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Drivers of genetic differentiation in a generalist insect-pollinated herb across spatial scales

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Abstract

The isolation-by-distance model (IBD) predicts that genetic differentiation among populations increases with geographic distance. Yet, empirical studies show that a variety of ecological, topographic and historical factors may override the effect of geographic distance on genetic variation. This may particularly apply to species with narrow but highly heterogeneous distribution ranges, such as those occurring along elevational gradients. Using nine SSR markers, we study the genetic differentiation of the montane pollination-generalist herb, *Erysimum mediohispanicum*. Because the effects of any given factor may depend on the geographic scale considered, we investigate the contribution of different environmental and historical factors at three different spatial scales. We evaluate five competing models that put forward the role of geographic distance, local environmental factors [biotic interactions (IBEb) and climatic variables (IBEa)], landscape resistance (IBR) and phylogeographic patterns (IBP), respectively. We find significant IBD regardless of the spatial scale and the genetic distance estimator considered. However, IBEa and IBP also play a prominent role in shaping genetic differentiation patterns at the larger spatial scales, and IBR is significant at the fine spatial scale. Overall, our results highlight the importance of combining different estimators, statistical approaches and spatial scales to disentangle the relative importance of the various ecological factors contributing to the shaping of genetic divergence patterns in natural populations.

Keywords: genetic structure, isolation by distance, landscape genetics, plant–pollinator interactions, spatial scale

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Introduction

Landscape genetics is concerned with explaining observed spatial genetic patterns across environments (Dobzhansky & Wright 1943; Manel *et al.* 2003; Holderegger & Wagner 2008). Plant species tend to occupy heterogeneous environments across their geographic range, and the magnitude and spatial distribution of their genetic variation is expected to vary accordingly (Hedrick 1986; Linhart & Grant 1996; Anderson *et al.* 2011). According to the isolation-by-distance model (IBD, Wright 1943), genetic differentiation increases with geographic distance because gene flow declines among increasingly distant populations. Since its formulation in the 1940s, this idea has been largely supported (Dobzhansky & Wright 1943; Imaizumi & Morton 1969; Relethford 1985; Sharbel *et al.* 2000). Yet, recent empirical studies have put forward that geographic distance by itself fails to

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fully explain geographic patterns of genetic variation in natural systems (Jenkins *et al.* 2010; Shafer & Wolf 2013). For example, phenological mismatches imposing assortative mating patterns in flowering trees have been shown to shape genetic differentiation at the fine scale (Oddou-Muratorio *et al.* 2005). At larger scales, past shifts in the distribution range of a species may also explain part of its genetic variation (Hewitt 2000). In fact, the role of the geographic, environmental (biotic and abiotic) and historical factors is not mutually exclusive but rather they may act as drivers of spatial genetic patterns simultaneously, with the relative impact of each factor changing at different spatial scales (Wang *et al.* 2013). However, empirical data documenting their relative role in defining genetic divergence patterns across spatial scales are scarce to date. Given that the genetic distance among populations determines important evolutionary traits, such as the effective population size, we require this type of studies to inform our knowledge of the ecological and evolutionary trends of natural populations inhabiting changing environments, such as mountain ranges.

An increasing number of landscape genetic studies identify the most relevant ecological factors determining genetic structure (Cushman *et al.* 2006; Orsini *et al.* 2013; Spurgin *et al.* 2014). Yet, our knowledge is limited and biased because most studies include few ecological factors and are restricted to a single spatial scale. Investigating the effect of different types of ecological and historical factors at different spatial scales is particularly relevant for plant species confined to restricted but heterogeneous distribution ranges, such as those occurring along elevational gradients. Many key abiotic factors controlling population dynamics change with elevation, such as temperature, precipitation and soil properties (Frei *et al.* 2014 and references therein). Biotic interactions such as pollination – largely ignored in landscape genetics studies – also vary along elevational gradients (Johnson & Steiner 2000; Thompson 2005). Therefore, in montane insect-pollinated plant species, the interplay between biotic and abiotic factors may override the influence of geographic distance on genetic differentiation. This is a timely question, because global change is eliciting fast-paced environmental shifts that imperil biotic interactions associated with plant population declines (Biesmeijer *et al.* 2006).

Here, we aim to investigate the influence of geographic, environmental and historical factors in determining patterns of genetic differentiation across spatial scales in the herb *Erysimum mediohispanicum* (Brassicaceae). Specifically, we test whether genetic differentiation in this plant may be explained by any of the following five competing scenarios (Table S1, Supporting information):

1. Isolation by distance (IBD). Gene flow declines with geographic distance imposing strong genetic differentiation patterns among distant populations.
2. Isolation by resistance (IBR). Topographic variation across the landscape hinders gene flow, which may then be restricted to a few particular pathways (McRae 2006).
3. Isolation by abiotic ecological factors (IBEa). Genetic differentiation is driven by contrasting abiotic climatic factors, such as temperature or precipitation, either due to local adaptation or to limited dispersal among sites (Sexton *et al.* 2014).
4. Isolation by biotic ecological factors (IBEb). This scenario tests whether genetic differentiation can be explained by similarity in pollinator compositions, reflecting either between-population gene flow or common within-population selective pressures.
5. Isolation by phylogeography (IBP). Historical migrations and demographic shifts (such as bottlenecks, colonization and migration events) impose strong genetic differentiation patterns that persist in current times despite proximity between populations.

Erysimum mediohispanicum (Brassicaceae) is a biennial herb endemic to the Iberian Peninsula with an elevational distribution ranging between 600 and 2200 m a.s.l. (Nieto-Feliner 1993). The evolutionary history of the species is complex, probably influenced by the isolation and hybridization of different evolutionary lineages (Muñoz-Pajares 2013a). *E. mediohispanicum* interacts with a diverse array of floral visitors, most of them acting as effective pollinators due to the open morphology of the flower and the accessibility of the reproductive organs (Gómez *et al.* 2009a). Pollinators are known to exert significant selection on several *E. mediohispanicum* phenotypic traits (Gómez *et al.* 2009a). In great contrast to pollination, seed dispersal occurs abiotically in *E. mediohispanicum* and is mostly restricted to a few metres from the source plant (Gómez 2007). The exhaustive background information on the ecological (Gómez 2003, 2005; Gómez *et al.* 2007) and phylogeographic patterns available for this species (Muñoz-Pajares 2013a) makes *E. mediohispanicum* an ideal system to gauge the influence of environmental and historical factors on spatial genetic patterns.

Materials and methods

Study system

Although partially self-compatible, *Erysimum mediohispanicum* needs pollinators to achieve complete seed set (Gómez 2003; Abdelaziz *et al.* 2014). Populations of this plant are usually composed of few dozens to several

hundreds of individuals at relatively low densities (around 10 individuals/50 m²; Gómez 2005). Depending on the population, these individuals are either diploid ($2n = 14$) or hypotetraploid ($2n = 26$; Nieto-Feliner 1993; Muñoz-Pajares 2013a). However, individuals with intermediate ploidy levels have scarcely been found, suggesting that gene flow between different cytotypes is negligible (A. J. Muñoz-Pajares, F. Perfectti, J. Loureiro, M. Abdelaziz, P. Biella, M. Castro, S. Castro & J. M. Gómez, in preparation). For this reason, and to avoid uncertainty associated with the assessment of allele dosage in polyploid individuals (Nybom 2004), we focus on the diploid cytotype.

Sampling design

Between 2006 and 2010, we sampled 30 diploid *E. mediohispanicum* populations (Fig. 1), encompassing the distribution of the diploid cytotype (restricted to southern Spain; A. J. Muñoz-Pajares, F. Perfectti, J. Loureiro, M. Abdelaziz, P. Biella, M. Castro, S. Castro & J. M. Gómez, in preparation). We studied population differences at three spatial scales: (i) large scale (including different mountain ranges, 30 populations; maximum between-population distance: 250 km); (ii) meso-scale (including different sites within the Sierra Nevada range, 26 populations; maximum distance: 115 km); and (iii) fine scale (including different populations within each of two sites in Sierra Nevada: *Dornajo*, 11 populations; maximum distance: 11 km; and *Cortijuela*, eight populations; maximum distance: 7 km) (Fig. 1).

DNA extraction and genotyping

We collected fresh leaf tissue from 15 individuals per population. Leaves were silica gel-dried in labelled envelopes and stored until processing. We extracted total genomic DNA from ca. 60 mg of dry leaf tissue using GenElute Plant Genomic DNA Miniprep Kit from Sigma-Aldrich (Darmstadt, Germany). Samples were amplified with nine specifically designed polymorphic microsatellite markers following the procedure described in Muñoz-Pajares *et al.* (2011). PCR products were diluted to 10 ng/μL and sent to MACROGEN (Geum-chun-gu, Seoul, Korea; http://www.macrogen.com) for microsatellite fragment separation. We analysed the electropherograms and called alleles with PEAK SCANNER Software version 1.0 (Applied Biosystems). The frequency of genotyping errors for these markers has been estimated at around 0.1% (A. J. Muñoz-Pajares, in preparation), basically due to allelic dropout in heterozygous loci, a common outcome in microsatellite

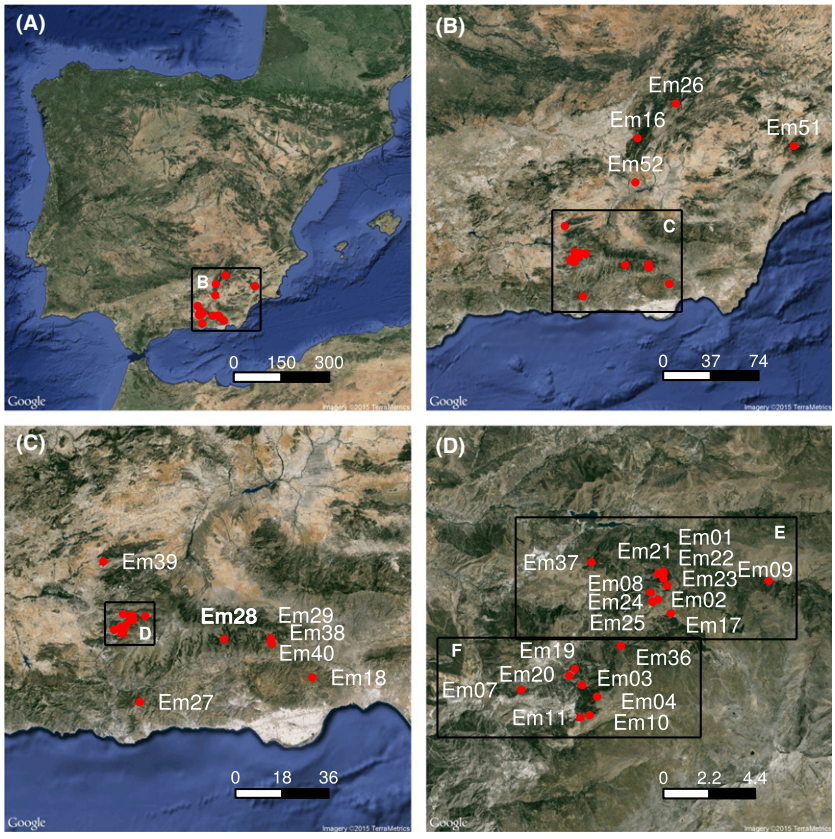

Fig. 1 Sampling design. (A) Location of the study populations within the Iberian Peninsula; (B) large scale ($N = 30$); (C) meso-scale ($N = 26$); (D) fine scale at Dornajo (E; $N = 11$); and Cortijuela (F; $N = 8$) [Colour figure can be viewed at wileyonlinelibrary.com]

genotyping (Hoffman & Amos 2005; Pompanon *et al.* 2005).

Estimate of genetic differentiation

We computed population genetic differentiation using both Nei's distance (D_S, Nei 1972) and conditional genetic distances (cGD, Dyer & Nason 2004), which consider genetic covariation among all study populations. We estimated D_S and cGD using the R packages 'ADEGENET' vs. 1.3–9.2 (Jombart 2008) and 'GSTUDIO' vs. 1.3 (Dyer 2014), respectively. Because cGD uses the multilocus genotype of the full study sample, its sensitivity for landscape genetic studies is expected to be high in comparison with pairwise estimates (Dyer *et al.* 2010). The information contained in the cGD can be visualized as a network (Fig. 2) that captures the structure of genetic covariation among populations (Dyer & Nason 2004; Dyer *et al.* 2010). These analyses were conducted and plotted with the R packages 'POPGRAPH' vs. 1.4 (Dyer 2014) and 'GGMAP' vs. 2.3 (Kahle & Wickham 2013).

Exploring the five scenarios

Each scenario was explored by quantifying the variables described below (Table S1, Supporting information).

Isolation by distance (IBD). We recorded the latitude and longitude of each population using a GPS Garmin e-trex (GARMIN Ltd, Canton of Schaffhaunsen, Switzerland). To test the occurrence of IBD, we used geographic coordinates to estimate between-population Euclidean geographic distances.

Isolation by resistance (IBR). Although various topographic factors may impose resistance to *E. mediohispanicum* gene flow, we have focused on elevation. We defined

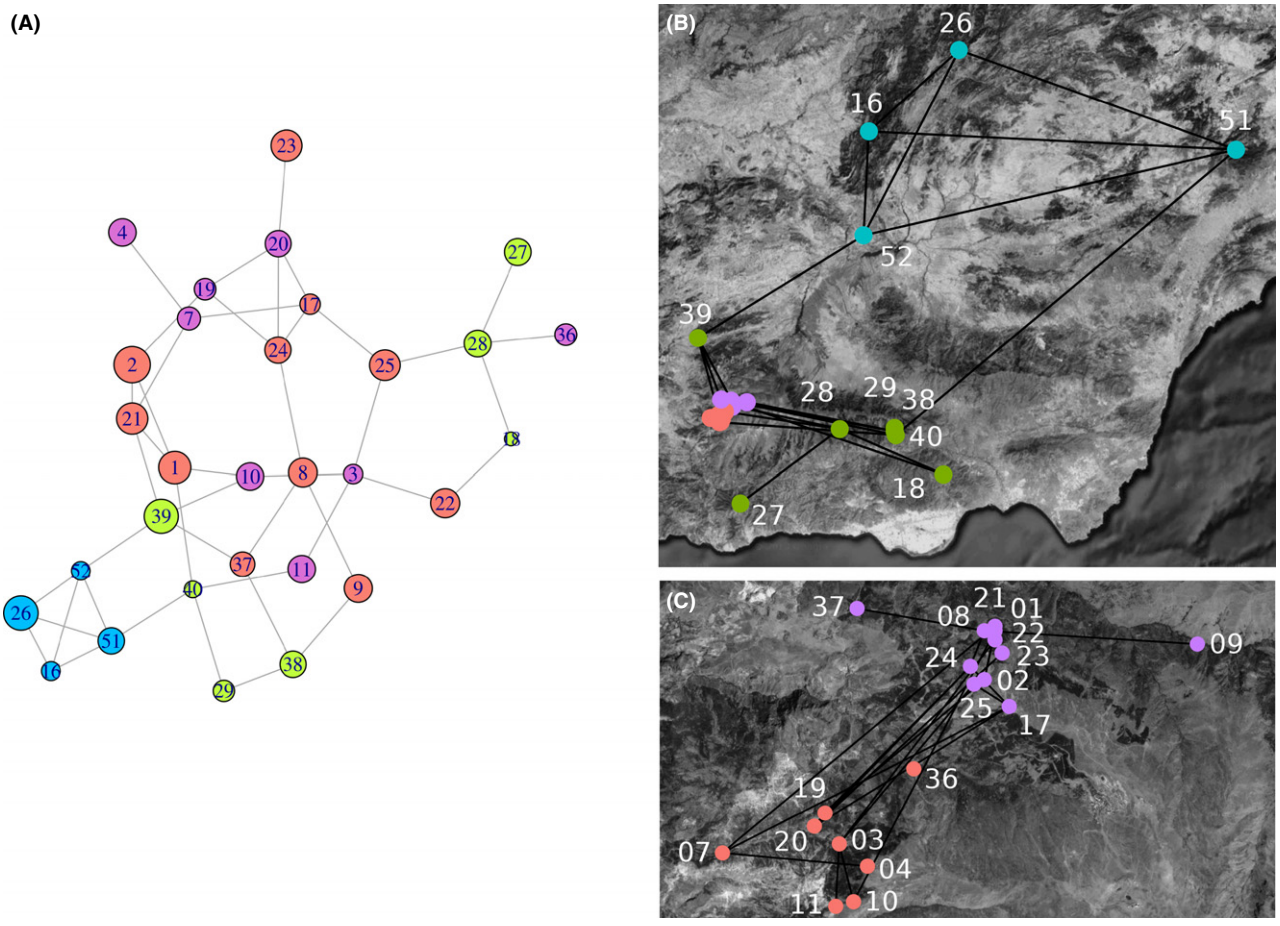

Fig. 2 Population network. Node coordinates are represented according to the Fruchterman–Reingold algorithm (A) and according to geographic location at large scale (B) and fine scale (C). Different colours represent the geographic scale at which each population is included: blue, populations included only in large-scale analyses; green, populations included in large-scale and meso-scale analyses. The remaining populations were included in large-, meso- and fine-scale analyses at Dornajo (purple) and Cortijuela (pink) [Colour figure can be viewed at wileyonlinelibrary.com]

geographic areas suitable for *E. mediohispanicum* based on its recorded elevational distribution range (between 700 and 2200 m a.s.l. in the study mountain ranges). We used the R package 'RASTER' vs. 2.3–40 (Hijmans 2015) to download 90-m-resolution data on elevation from SRTM (http://srtm.csi.cgiar.org/) and created a raster grid of resistances, giving different values (100 and −999, respectively) to areas within and outside the species distribution elevational limits. Then, we used the CIRCUITSCAPE software vs. 4.0 (McRae *et al.* 2013) to transform the raster grid into the resistance matrix that we used to test the occurrence of IBR.

Isolation by abiotic ecological factors (IBEa). Based on geographic coordinates, we obtained interpolated climatic conditions from the KMNI Climate explorer database (http://climexp.knmi.nl/) for each population. We used records of the last 50 years to obtain the following monthly variables: (i) precipitation (Pr; mm/day); (ii) mean temperature (T_m; °C); and (iii) temperature range (T_r; estimated as the difference between the maximum and the minimum monthly mean temperatures) (Haylock *et al. 2008*). We summarized all the monthly climatic variables in one synthetic variable called *climatic Euclidian weighting distance* [CEWD, Hübner *et al.* (2009)].

Isolation by biotic ecological factors (IBEb). Pollinator composition at a given population may vary throughout the flowering period and between years. To minimize the effects of this variation, pollinator surveys were conducted during peak bloom, when most pollinator–plant interactions occur. In most populations, surveys were conducted on 2 years or more (see Supporting information for details). At any rate, differences in pollinator assemblage have been shown to be much more important at the spatial than at the temporal level in *E. mediohispanicum* (Gómez *et al.* 2009a but see Valverde *et al.* 2016 for a detailed study on temporal variation in *E. mediohispanicum* pollinator interactions). In each survey, an observer walked through one or more pre-established transects across the *E. mediohispanicum* population and recorded all insects contacting the reproductive organs of the flowers. Transect length varied according to population so as to cover an area representative of the entire population. We tried to obtain 130–150 plant–pollinator interactions for each populations, as this sample size has been shown to provide an accurate estimate of *E. mediohispanicum* local pollinator assemblages (Gómez *et al.* 2009a). Pollinators were particularly scarce in a few locations, and therefore, our sampling effort could not be entirely homogeneous across populations. We nonetheless have included all populations in our analyses because the results are similar with and without these populations. Some individual pollinators were captured for later identification in the laboratory. Pollinators were classified into functional groups (Fenster *et al.* 2004), based on body and proboscis sizes, foraging behaviour and feeding habits. We established nine functional groups: (i) ants: Formicidae; nectar collectors; (ii) beetles: Coleoptera collecting nectar and/or pollen; (iii) beeflies: long-tongued Bombyliidae, nectar collectors; (iv) butterflies: Lepidoptera, nectar collectors; (v) hoverflies: Syrphidae and short-tongued Bombyliidae pollen/nectar collectors; (vi) large bees: Apiformes, females over 10 mm in body length, pollen/nectar collectors; (vii) small bees: Apiformes, females under 10 mm, in body length, pollen/nectar collectors; (viii) wasps: wasps and cleptoparasitic bees, collecting only nectar; (ix) others: nectar-collecting flies, grasshoppers and bugs. To evaluate the effect of differences in the pollinator assemblage on *E. mediohispanicum* genetic differentiation, we estimated between-population similarity of the pollinator assemblages at the functional group level using the Chao index (Chao *et al.* 2005) as implemented in the VEGAN package in R (Oksanen *et al.* 2013).

Isolation by phylogeography (IBP). We compared genetic markers evolving at different rates, thus accounting for recent (microsatellites) and historical (plastidial DNA) events. We used the pairwise genetic distance matrix obtained by Muñoz-Pajares (2013a) as our phylogeographic hypothesis. To obtain this matrix, genetic distances based on substitutions were estimated using the R package 'APE' vs. 3.0-9 (Paradis *et al.* 2004), and genetic distances based on indels were estimated using the R package 'SIDIER' vs. 2.3 (Muñoz-Pajares 2013b). Both distance matrices were combined (same weight given to each matrix) to obtain a haplotype pairwise distance matrix. Population pairwise distances were estimated using haplotype pairwise distances and the frequency of haplotypes found in each population (Muñoz-Pajares 2013a).

Statistical analyses

Our response variables were the matrices of genetic distance (D_S and cGD) among populations. As explanatory variables, we used the various distance matrices described above. Correlations among genetic and environmental distances were tested according to the various scenarios considered by applying two distance-based approaches: (i) Mantel and partial Mantel tests (the latter controlling for autocorrelation using the geographic distance matrix because we assume that spatial autocorrelation is inherent to most of the factors evaluated in this study); and (ii) multiple

regression on distance matrices (MRM, Lichstein 2007). We performed Mantel tests using the R package 'VEGAN' vs. 2-0-10 (Oksanen *et al.* 2013) and estimated significance based on 10 000 permutations. We performed MRM as implemented in the R package 'ECODIST' vs. 1.2-9 (Goslee & Urban 2007). To do this, we built an initial model and, for each variable, we estimated regression coefficients and associated *P*-values based on 10 000 permutations. Because we were interested only in those variables that significantly contribute to explain genetic distances, we performed model selection following a backward elimination procedure as described in Legendre & Legendre (2012). We compared six initial models. Initial model #1 included all variables, that is resistance, abiotic factors, biotic factors, phylogeography and geography. The remaining initial models, #2 to #6, each lacked one different variable. For each initial model, the variable showing the highest nonsignificant *P*-value was removed. We repeated this procedure until all variables included in the analysis showed *P*-values lower than 0.05. Finally, we compared the six final models based on their *F*-values.

We corrected *P*-values obtained for Mantel tests and MRM models for multiple testing using the Benjamini & Hochberg (1995) method as implemented in the 'STAT' package in R. Following the rationale of Moran (2003), we tried to 'inject some logic into our interpretation of statistical results'. Thus, we discuss not only results achieving standard pre-established significance levels following the mentioned correction, but also results that through their consistent association with relatively low *P*-values suggest some common patterns.

Results

Pattern of genetic covariance among populations

The population graph shows congruency between geographic distance and the magnitude of the genetic covariance among populations. The four outermost populations (51, 52, 16, 26) formed a peripheral module within the population network graph suggesting that the most remote populations are also the most distinctive ones (Fig. 2A). The remaining populations become connected in the network regardless their geographic distance. Thus, populations at Cortijuela and Dornajo are linked intermingled in the graph with populations belonging to geographically distant areas included in the meso-scale analyses (Fig. 2A).

Mantel tests

D_S and cGD coincided in highlighting the importance of IBD both at the large scale and meso-scale, but at the fine scale, only the Dornajo site showed IBD when genetic distance was expressed as D_S (Table 1). In contrast, IBD was not found for the Cortijuela fine scale site, suggesting that the effect of the geographic distance in imposing genetic isolation is site-dependent at the fine scale. A significant IBR was also detected at Dornajo site according to D_S, and at the large and meso-scales according to cGD. Significant IBEa and IBP were also found for cGD at the large scale and the meso-scale (Table 1).

After correcting for the effect of the geographic distance, only the IBP scenario remained significant at the large and the meso-scales (partial Mantel tests, Table 1).

Table 1 Results of Mantel and partial Mantel correlation tests

	Nei's distances (D_S)					Conditional genetic distances (cGD)			
	Large	Meso	Fine (Dornajo)	Fine (Corituela)		Large	Meso	Fine (Dornajo)	Fine (Corituela)
Simple mantel tests									
IBD	**0.2727**	***0.4671***	**0.5398**	0.2245	IBD	***0.5073***	**0.2670**	0.2136	0.2378
IBR	0.1997	0.3070	**0.3945**	0.1686	IBR	***0.4941***	***0.3365***	0.0061	0.2753
IBEa	0.1073	0.2063	0.0886	−0.1846	IBEa	***0.3378***	**0.2005**	−0.2192	−0.5099
IBEb	0.0061	−0.0049	−0.0921	−0.2717	IBEb	0.0130	−0.0790	0.1283	−0.1485
IBP	−0.0198	−0.0182	0.2899	0.2928	IBP	***0.3378***	**0.1991**	−0.0652	0.5263
Partial mantel tests: controlling by geographic distances									
IBR	−0.0876	−0.0756	−0.1617	−0.0311	IBR	0.1170	0.2137	−0.3557	0.1440
IBEa	−0.2636	−0.2020	0.3617	−0.2186	IBEa	−0.2299	0.0158	−0.1558	−0.5582
IBEb	−0.0838	−0.0359	−0.2150	−0.3177	IBEb	0.0130	−0.0790	0.1283	−0.1485
IBP	−0.1425	−0.0646	0.1381	0.2183	IBP	**0.1763**	**0.1843**	−0.1483	0.4834

For each test, r_M is provided. Significant results before *P*-value correction represented in bold; Significant results after *P*-value correction represented in bold and italized.

Table 2 Best models obtained with backward elimination MRM procedures using two genetic distance estimators (D_S and cGD)

Geographie scale	Genetic distance estimator	Initial model	R^2	F	Corrected P value	IBD	IBR	IBEa	IBEb	IBP
	cGD	3	0.2573	150.05	0.0005	10.75 ($P = 0.0001$)		N/A		
Large	D_S	3	0.0744	34.79	0.0510	$2.56{\cdot}10^{-5}$ ($P = 0.0446$)		N/A		
	D_S	1	0.1387	34.78	0.0449	$6.63{\cdot}10^{-5}$ ($P = 0.0005$)		$-1.33{\cdot}10^{-7}$ ($P = 0.035$)		
	cGD	1	0.1132	41.25	0.0064		0.048 ($P = 0.002$)			
Meso	D_S	1	0.2182	90.13	0.0072	$9.03{\cdot}10^{-5}$ ($P = 0.0042$)				
	cGD	1	0.1664	5.19	0.1106	274.41 ($P = 0.0270$)	-0.270 ($P = 0.047$)			
Fine (Doinajo)	D_S	1	0.2914	21.79	0.0304	$6.53{\cdot}10^{-4}$ ($P = 0.0207$)				
Fine (Cortijuela)	cGD & D_S	N/A	N/A	N/A	N/A					

For every model, R^2, F and P-values after correction for the number of analyses are shown. Coefficients and P-values for the variables retained in the best models are also provided. All best models resulted from initial models #1 (containing all variables) and #3 (lacking IBEa, indicated by 'N/A').

Finally, after correcting P-values for the number of analyses performed, only correlations observed with simple Mantel tests at the large (IBD, IBR, IBEa and IBP, using cGD) and the meso-scale (IBR using cGD and IBD using D_S) maintained significant (Table 1).

Multiple regression on distance matrices (MRM)

Geographic distance was the only factor retained in the best models at the three spatial scales when we used D_S as a genetic differentiation metric (Table 2). However, at the large scale, a second model including geographic abiotic distances showed similar F-values, suggesting that climatic variables may also significantly affect genetic differentiation at this spatial scale. In terms of cGD, IBR played a dominant role explaining genetic differentiation at the meso-scale and fine scale (in the latter case, together with IBD). IBEb and IBP did not contribute to any of the best models explaining genetic differentiation regardless of the spatial scale considered (Table 2).

Discussion

Factors shaping genetic differentiation patterns in E. mediohispanicum

Our results reveal the importance of geographic and environmental distances, namely topography and climatic variables, in shaping the pattern of genetic differentiation in *E. mediohispanicum*. Although IBD affected genetic differentiation across all spatial scales, other factors act in a scale-dependent manner. Thus, IBEa explained genetic differentiation at the large scale, whereas IBR was more important at the finer spatial scales. In sum, we found that some factors were more relevant than others, and among those playing a significant role, their influence varied across spatial scales.

All the analyses coincided to highlight IBEb as the scenario contributing the least to the observed patterns of genetic differentiation. IBP, on the other hand, seems to influence genetic distances at the large scale and meso-scale. In fact, IBP was the only scenario showing significant P-values after controlling by geographic distances indicating that distant populations from the evolutionary point of view also tend to differ genetically regardless of their geographic distance. This result confirms that population differentiation arises even at early speciation stages and with a recent colonization history (Orsini *et al.* 2013).

The strong effect of IBP at the larger scales was probably due to the resulting admixture of different evolutionary lineages hypothesized to happen in the Iberian Peninsula. Although the origin of *Erysimum* has been estimated to occur during the Pliocene, about 3.5 Mya, the genus appears to have reached the Iberian Peninsula around 1.1 to 0.5 Mya (Moazzeni *et al.* 2014). This colonization coincides with the glacial and interglacial periods leading to recurrent intervals of isolation and gene flow (Muñoz-Pajares 2013a). In fact, several Iberian *Erysimum* species may hybridize in natural (Clot 1991) and controlled conditions (Abdelaziz 2013).

Accordingly, we found that populations at the fine scale and meso-scale share genetic covariation structures (Fig. 2A). The recent origin of *E. mediohispanicum* may partially explain this low divergence among relatively distant populations. Gene flow and geographic similarity in selective pressures can also account for the low divergence observed among *E. mediohispanicum* populations.

First, contemporary gene flow can occur via pollen dispersal (carried by pollinators between adjacent populations) or via seed dispersal (mediated by abiotic vectors). While the former process may occur at a relatively high rate (on a yearly time frame), the second is less frequent (on a decadal time frame), occurring when specific climatic conditions lead to long seeds dispersal and establishment of individuals in unusual geographic areas, thus connecting populations that are normally isolated (authors' observation). Second, low divergence is also expected if pollinators' selective redundancy constrains divergence between populations by exerting similar selective pressures. In that case, different functional groups showing similar preferences in different populations could select similar genotypes, thus leading to low divergence between populations even in absence of gene flow. Previous studies on *E. mediohispanicum* found, however, that at dornajo site, spatial variation in the identity of pollinators causes geographic mosaic of selection and local adaptation (Gómez *et al.* 2009a,b). The lack of significant IBEb found in this work suggests that, despite the existence of these selective mosaics, neutral genetic divergence is explained by factors other than pollinator composition. Contrasting with the low contribution of pollinator assemblages to plant genetic divergence, pollinator composition is an important factor explaining within-population genetic diversity in *E. mediohispanicum* (A. J. Muñoz-Pajares, F. Perfectti, J. Loureiro, M. Abdelaziz, P. Biella, M. Castro, S. Castro & J. M. Gómez, in preparation).

Differences in flowering phenology among close populations from different altitudes may further limit insect-mediated pollen flow, which will be restricted to populations with overlapping blooming. Differences among populations in flowering time may also explain the contrasting IBD patterns observed at the two fine-scale sites. Pollen flow is expected to be hindered at Dornajo, a site with steeper slope (average slope: 0.18) compared to Cortijuela, with a weaker slope (average slope: 0.09). Therefore, by promoting or hampering gene flow among neighbouring locations, environmental factors, such as topography or phenology, might override the IBD pattern at the fine scale.

The importance of using different statistical methods and genetic distance metrics

Patterns inferred using cGD and D_S were more congruent in MRM analyses than in Mantel tests. This result supports the idea that MRM provides more consistent inferences of the effects of ecological factors at the landscape level (Balkenhol *et al.* 2009). We found two main discrepancies between both genetic distance metrics in Mantel test results. First, in the IBD models, the magnitude of the correlation increases with geographic distances according to cGD but decreases according to D_S. Second, we found significant IBEa and IBP only according to cGD. These results may be explained by the fact that cGD is not a pairwise distance, but rather takes into account the structure of the genetic covariation among all study populations (see Dyer *et al.* 2010). This emphasizes the importance of using different genetic distances as metrics of genetic differentiation when accounting for spatial genetic patterns at different spatial scales. Nonetheless, numerical simulations would be needed to accurately ascertain the advantages and drawbacks of both types of metrics in approaching landscape genetic studies (e.g. the impact of incomplete sampling on correlations performed using cGD estimates).

Conclusions

Patterns of genetic differentiation along environmental gradients and across regions are complex and difficult to explain as a result of the action of a single factor. We have disentangled the relative importance of the various factors in shaping the genetic differentiation of natural populations across spatial scales by combining different statistical approaches to test competing scenarios. Although adaptive responses to local environmental conditions also result in genetic differentiation, quantifying the amount of neutral genetic variation provides valuable insights into understand how genetic variation becomes spatially structured. On the one hand, as previously found in similar studies (Orsini *et al.* 2013; Wang *et al.* 2013), we can conclude that IBD is pervasive across different spatial scales and metrics whereas the influence of environmental factors (including topography and climatic variables) changes with the geographic scale considered. In addition, the evolutionary history influences genetic distance at the large scale. On the other hand, we found a negligible influence of the pollinator assemblage on population genetic differentiation on this generalist herb, but it would be interesting to test whether specialist pollinator assemblages render similar or contrasting results. Biotic interactions are typically ignored in landscape studies, but they drive connectivity patterns via pollen and seed dispersal for many plant species (Jordano 2000). More interesting, the topology of these interaction networks changes across the landscape, and so do their effectiveness (Thompson 2005) and, therefore, it is expected that they impact the genetic distance among populations. In addition, other components of the genetic variation, such as the genetic diversity, might be more sensitive to the effect of biotic interactions, which should be explored in forthcoming studies. In spite the fact that we fail to detect any effect of the pollinator assemblage, we encourage researchers

to make an effort to integrate biotic interactions into landscape genetics studies, particularly those entailing pollen and seed dispersal services.

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Data accessibility

Data used to perform this study have been uploaded as online Supporting information.

Field sampling was performed by A.J.M.-P., M.A.M., J.M.G., F.P. and J.B.; Individual plants were genotyped by A.J.M.-P.; Statistical analyses were performed by A.J.M.-P. and C.G. All authors contributed to writing the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Scenarios set in this study to test the relationship between genetic distance and geographic, ecological and phylogenetic factors.