r = 0.04$, $P = 0.896$; PC1 and PC3: $r = -0.43$, $P = 0.188$; PC2 and PC3: $r = 0.40$, $P = 0.223$). Bioclimatic variables were extracted from sampling sites, occurrence data from the extant literature (Llucia-Pomares, 2002; Defaut, 2011) and 1000 random points within the study area using ArcGis 10.0 (ESRI, Redlands, CA, USA). This procedure allowed us to capture the environmental variation in the study area and avoid the potential biases resulting from considering exclusively the environmental conditions of our sampling sites. Then, we obtained for each population the PC scores of the first three PC (eigenvalues > 1), which accounted for 63.43%, 20.57% and 8.48% of environmental variance, respectively. Additionally, we extracted the elevation (in metres) of each sampling site from a 90-m resolution digital elevation model obtained from NASA Shuttle Radar Topographic Mission (SRTM Digital Elevation Data, <http://srtm.csi.cgiar.org/>).

We tested the relationship between phenotypic differentiation and geography, environment and elevation using distance-based redundancy analyses (dbRDAs) (Legendre & Anderson, 1999). We performed dbRDAs using the 'capscale' function in the R package VEGAN (Oksanen *et al.*, 2016). Phenotypic distance matrices (pairwise P_{ST} values) were tested against the following predictors: (i) interpopulation geographical distances transformed by principal coordinates analyses using the 'pcnm' function in the package VEGAN, (ii) elevation and (iii) population PC scores of the first three axes from the PCA on bioclimatic data. We assessed the significance of the predictors using multivariate F -statistics with 9999 permutations using the 'anova.cca' function included in VEGAN. dbRDAs were conducted separately for males and females. Initially, we analysed the relationship between the P_{ST} matrices and each variable separately (marginal test) and then we performed a partial dbRDA (conditional test) for each variable while controlling for the influence of geographical distance (fitted as covariate).

Dispersal-related morphology in relation to climatic suitability stability, distance to the distribution core and genetic variability

We modelled the distribution of the Pyrenean Morales grasshopper at different time periods to estimate the stability of climatically suitable habitats for this species during the last 120 kya. First, we used MAXENT 3.3.3 (Phillips *et al.*, 2006; Phillips & Dudik, 2008) to model the present distribution of the species using the 19 bioclimatic variables available in WorldClim at 30 arc-sec resolution (Hijmans *et al.*, 2005) and 47 occurrence points obtained from the extant literature and our own records (Llucia-Pomares, 2002; Defaut, 2011). We estimated the distribution of climatically suitable habitats for the study species in the last glacial maximum (LGM, *c.* 21 kya BP; CCSM3 model; Collins *et al.*, 2006) and the last interglacial (LIG, *c.* 120 kya BP; Otto-Bliesner *et al.*, 2006) projecting contemporary species-climate relationships into these periods. Model and variable selection was performed as detailed in Noguerales *et al.* (2016). The final model was built with six bioclimatic variables: annual mean temperature (*Bio1*), mean temperature of the coldest quarter (*Bio11*), annual precipitation (*Bio12*), precipitation of the driest month (*Bio14*), precipitation seasonality (*Bio15*) and precipitation of the warmest quarter (*Bio18*). The final climate niche model showed an overall good performance (AUC = 0.919 ± 0.067 SD). We summed current, LGM and LIG suitability layers to generate a map of climate suitability stability, with pixel values ranging from 0 to 3 (minimum and maximum climate suitability in all periods, respectively). Average climate suitability stability (CLIM_{STA}) for each population was estimated in an area of 10 km² around sampling locations.

We estimated the core of the current distribution range of the Pyrenean Morales grasshopper by calculating the central point of the minimum convex polygon that included all known occurrences of the species. Then, we calculated the Euclidean geographical distance between the species distribution core and each sampled population (DIST_{COR}). All GIS calculations were conducted in ArcGIS 10.0.

In order to visualize the spatial patterns of genetic variation, we conducted a genetic landscape shape interpolation analysis using Alleles in Space 1.0 (Ais) software (Miller, 2005). We employed the Delaunay triangulation-based connectivity network to link neighbouring sampling sites and calculate genetic distances based on microsatellites. We used residual genetic distances to avoid the possible effects of isolation by distance and their values were extracted from sampling sites (GEN_{VAR}) to be used in further analyses. By this approach, a genetic surface of interindividual genetic distances (i.e. genetic variability between nearby demes) is expressed as 'surface heights' and visually displayed as a 3D graph.

We assessed the interdependence between population genetic variability (GEN_{VAR}), climate suitability stability ($CLIM_{STA}$) and the distance from each population to the species distribution core ($DIST_{COR}$) by means of Pearson's rank correlations. Finally, we used generalized linear mixed models (GLMMs) and an information-theoretic model selection approach (Burnham & Anderson, 2002) to analyse the association between individual-based morphological traits related to dispersal capability (FL, FWL and RFWL) and $CLIM_{STA}$, $DIST_{COR}$ and GEN_{VAR} . We built GLMMs using a Gaussian error distribution and an identity link function. We fitted separated models for males and females and included locality as a random effect to control for the statistical dependence of individuals from the same population. GLMMs were built in the R package `LME4` (Bates *et al.*, 2015) and model selection and averaging were performed using the R package `MuMIn` (Barton, 2015) as detailed in Noguerales *et al.* (2015) and Ortegó *et al.* (2015a).

Results

Genetic and phenotypic divergence

Global F_{ST} and F_{STNA} values were 0.128 and 0.094, respectively (Table S2). Pairwise F_{ST} and F_{STNA} values ranged from 0.021 to 0.216 and 0.014 to 0.148, respectively (see Supplementary Material in Noguerales *et al.*, 2016) and both genetic matrices were highly correlated (Mantel $r = 0.941$; $P < 0.001$).

We found that FL, FWL and RFWL traits differed significantly among populations in both sexes (all $P_s < 0.05$, see Fig. S2). RW analyses of shape variation showed a clustering pattern that roughly grouped individuals from the same populations (Fig. S3). Accordingly, CVA revealed significant differences in forewing shape between populations for both sexes. After Bonferroni correction, Mahalanobis distances (D_2) exhibited statistical significance for 89% and 84% of the total pairwise comparisons in males and females, respectively (Table S3). Accordingly, global and pairwise P_{ST} values indicated a high degree of phenotypic divergence for both sexes and all morphological traits (Table S2 and Table S4). Pairwise P_{ST} values were not correlated with pairwise F_{ST} or F_{STNA} values (Mantel tests: all $r < 0.119$, all $P_s > 0.193$; Table S5) or geographical distances (Mantel tests: all $r < 0.309$, all $P_s > 0.055$; Table S5) in any morphological trait, which suggests that morphological variation does not conform with that expected under a pattern of neutral genetic differentiation due to population isolation. Additionally, 95% confidence intervals of global P_{ST} values for any morphological trait did not overlap with those obtained for global F_{ST} or F_{STNA} (Fig. 2; Table S2), suggesting a predominant effect of divergent selection on the observed patterns of phenotypic differentiation among populations. Sensitivity

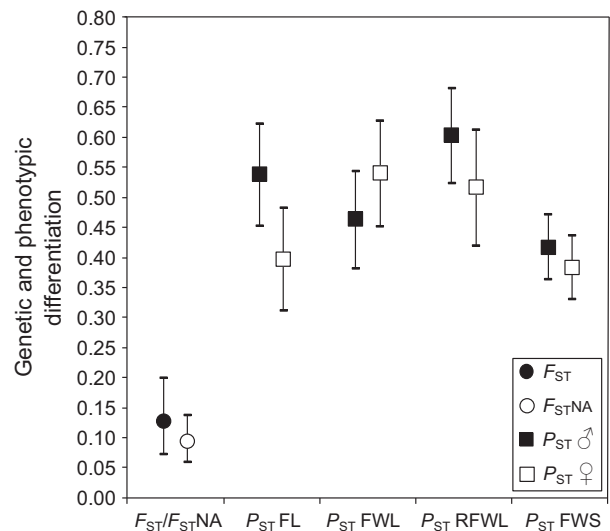


Fig. 2 Global F_{ST} , F_{STNA} corrected for null alleles, and P_{ST} for four morphological traits (FL: femur length; FWL: forewing length; RFWL: forewing length relative to femur length; FWS: forewing shape) and their respective 95% confidence intervals across all populations of the Pyrenean Morales grasshopper. Global P_{ST} values are shown for each sex separately. The reported P_{ST} values are those obtained assuming $c = h^2 = 0.5$.

analyses showed that our results remained similar even under very conservative scenarios considering small values of c/h^2 ratio (Fig. S4).

Environmental effects on phenotypic divergence

After controlling for the influence of geographical distance, dbRDA tests for males showed a significant association between population divergence of forewing length (P_{ST} FWL) and elevation and climate PC2, and between population divergence of forewing length relative to femur length (P_{ST} RFWL) and climate PC3 (all $P_s < 0.044$), which explained 23.62%, 31.70% and 28.81% of variation, respectively (Table 1). Analyses focused on females indicated that climate PC3 significantly explained 31.76% of morphological variation in forewing length relative to femur length (P_{ST} RFWL) (Table 1). Climate PC2 was explained by a pool of bioclimatic variables related to annual temperature variation (*Bio2*, *Bio3* and *Bio7*), whereas climate PC3 is mainly associated with mean temperature during the driest period of the year (*Bio8*) (Table S6).

Dispersal-related morphology in relation to climatic suitability stability, distance to the distribution core and genetic variability

Landscape interpolation of interindividual genetic distances revealed the existence of an area with higher genetic diversity at the core of the species distribution

Table 1 Results of distance-based redundancy analyses (dbRDAs) testing the effects of geography, elevation and climate on phenotypic differentiation (pairwise P_{ST} values) quantified for four morphological traits (FL: femur length; FWL: forewing length; RFWL: forewing length relative to femur length; FWS: forewing shape) in 11 populations of the Pyrenean Morales grasshopper. In marginal tests, each predictor was tested separately, whereas in conditional tests geography was always included as covariate. The proportion of the multivariate phenotypic variation explained (% var) by a given predictor or set of predictors is indicated. Predictors with $P < 0.05$ after controlling for geographical influence are highlighted in boldface.

Males				Females											
Marginal tests			Conditional tests (vs. geography)			Marginal tests			Conditional tests (vs. geography)						
Variable	<i>F</i>	<i>P</i>	% var	Variable	<i>F</i>	<i>P</i>	% var	Variable	<i>F</i>	<i>P</i>	% var	Variable	<i>F</i>	<i>P</i>	% var
P_{ST} FL				P_{ST} FL											
Geography	0.697	0.685	7.18					Geography	0.750	0.605	8.57				
Elevation	1.565	0.125	14.81	Elevation	1.815	0.100	17.16	Elevation	0.952	0.449	10.63	Elevation	1.012	0.411	11.55
Climate PC1	1.403	0.180	13.49	Climate	1.162	0.305	11.77	Climate	0.342	0.936	4.10	Climate	0.785	0.551	9.21
				PC1				PC1				PC1			
Climate PC2	0.430	0.962	4.56	Climate	1.500	0.153	14.65	Climate	1.535	0.176	16.10	Climate	1.058	0.376	12.04
				PC2				PC2				PC2			
Climate PC3	0.702	0.713	7.23	Climate	0.747	0.619	7.93	Climate	0.384	0.953	4.58	Climate	1.783	0.173	18.56
				PC3				PC3				PC3			
P_{ST} FWL				P_{ST} FWL											
Geography	0.849	0.535	8.62					Geography	2.085	0.092	20.68				
Elevation	2.628	0.044	22.60	Elevation	2.789	0.044	23.62	Elevation	0.648	0.686	7.49	Elevation	0.708	0.577	7.29
Climate PC1	1.066	0.410	10.59	Climate	1.327	0.266	13.00	Climate	1.108	0.372	12.1	Climate	0.556	0.759	5.83
				PC1				PC1				PC1			
Climate PC2	1.256	0.272	12.25	Climate	4.251	0.007	31.70	Climate	0.846	0.524	9.56	Climate	0.346	0.934	3.74
				PC2				PC2				PC2			
Climate PC3	1.095	0.338	10.85	Climate	1.577	0.193	15.04	Climate	4.288	0.001	34.90	Climate	2.57	0.055	32.32
				PC3				PC3				PC3			
P_{ST} RFWL				P_{ST} RFWL											
Geography	0.881	0.527	8.91					Geography	1.366	0.251	14.59				
Elevation	0.824	0.545	8.39	Elevation	0.811	0.601	8.38	Elevation	0.563	0.799	6.57	Elevation	0.542	0.849	6.16
Climate PC1	0.945	0.484	9.50	Climate	1.187	0.309	11.77	Climate	1.223	0.313	13.27	Climate	0.607	0.778	6.82
				PC1				PC1				PC1			
Climate PC2	0.617	0.722	6.42	Climate	0.831	0.574	8.57	Climate	0.845	0.533	9.654	Climate	0.725	0.658	8.02
				PC2				PC2				PC2			
Climate PC3	2.032	0.055	18.42	Climate	3.702	0.001	28.81	Climate	4.288	0.002	34.90	Climate	4.143	0.004	31.76
				PC3				PC3				PC3			
P_{ST} FWS				P_{ST} FWS											
Geography	0.216	0.993	2.34					Geography	1.495	0.152	15.75				
Elevation	1.019	0.400	10.18	Elevation	0.839	0.562	9.27	Elevation	0.276	0.988	3.34	Elevation	0.285	0.975	3.30
Climate PC1	0.869	0.529	8.81	Climate	1.095	0.388	11.76	Climate	0.743	0.737	8.49	Climate	0.435	0.934	4.93
				PC1				PC1				PC1			
Climate PC2	0.449	0.908	4.75	Climate	0.455	0.907	5.25	Climate	0.649	0.781	7.50	Climate	0.491	0.871	5.52
				PC2				PC2				PC2			
Climate PC3	1.564	0.110	14.81	Climate	1.855	0.094	18.38	Climate	1.693	0.105	17.47	Climate	0.762	0.660	8.27
				PC3				PC3				PC3			

range, whereas western and eastern margins of the distribution were characterized by lower genetic diversity (i.e. higher genetic similarity between nearby demes) (Fig. 3). Accordingly, GEN_{VAR} was negatively correlated with $DIST_{COR}$ ($r = -0.668$; $P = 0.025$). However, we did not find a significant correlation between $CLIM_{STA}$ and GEN_{VAR} ($r = 0.172$; $P = 0.613$). Likewise, $CLIM_{STA}$ and $DIST_{COR}$ were not significantly correlated ($r = 0.241$; $P = 0.474$). Model selection results showed that $CLIM_{STA}$, $DIST_{COR}$ and GEN_{VAR} were all included in the best ranked models ($\Delta AIC_c \leq 2$) for all the

analysed morphological traits (Table S7). However, exclusively $DIST_{COR}$ had a significant and positive effect on FWL and RFWL in both sexes (Table 2; Fig. 4). None of the predictors included in the averaged model for FL had a significant effect on this trait (i.e. all unconditional CIs crossed zero; Table 2).

Discussion

Studies combining information on genetic and ecological data provide a powerful approach to assess the

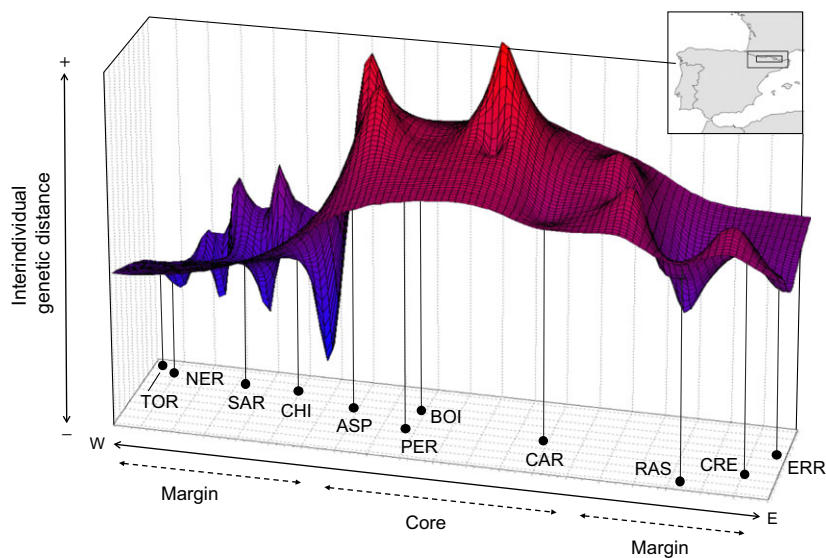


Fig. 3 Genetic landscape shape interpolation analysis based on 100×60 grids and a distance-weighted value of 2. Surface plot heights are proportional to interindividual genetic distances. Peaks (red) and troughs (blue) reflect areas with high or low genetic distances (i.e. regions with high or low levels of genetic variability) between neighbouring demes, respectively. Population codes are described in Table S1.

relative role of natural selection and neutral mechanisms in shaping phenotypic variation in natural populations and infer the proximate factors involved in such evolutionary processes (Storz, 2002; Hangartner *et al.*, 2012; Defaveri & Merilä, 2013). Although populations of the Pyrenean Morales grasshopper exhibit a strong spatial genetic structure (Noguerales *et al.*, 2016), our results indicate that phenotypic differentiation is not driven by genetic drift and point to a more important role of local adaptation processes across the environmentally heterogeneous landscape characterizing the distribution range of this species (Whitlock, 2008). Accordingly, phenotypic differentiation (P_{ST}) for all the analysed traits largely exceeded the background level of genetic neutral differentiation (F_{ST}), which points to divergent selection as the main evolutionary process explaining interpopulation phenotypic variation (Leinonen *et al.*, 2006; Lehtonen *et al.*, 2009; see however, Edelaar *et al.*, 2011). Our results are in agreement with previous studies where it has been shown a predominant role of selection, rather than drift, in morphological differentiation across many taxonomic groups (Merilä & Crnokrak, 2001; McKay & Latta, 2002; Leinonen *et al.*, 2008). At this point, we must note that our results should be taken cautiously due to the limitations and potential biases underlying P_{ST} - F_{ST} comparisons (Brommer, 2011). On the one hand, it must be emphasized that because we do not know the exact value of the c/h^2 coefficient, any inferences made on the basis of P_{ST} estimates can be only considered indicative. Regarding this, we consider unlikely that the variation among populations is strongly affected by differences in the environmental conditions experienced across populations (i.e. nonadditive genetic effects), because the studied traits are heritable ($h^2 \sim 0.5$; Butlin & Hewitt, 1986; Roff, 1986; Mousseau &

Roff, 1987; see also Berggren *et al.*, 2012). Even so, our conclusions remained valid when considering other more conservative scenarios ($c < h^2$) (Fig. S4). On the other hand, it has been suggested that the employment of hypervariable markers such as microsatellite loci can diminish the estimates of neutral genetic differentiation (F_{ST}) and thus induce a bias towards inferring divergent selection (Whitlock, 2008; Edelaar & Björklund, 2011; Edelaar *et al.*, 2011). However, our analyses based on pairwise comparisons lack the caveats associated with overall F_{ST} - P_{ST} comparisons and suggest that genetic drift cannot solely explain the morphological trait variation (Lehtonen *et al.*, 2009).

Our analyses aimed to identify the proximate factors shaping phenotypic variation revealed that differentiation in dispersal-related morphology is associated with temperature gradients. Many environmental factors, such as climate and elevation, have been found to be involved in local adaptation processes and shape morphological and life-history trait variation in insects (e.g. Berner *et al.*, 2004; Wojcieszek & Simmons, 2012; Keller *et al.*, 2013; Laiolo & Obeso, 2015). With regard to temperature influence, our distance-based redundancy analyses indicate that temperature gradients significantly explained the variation in population differentiation in forewing length and forewing length relative to body size (Pitchers *et al.*, 2013). Different hypotheses have been proposed to explain the influence of temperature on the evolution of more developed wings in insects. The most accepted hypothesis argues that larger wings (i.e. lower wing loadings) would facilitate flight in ectotherms organisms under cooler conditions because low temperatures impair locomotor performance (Gilchrist & Huey, 2004; Dillon *et al.*, 2006; Pitchers *et al.*, 2013). Alternatively, the

Table 2 Generalized linear mixed models (GLMMs) for three dispersal-related traits (FL: femur length; FWL: forewing length relative to femur length) in the Pyrenean Morales grasshopper testing the effects of climate stability during the last 120 kya (CLIM_{STA}), distance from each population to the distribution core (DIST_{COR}) and population genetic variability (GEN_{VAR}). Model averaging was performed for the best ranked equivalent models ($\Delta AIC_c \leq 2$) in order to obtain parameter estimates and unconditional standard errors (SE) (for the model selection results, see Table S7). The relative importance of each predictor is indicated ($\sum \omega_i$, sum of Akaike weights of models with $\Delta AIC_c \leq 2$ in which the predictor was present). Bold type indicates predictors excluding the value 0 in their 95% CI and whose effect is considered significant. Models were fitted and averaged separately for each sex.

		Males					Females				
Model	Predictor	Estimate ± SE	$\sum \omega_i$	Lower 95% CI	Upper 95% CI	Model	Predictor	Estimate ± SE	$\sum \omega_i$	Lower 95% CI	Upper 95% CI
FL	Intercept	9.1052 ± 0.5981				FL	Intercept	12.5133 ± 0.3449			
	CLIM _{STA}	0.5192 ± 0.4203	0.78	-0.3056	1.3442	CLIM _{STA}	CLIM _{STA}	-0.8124 ± 0.1913	0.23	-0.4562	0.2937
	DIST _{COR}	-0.0012 ± 0.0027	0.33	-0.0071	0.0044	DIST _{COR}	DIST _{COR}	0.0004 ± 0.0011	0.21	-0.3746	0.3754
	GEN _{VAR}	2.4626 ± 3.7148	0.43	-4.8188	9.7439	GEN _{VAR}	GEN _{VAR}	-	-	-	-
FWL	Intercept	9.4348 ± 0.2029				FWL	Intercept	11.2895 ± 0.5737			
	CLIM _{STA}	-				CLIM _{STA}	CLIM _{STA}	-0.1196 ± 0.2618	0.24	-0.6328	0.9353
	DIST _{COR}	0.0071 ± 0.0033	1.00	0.0019	0.0165	DIST _{COR}	DIST_{COR}	0.0122 ± 0.0048	0.77	0.0027	0.0216
	GEN _{VAR}	1.8227 ± 3.1316	0.37	-4.3146	7.9601	GEN _{VAR}	GEN _{VAR}	-3.0428 ± 5.104	0.23	-13.0475	6.9618
RFWL	Intercept	1.0096 ± 0.0488				RFWL	Intercept	0.9054 ± 0.0363			
	CLIM _{STA}	-0.0501 ± 0.0355	0.79	-0.1198	0.0185	CLIM _{STA}	CLIM _{STA}	-	-	-	-
	DIST _{COR}	0.0012 ± 0.0003	1.00	0.0007	0.0018	DIST _{COR}	DIST_{COR}	0.0007 ± 0.0003	0.70	9.7175 × 10⁻⁶	1.4808 × 10⁻³
	GEN _{VAR}	0.2184 ± 0.3259	0.39	-0.4191	0.8553	GEN _{VAR}	GEN _{VAR}	0.0002 ± 0.0173	0.50	-0.0921	0.0927

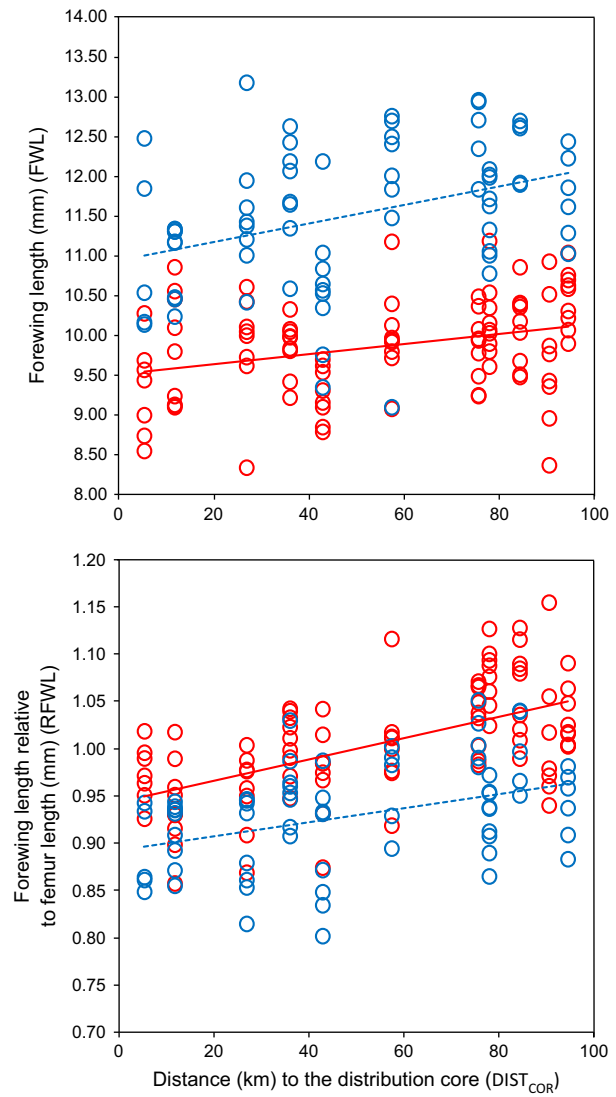


Fig. 4 Relationships between forewing length (FWL) and forewing length relative to femur length (RFWL) and the distance to the distribution range core (DIST_{COR}) in 11 populations of the Pyrenean Morales grasshopper. Regression lines for each sex (males: red dots and solid red line; females: blue dots and dashed blue line) are shown.

thermoregulatory hypothesis posits that the evolution of wings in insects is associated with its thermoregulatory function as heat absorption capability increases with wing size (Kingsolver & Koehl, 1985; Lewin, 1985).

Several studies on insect taxa including orthopterans have examined evolutionary responses to climate along latitudinal or altitudinal gradients (Shelomi, 2012). The most frequently reported pattern is the existence of a negative association between body size and temperature and/or elevation (converse Bergmann's rule; e.g. Blanckenhorn & Demont, 2004; Laiolo *et al.*, 2013;

Levy & Nufio, 2015). Body size is a key trait associated with thermoregulation and fecundity in insects (Gilchrist, 1990; Honek, 1993; Blanckenhorn, 2000; Whitman, 2008), and this pervasive trend has been traditionally explained as an adaptation to short growing seasons in cooler climates and/or a consequence of resource shortage under harsh environmental conditions (Hodkinson, 2005; Roff & Mousseau, 2005; Dillon *et al.*, 2006). Here, we failed to find an altitudinal or environmental cline for body size in this species. The absence of environmental effects on body size variation among populations of the Pyrenean Morales grasshopper may be a consequence of the faster development rates that orthopterans inhabiting montane habitats exhibit (Telfer & Hassall, 1999; Berner *et al.*, 2004; Berner & Blanckenhorn, 2006). Alternatively, the studied environmental variables become more relevant at a microgeographical scale (e.g. microhabitat sun exposure, slope,) or body size evolution may be driven by other agents not considered in our study (e.g. sexual selection, habitat structure, predation risk; Basolo & Wagner, 2004; Berner *et al.*, 2004; Grace *et al.*, 2010; Heidinger *et al.*, 2010).

Regarding forewing shape, there was neither a significant association between this variable and elevation or climate. This is in contrast to previous studies in flying insects where it has been shown the existence of environmental clines for wing morphology (Pitchers *et al.*, 2013; Perrard *et al.*, 2014). As far as we know, no other study has investigated FWS in a grasshopper species, which prevent us from determining whether this lack of variability is widespread in this group or not. In many grasshoppers, including our study species, males produce conspicuous songs by rubbing the inner part of hind legs against forewings, whereas females emit short syllables if the specific song of a male matches with their preferences (Harz, 1975; Lampe *et al.*, 2012). Thus, it would be reasonable to expect that stabilizing (sexual) selection against immigrants exhibiting different forewing shapes impacts phenotypic divergence on this trait regardless of the studied environmental factors (Wojcieszek & Simmons, 2012; Oneal & Knowles, 2013).

Dispersal capability plays a key role in determining gene flow, ultimately shaping range-wide patterns of genetic variability and spatial genetic structure (Kanuch *et al.*, 2012; García-Navas *et al.*, 2014; Dussex *et al.*, 2016). We found support to our hypothesis predicting a higher development of dispersal-related traits in populations located in the periphery of the species distribution range. Thus, our findings are consistent with previous studies reporting that individuals from populations located at range boundaries, fragmented habitats, and expanding edges possess a higher dispersal capacity than those inhabiting core areas or stable/continuous habitats (Thomas *et al.*, 2001; Hughes *et al.*, 2003; Kanuch *et al.*, 2012; Therry *et al.*, 2014a,b; Fountain *et al.*, 2016). According to theory on evolutionary stable

dispersal strategies, selection for increasing dispersal capability should be stronger under the conditions of high population turnover (Comins *et al.*, 1980; Berggren *et al.*, 2012). Under this scenario, individuals with more 'willing to disperse' phenotypes would be able to settle at the dynamic range boundaries (Shine *et al.*, 2011), where habitat suitability may experience stronger temporal changes than in more stable populations at the core of the distribution range (Hardie & Hutchings, 2010). The notion that peripheral populations of the Pyrenean Morales grasshopper are less stable than those located in the core area is also reinforced by the lower levels of genetic diversity observed in these populations, which indicates that they sustain smaller effective population sizes according to the central-marginal hypothesis (Eckert *et al.*, 2008; Lira-Noriega & Manthey, 2014). The fact that we did not find a direct relationship between habitat suitability stability and our estimates of dispersal-related morphology or genetic variability may be consequence of the uncertainties associated with the projection of the current species' distribution models into past climates (e.g. Diniz-Filho *et al.*, 2015). Alternatively, it is also plausible that past demographic dynamics are not reflected in current evolutionary processes, which could be happening at much shorter temporal scales than those reflected by our long-term habitat stability estimates (Thomas *et al.*, 2001; Simmons & Thomas, 2004; Fountain *et al.*, 2016).

Conclusions

Studying ongoing differentiation processes in small-scale situations is essential to gain insight into the mechanisms driving adaptive divergence. Here, we sustain local adaptation, rather than genetic drift, as the main factor shaping phenotypic divergence in our study system. Analyses of the proximate environmental factors potentially determining range-wide patterns of phenotypic variation indicate that temperature gradients seem to be involved in morphological divergence. Our data also provide evidence for a higher development of dispersal-related traits in populations located at range boundaries characterized by higher population turnover, unpredictable habitat dynamics and lower levels of genetic diversity in comparison with those located at the core of the species distribution. Overall, our study highlights the importance of integrating genetic and environmental data to obtain robust inferences about the different evolutionary processes and selective agents shaping the phenotypic variation across a species' distribution range.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Geographical location, elevation and number of genotyped individuals for the studied populations of the Pyrenean Morales grasshopper.

Table S2 Global genetic and phenotypic differentiation for four morphological traits across 11 populations of the Pyrenean Morales grasshopper.

Table S3 Results of Canonical Variates Analyses testing for forewing shape differentiation between populations of Pyrenean Morales grasshopper.

Table S4 Phenotypic differentiation (P_{ST}) between 11 populations of the Pyrenean Morales grasshopper for four morphological traits.

Table S5 Results of Mantel tests analysing the association between pairwise P_{ST} values for four morphological traits and Euclidean geographical distances, F_{ST} and F_{STNA} .

Table S6 Factor loadings for the first three principal components (PC) axes and the 19 bioclimatic variables obtained from the WorldClim data set.

Table S7 Model selection testing the association between three dispersal-related traits and climate stability during the last 120 kya, distance from each population to the distribution core and population genetic variability.

Figure S1 Sampling sites of the Pyrenean Morales grasshopper plotted on an elevation map of the study area.

Figure S2 Population mean (\pm SD) values for linear morphological traits.

Figure S3 Forewing shape variation along the two first relative warps.

Figure S4 P_{ST} values and their 95% confidence intervals for varying values of clh^2 ratio and different morphological traits.

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