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Ecological and historical determinants of population genetic structure and diversity in the Mediterranean shrub *Rosmarinus officinalis* (Lamiaceae)

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Population genetic studies of widespread Mediterranean shrubs are scarce compared with those of trees and narrow endemics or studies from phylogeographical perspectives, despite the key role these species may play in Mediterranean ecosystems. Knowledge on the effect of ecological factors in shaping their genetic patterns is also limited. In this study we investigate genetic diversity and population structure across 18 populations of *Rosmarinus officinalis*, a Mediterranean shrubland plant. Populations were sampled along two elevational gradients, one each on calcareous and siliceous soils in a mountain system in the eastern Iberian Peninsula, to decipher the effect of ecological factors on the genetic diversity and structure based on 11 microsatellite loci. We found overall high levels of genetic diversity and weak population structure. Genetic diversity increased with elevation, whereas population differentiation was stronger among populations growing on siliceous soils. The nested analysis of elevational gradients within soil types revealed that these general patterns were mostly driven by siliceous populations, whereas calcareous populations were more homogeneous along elevational belts. Bayesian analysis of population structure revealed genetic membership of lowland and high-elevation populations to different genetic clusters and a higher admixture of intermediate-elevation populations to both clusters. High-elevation populations were less differentiated from a hypothetical ancestral cluster, suggesting the persistence of their gene pool during the Pleistocene glaciations. In contrast, lowland populations resulted from more recent divergence. We propose that life-history and reproductive traits mostly contribute to explain the high levels of genetic diversity and weak population structure, whereas ecological and historical factors mostly contribute to the stronger differentiation of siliceous populations and a rapid expansion of *R. officinalis* on calcareous soils possibly mediated by human landscape transformations.

ADDITIONAL KEYWORDS: ecological gradients – elevational gradients – gene flow – microsatellite – rosemary – soil type

INTRODUCTION

The Mediterranean basin is one of the major hotspots of plant biodiversity (Médail & Quézel, 1997). Climatic and geological characteristics have shaped the assembly of its flora and have played a major role during the evolutionary history of plant lineages (Hewitt, 1996, 1999; Médail & Diadema, 2009; Molina-Venegas *et al.*, 2014). Many molecular studies have addressed the phylogeography of woody and herbaceous Mediterranean plant species and have revealed complex patterns of colonization, population expansion and local extinction or survival in refugia in relation to Quaternary glaciations (e.g. Migliore *et al.*, 2012; Martínez-Nieto

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et al., 2013; García-Castaño *et al*., 2014) or have addressed population genetic studies of Mediterranean narrow endemics from a conservation perspective (e.g. Migliore *et al*., 2011). However, few studies have addressed population genetic studies on widespread Mediterranean species or have investigated population genetic diversity levels in relation to ecological factors (but see Gil-López, Segarra-Moragues & Ojeda, 2014; Giovino *et al*., 2014), despite the key role these species may play in Mediterranean ecosystems. Thus, an important part of species diversity and how it is influenced by climatic, geological and historical factors remains poorly understood.

The Mediterranean genus *Rosmarinus* L. (Lamiaceae) includes only three species. Two, the Iberio-Maghrebian endemic *R. eriocalix* Jord. & Fourr. and the southern Iberian, narrow endemic *R. tomentosus* Hub.-Mor. & Maire., have narrow distribution areas and are known from few populations (Morales, 2010). In contrast, the third species, *R. officinalis* L., is widespread and ubiquitous in western Mediterranean shrublands from sea level to 1600 m a.s.l., where it colonizes more luxuriantly calcareous soils (Morales, 2010). All three species are small, aromatic evergreen shrubs with small white to blueish hermaphrodite flowers.

Rosmarinus officinalis has been traditionally valued and cultivated for its medicinal, culinary and ornamental properties. The distributional and ecological amplitude of this major constitutive species of Mediterranean shrublands is paralleled by extensive variation in morphology. Some attempts have been made to establish morphological boundaries to this variation from a taxonomic point of view (Ferrer-Gallego *et al*., 2014). However, other authors have considered all subordinate taxa as part of an extraordinarily high phenotypic plasticity of the species (Morales, 2010). Variation in flower size has also been observed in elevational gradients, with plants growing at higher elevations producing larger corollas (Herrera, 2005). This clinal variation has been associated with the less stressful ecological conditions at higher elevations allowing for larger corollas, potentially coupled with local adaptation to larger-bodied pollinator guilds (Herrera, 2005). Biochemical studies have noted the correlation among particular habitat environmental variables and the quantitative and qualitative composition in essential oils, resulting in different ecologically segregated chemotypes (Zaouali *et al*., 2005). Similarly, population differentiation according to allozyme markers was also correlated to population differentiation based on essential oils (Zaouali & Boussaïd, 2008).

Whether morphological variation and ecological amplitude are the consequence of similarly high levels of genetic variation and how life-history traits affect genetic diversity and structure have not yet been addressed in *R. officinalis*. Phylogeographical studies based on plastid microsatellites, however, revealed a small number of haplotypes across the distribution range of the species (Mateu-Andrés *et al*., 2013), which is consistent with a rapid population expansion scenario but is in strong contrast to other studies indicating higher amounts of genetic variation at nuclear loci (Zaouali & Boussaïd, 2008; Segarra-Moragues & Gleiser, 2009). *Rosmarinus officinalis* populations inhabit fire-prone habitats and are therefore likely to have short generation spans. This, combined with an extended flowering phenology, massive blooming and generalist pollination, could influence genetic diversity, gene flow and population structure at local and wider spatial scales (Hamrick & Godt, 1996).

In this study we investigate genetic diversity and population structure in *R. officinalis* across ecological gradients, established according to elevation and soil characteristics (calcareous vs. siliceous habitats). We restrict the study to one mountain system in the eastern Iberian Peninsula to decrease potential phylogeographical effects on the observed levels of genetic variation. We hypothesize that environmental heterogeneity related to edaphic variation and elevation can impact the population genetics of *R. officinalis* through the combined effects of historical processes and differential selection in different habitats, potentially leading to local adaptation or variation in reproductive traits. Specifically we test if genetic diversity and population structure have been affected by three types of factors. (1) Calcareous and siliceous soils are characterized by different physical and chemical attributes and these are likely to affect plant colonization and fitness. A number of studies have reported that variation in chemotypes in Mediterranean aromatic plants, including *R. officinalis*, is among other factors related to hydric stress and edaphic factors and that this variation is correlated to genetic diversity (Gouyon *et al*., 1986; Zaouali *et al*., 2005; Figueiredo *et al*., 2008; Zaouali & Boussaïd, 2008). In the study area siliceous substrates occur as fragmented outcrops embedded in a continuous matrix of calcareous substrates. Thus, in this regional context, siliceous areas can be considered as edaphic islands with different ecological and spatial characteristics compared with calcareous areas. Accordingly, the stronger fragmentation of siliceous areas and different selective pressures would favour higher differentiation among their populations compared with populations on calcareous habitats. This pattern is likely to be favoured because of a combination of geographical isolation and local adaptation. (2) Historical demographic changes during Quaternary glaciation could have produced clinal variations in genetic diversity. However, although our study area is unlikely to have been affected directly by ice sheets

during the Last Glacial Maximum (LGM), it is assumed that the bioclimatic belts descended about 1000 m in elevation as a result of the drop in temperatures (Badal-García *et al*., 2012) and high-elevation populations of *R. officinalis* could thus be the result of post-glacial colonization. (3) Plant phenology, reproductive traits (such as corolla size) and pollinator guilds are likely to vary across elevational gradients (Herrera, 2005; Giménez-Benavides, Escudero & Iriondo, 2007). Such variation could contribute to reproductive isolation between populations at the two elevational extreme ranges (i.e. lowland and high-elevation populations), thereby increasing genetic differentiation between the groups. However, populations at intermediate elevations may play a crucial role in establishing the connection between lowland and high-elevation populations, thus blurring genetic differentiation between elevational groups, if variation in ecological and reproductive traits occurs continuously along the elevational gradient.

MATERIAL AND METHODS

Population sampling

Eighteen populations of *R. officinalis* were selected along an elevational gradient in the eastern Iberian Peninsula (Fig. 1). The selected populations were similar in extension and were composed of several thousand individuals. Population sampling of *R. officinalis* in this area was structured so as to include as much climatic and ecological variability as possible. Thus, six populations were sampled in each of three bioclimatic belts as defined by Costa (1982): thermo-Mediterranean (*ThM*) (0–450 m a.s.l.), meso-Mediterranean (*MsM*) (450–950 m.a.s.l.) and supra-

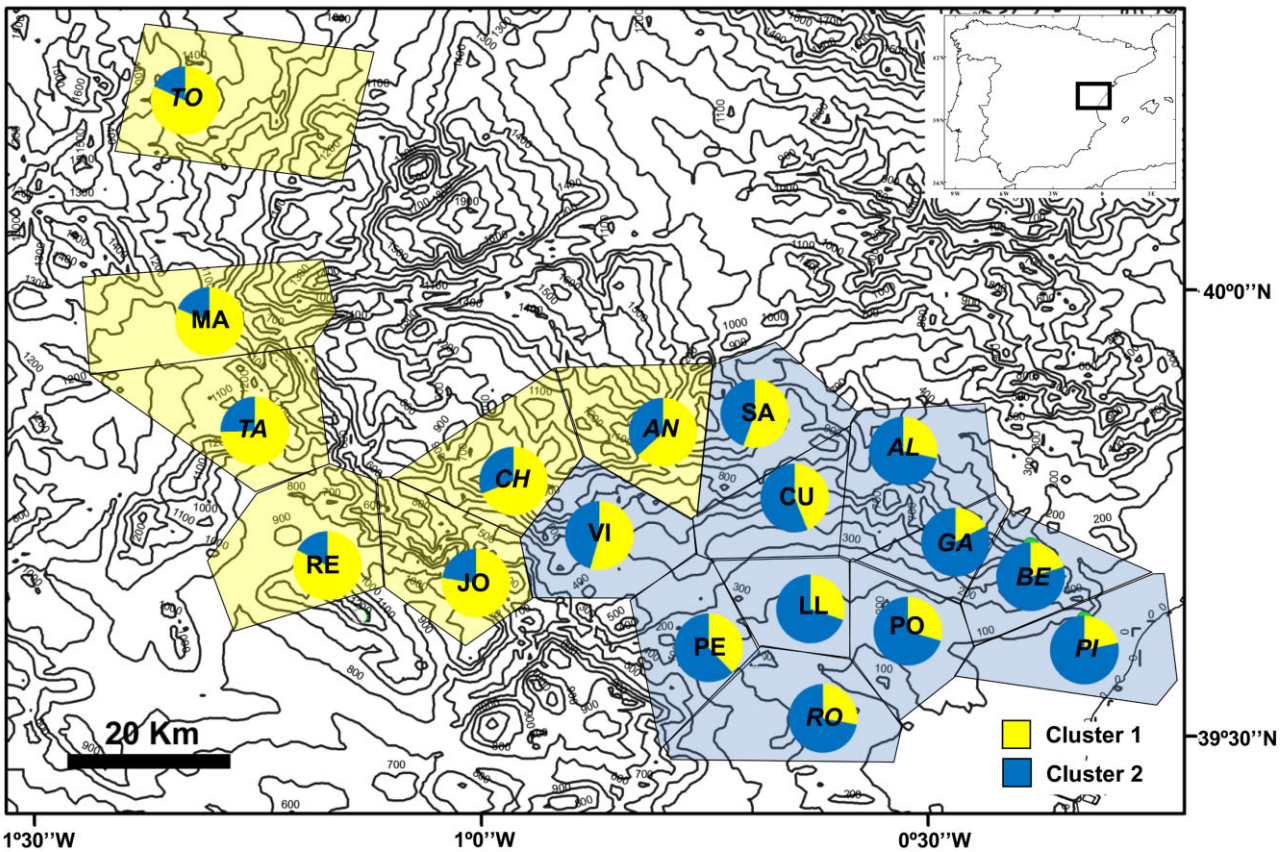

Figure 1. Geographical location and population genetic structure of the 18 sampled populations of *Rosmarinus officinalis* resulting from STRUCTURE and BAPS analyses. The proportion of membership to two genetic clusters ($K = 2$) inferred from Bayesian analysis with STRUCTURE is indicated by pie charts for each population. Voronoi tessellation for the $K = 2$ most likely number of genetic clusters obtained from BAPS has been projected on the map. Thermo-Mediterranean, calcareous (*ThM-Ca*): LL, PE, PO; thermo-Mediterranean, siliceous (*ThM-Si*): BE, PI, RO; meso-Mediterranean, calcareous (*MsM-Ca*): CU, JO, VI; meso-Mediterranean, siliceous (*MsM-Si*): AL, CH, GA; supra-Mediterranean, calcareous (*SuM-Ca*): MA, RE, SA; supra-Mediterranean, siliceous (*SuM-Si*): AN, TA, TO. Population codes are as in Table 1. Elevations in the studied area are represented by 100-m a.s.l. curves.

Mediterranean (*SuM*) (950–1600 m a.s.l.). The last of these includes the upper limit of climatic tolerance of *R. officinalis*. Within each bioclimatic belt, three populations were sampled on calcareous (Cretaceous and Jurassic limestone-derived) soils (*Ca*) and three on siliceous (Triassic sandstone-derived) soils (*Si*).

DNA extraction, microsatellite amplification

Leaves from 96 individuals in each population (totalling 1728 individuals) were collected for microsatellite genotyping and dried in silica gel until DNA extraction. High levels of microsatellite allelic diversity in *R. officinalis* were anticipated by Segarra-Moragues & Gleiser (2009) and large sample sizes within populations were thus deemed necessary to avoid biased estimates of genetic diversity due to small sample sizes. The geographical coordinates of each individual were recorded using a GPS (PC5L; Corvallis Microtechnology). Approximately 50 mg dry weight per sample was used for DNA extraction. Dry material was reduced to fine powder using 2.3-mm stainless steel beads in a Mixer Mill MM400 cell disrupter (Retsch, Biometa Tecnología y Sistemas). DNA was extracted using the Invisorb DNA plant HTS 96 Kit (Stratec Molecular) and eluted in 50 μL Tris-EDTA 0.1 × buffer. Crude DNA extracts were used for PCR amplification without dilution. Individuals were genotyped for 11 nuclear microsatellite loci from those described by Segarra-Moragues & Gleiser (2009) in four multiplex groups: I, Roff101-6FAM, Roff135-NED and Roff203-HEX at 61 °C annealing temperature; II, Roff237-PET, Roff246-VIC and Roff335-PET at 55 °C annealing temperature; III, Roff405-NED, Roff424-HEX and Roff438-HEX at 56 °C annealing temperature; and IV, Roff515-6FAM and Roff850-NED at 55 °C annealing temperature. PCRs were performed in a 20-μL volume including 1× Taq Buffer (Biotools), 2 mM $MgCl_2$, 0.4 mM each dNTP, 0.5 mg mL^{-1} bovine serum albumin, 3–5 pmol forward and reverse primers depending on locus and 1.5 U Taq DNA polymerase (Biotools). PCRs were carried out on PE2720 (Applied Biosystems) and PTC100 (MJ Research) thermal-cyclers and the programme followed Segarra-Moragues & Gleiser (2009), except for annealing temperatures indicated above for multiplex amplification. PCR products of multiplex groups were combined for two separate runs on an ABI3730 automated sequencer (Applied Biosystems) using ROX400HD (multiplex groups I + III) or LIZ500 (multiplex groups II + IV) as internal lane size standards. Fragments were assigned to allele classes using Genemarker v. 1.85 software (Softgenetics). Possible effects of amplification artefacts (e.g. null alleles) and patterns of selection as potential biases to allele frequencies among loci and populations were investigated in our genotypic matrices using MICRO-CHECKER v. 2.2.3 (van Oosterhout *et al.*, 2004) and LOSITAN (Antao *et al.*, 2008), respectively. LOSITAN software was used to perform F_{ST} outlier tests as described by Beaumont & Nichols (1996), using the 'neutral mean F_{ST}' option, and analyses were conducted for the whole dataset and for separate population groups according to soil types (*Ca*, *Si*) and elevational belts (*ThM*, *MsM* and *SuM*).

Analysis of microsatellite data

Allele frequencies, mean number of alleles per locus (N_A) and observed (H_O) and unbiased expected (H_E) heterozygosities (Nei, 1978) were calculated for each population using GENETIX v. 4.05 (Belkhir *et al.*, 2004). Wright's *F*-statistics were estimated according to Weir & Cockerham (1984) using GENEPOP'007 (Rousset, 2008) and tested for significance by Fisher's exact tests. This software was also used to check for departures from Hardy–Weinberg equilibrium (HWE) at each locus using Fisher's exact tests.

Groups of predefined populations according to soil type (*Ca* vs. *Si*), bioclimatic belts (*ThM* vs. *MsM* vs. *SuM*), and combined soil and bioclimatic attributes were tested for significant differences in pairwise comparisons of genetic diversity and population structure indices (*A*, average allelic richness per locus; H_O, observed heterozygosity; H_S, genetic diversity within populations; F_{IS}, inbreeding coefficient; and F_{ST}, population differentiation) using FSTAT v. 2.9.3.2 (Goudet, 2001). Significance of tests ($P < 0.05$) was assessed by permuting populations 1000 times between the predefined groups.

Population genetic structure was investigated through a Bayesian analysis implemented in STRUCTURE v. 2.1 (Pritchard, Stephens & Donnelly, 2000). Our analyses were based on an admixture ancestral model with correlated allele frequencies for a range of *K* values from 1 to 20 (the number of populations considered plus 2) to determine the optimal number of genetic clusters (*K*) according to Evanno, Regnaut & Goudet (2005). The proportion of membership of each individual and population to the inferred *K* clusters were then calculated. We used a burn-in period and a run length of the Monte Carlo Markov chain (MCMC) of 1×10^5 and 1×10^6 iterations, respectively. Ten runs were carried out for each *K* in order to quantify the amount of variation of the likelihood. Additionally, we used BAPS (Bayesian analysis of population structure) v. 5.4 (Corander & Marttinen, 2006), which uses stochastic optimization instead of MCMC to find the optimal number of *K* clusters. We performed ten replicates for each possible *K*. The analyses were performed with the spatial clustering of groups module, which takes into account

the geographical location of populations during the estimation of genetic assignments.

Fine-scale spatial genetic structure (SGS) was studied using the computation of pairwise kinship coefficients between all sampled individuals as a measure of the relatedness between individuals. Average and standard error in kinship coefficients were calculated using jackknifing over loci as correlations between allelic states, as indicated by Loiselle *et al*. (1995) using SPAGeDi v. 1.3 (Hardy & Vekemans, 2002). Twelve spatial distance classes were established, in which each distance class varied in length to account for a similar number of pairwise comparisons, covering the minimum and maximum pairwise spatial distances of sampled individuals.

Isolation by distance (IBD) was assessed by matrix correlation analyses between a matrix of all pairwise linearized F_{ST} values [i.e. $F_{ST}/(1 - F_{ST})$; Slatkin (1995)] computed with ARLEQUIN v. 3.5 (Excoffier & Lischer, 2010) and a matrix of log-transformed pairwise geographical distances between populations and separately for calcareous and siliceous population groups. Significance of the correlation was tested in each correlation analysis with 1000 permutation Mantel tests using NTSYSpc v. 2.1 (Rohlf, 2002).

RESULTS

Genetic diversity in *R. officinalis* populations

The 11 microsatellite loci were polymorphic in all 18 studied populations of *R. officinalis* totalling 231 different alleles (data stored on Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.55p22 and available upon request). The number of alleles per locus ranged from five (Roff237) to 34 (Roff135, Roff438 and Roff850), with a mean of 21 ± 11.4. The mean number of alleles per population was similar across populations, ranging from a minimum of 12.64 for *ThM-Si* PI population to a maximum of 15.27 for *MsM-Ca* CU population (Table 1). Average observed heterozygosities (H_O) ranged from 0.599 (population AN) to 0.660 (population SA) and unbiased expected heterozygosities (H_E) ranged from 0.760 (population PI) to 0.794 (population MA; Table 1). All populations showed significant deviation of HWE towards heterozygote deficiency, with positive F_{IS} ranging from +0.149 (population SA) to +0.233 (population AN, Table 1). Such significant heterozygote deficiency may indicate the presence of null alleles in some of the genotyped loci. The MICRO-CHECKER analysis detected the potential presence of null alleles in four to eight loci (5.89 in average) in the studied populations. However, only three loci (Roff101, Roff246 and Roff515) were identified as potentially affected by null alleles in all populations. Notwithstanding these estimations, our large number of genotyped individuals did not support a high incidence of null alleles, as no null homozygotes (i.e. no amplification failures) were detected in any of the genotyped loci. In addition, the estimation of null allele frequencies may be inaccurate in populations with strong deviations from HWE, as in our case, where no null homozygotes have been observed (Dabrowski *et al*., 2015). Furthermore, ongoing reproductive biology studies on six of these populations and based on large progeny arrays genotyped for this same set of microsatellite loci (unpublished results by the authors) did not reveal the presence of null alleles. Thus, even if null alleles exist in the studied populations for these microsatellite loci, their impact on the observed genetic patterns is expected to be negligible. Tunisian populations of *R. officinalis* genotyped at allozyme loci (Zaouali & Boussaïd, 2008) revealed similar levels of inbreeding as those found here, indicating that factors other than technical reasons may account for the observed patterns and that high F_{IS} values may be a general characteristic of *R. officinalis* populations throughout its distribution.

LOSITAN analyses revealed that for the whole dataset one locus (Roff246) was affected by divergent selection and another was affected by balancing selection (Roff850; Supporting Information Fig. S1A). Independent analyses with populations grouped according to soil types revealed that three loci (Roff101, Roff246 and Roff438) were potentially affected by divergent selection in *Si* populations but none was affected in *Ca* populations (Supporting Information Fig. S1B, C). Regarding elevational belts, locus Roff246 was potentially affected by divergent selection in *ThM* and *MsM* populations but none was affected by selection in *SuM* populations (Supporting Information Fig. S1D–F).

Genetic diversity indices did not differ among populations grouped according to soil characteristics (Table 2A). However, genetic diversity within populations tended to increase with elevation; those at lower elevations (*ThM*) showed significantly lower genetic diversity ($H_S = 0.770$ vs. $H_S = 0.781$, $P < 0.05$) than those at higher elevations (*SuM*) and intermediate elevation populations (*MsM*) did not differ significantly from populations at both elevation extremes (Table 2B). The comparison of populations grouped according to bioclimatic belts and nested within soil types revealed that elevation had no significant effect on genetic diversity of calcareous populations (Table 2C). Conversely, in siliceous populations allelic richness and genetic diversity within populations increased with elevation (Table 2D), indicating that the overall patterns observed in the comparison of population groups according to bioclimatic belts were largely influenced by the soil environment.

Table 1. Population data and genetic diversity indices in 18 populations of *Rosmarinus officinalis* based on 11 microsatellite loci

Code	Population (voucher)	Geographical coordinates		Elevation	Bioclimatic belt	Soil type	N_A	H_O	H_E	F_{IS}
		Latitude (N)	Longitude (W)	(m a.s.l.)						
LL	Lliria (VAL-227059)	39°38′18.99″	0°36′38.08″	200	*ThM*	Ca	14.55	0.617 ± 0.177	0.769 ± 0.178	+0.199***
PE	Pedralba (VAL-217230)	39°35′05.27″	0°41′30.76″	210	*ThM*	Ca	13.91	0.655 ± 0.145	0.786 ± 0.175	+0.167***
PO	Bétera, Porta-Coeli (VAL-227061)	39°39′43.02″	0°29′35.48″	240	*ThM*	Ca	14.64	0.623 ± 0.177	0.777 ± 0.191	+0.198***
CU	Alcublas, masía Cucalón (VAL-227063)	39°46′30.06″	0°38′27.70″	665	*MsM*	Ca	15.27	0.651 ± 0.161	0.786 ± 0.190	+0.173***
JO	Calles, fuente Jórgola (VAL-227065)	39°40′04.16″	0°59′08.92″	690	*MsM*	Ca	13.64	0.616 ± 0.204	0.780 ± 0.194	+0.212***
VI	Villar del Arzobispo (VAL-227062)	39°43′29.94″	0°51′15.19″	695	*MsM*	Ca	14.09	0.606 ± 0.189	0.781 ± 0.183	+0.225***
SA	Sacañet (VAL-227064)	39°51′27.14″	0°42′45.58″	995	*SuM*	Ca	14.09	0.660 ± 0.195	0.775 ± 0.202	+0.149***
MA	Manzaneruela (VAL-227068)	39°56′59.41″	1°18′49.57″	1050	*SuM*	Ca	14.36	0.646 ± 0.199	0.794 ± 0.186	+0.187***
RE	Benagéber, ermita del Remedio (VAL-105575)	39°38′04.01″	1°08′08.17″	1240	*SuM*	Ca	13.73	0.617 ± 0.221	0.770 ± 0.204	+0.200***
PI	Puçol, monte Picaio (VAL-199443)	39°37′52.27″	0°19′29.86″	110	*ThM*	Si	12.64	0.635 ± 0.180	0.760 ± 0.188	+0.164***
RO	Villamarxant, Les Rodanes (VAL-227058)	39°32′13.48″	0°36′30.88″	240	*ThM*	Si	13.18	0.604 ± 0.157	0.763 ± 0.186	+0.209***
BE	Beselga (VAL-2906)	39°42′50.32″	0°23′07.79″	285	*ThM*	Si	13.36	0.623 ± 0.161	0.770 ± 0.184	+0.192***
AL	Altura (VAL-227060)	39°48′25.04″	0°31′39.05″	650	*MsM*	Si	14.18	0.629 ± 0.153	0.782 ± 0.170	+0.197***
CH	Chelva (VAL-227066)	39°46′06.88″	0°57′37.30″	655	*MsM*	Si	13.91	0.614 ± 0.188	0.774 ± 0.187	+0.208***
GA	Gátova (VAL-53557)	39°43′56.80″	0°28′14.15″	750	*MsM*	Si	13.55	0.622 ± 0.174	0.764 ± 0.203	+0.186***
TA	Talayuelas (VAL-227067)	39°49′02.03″	1°15′36.01″	990	*SuM*	Si	15.09	0.640 ± 0.182	0.793 ± 0.170	+0.193***
AN	Andilla (VAL-71956)	39°50′49.26″	0°47′35.09″	1055	*SuM*	Si	15.00	0.599 ± 0.206	0.781 ± 0.209	+0.233***
TO	Tormón (VAL-227069)	40°13′44.31″	1°19′33.50″	1200	*SuM*	Si	14.09	0.619 ± 0.212	0.776 ± 0.188	+0.203***

ThM, thermo-Mediterranean; *MsM*, meso-Mediterranean; *SuM*, supra-Mediterranean; Ca, calcareous, Si, siliceous. Significance: ***$P < 0.001$.

Table 2. Comparison of genetic diversity and differentiation between *R. officinalis* populations grouped according to: A, soil type; B, bioclimatic belt; and C, D, bioclimatic belts within soil types

A		Soil		B	Bioclimatic belt		
		Calcareous ($N = 9$)	Siliceous ($N = 9$)		*ThM* 110–285 m a.s.l. ($N = 6$)	*MsM* 650–750 m a.s.l. ($N = 6$)	*SuM* 990–1240 m a.s.l. ($N = 6$)
	A	14.25[a]	13.89[a]		13.71[a]	14.11[a]	14.39[a]
	H_O	0.631[a]	0.620[a]		0.625[a]	0.622[a]	0.629[a]
	H_S	0.780[a]	0.773[a]		**0.770[a]**	**0.777[ab]**	**0.781[b]**
	F_{IS}	0.190[a]	0.199[a]		0.188[a]	0.200[a]	0.195[a]
	F_{ST}	**0.016[a]**	**0.024[b]**		0.016[a]	0.018[a]	0.019[a]
C		Calcareous		D	Siliceous		
		ThM 200–240 m a.s.l. ($N = 3$)	*MsM* 665–695 m a.s.l. ($N = 3$)	*SuM* 995–1240 m a.s.l. ($N = 3$)	*ThM* 110–285 m a.s.l. ($N = 3$)	*MsM* 650–750 m a.s.l. ($N = 3$)	*SuM* 990–1200 m a.s.l. ($N = 3$)
	A	14.36[a]	14.33[a]	14.06[a]	**13.06[a]**	**13.88[ab]**	**14.73[b]**
	H_O	0.632[a]	0.624[a]	0.641[a]	0.621[a]	0.622[a]	0.620[a]
	H_S	0.778[a]	0.783[a]	0.780[a]	**0.765[a]**	**0.774[ab]**	**0.784[b]**
	F_{IS}	0.188[a]	0.203[a]	0.179[a]	0.188[a]	0.197[a]	0.210[a]
	F_{ST}	0.009[a]	0.009[a]	0.016[a]	0.022[a]	0.021[a]	0.019[a]

Values of genetic diversity indices which showed statistically significant values ($P < 0.05$) based on 1000 permutations for some of the compared population groups are indicated in bold. Different superscript letters indicate statistically significant ($P < 0.05$) differences in pairwise comparisons of population groups.
ThM, thermo-Mediterranean; *MsM*, meso-Mediterranean; *SuM*, supra-Mediterranean. *N*, number of populations, each composed of 96 individuals analysed.

POPULATION GENETIC STRUCTURE IN *R. OFFICINALIS* POPULATIONS

The estimation of the most likely number of genetic clusters (K) in *R. officinalis* following Evanno *et al*. (2005) revealed a maximum modal value of $\Delta K = 182.43$ for $K = 2$. In this clustering, *ThM* populations had a higher proportion of membership to cluster 2, whereas *SuM* populations had a higher proportion of membership to cluster 1 (Fig. 1). *MsM* populations showed diverse patterns with some populations showing a higher membership to cluster 2 (populations CU, AL, GA) or to cluster 1 (populations JO, VI, CH), indicating a closer relationship to *ThM* or *SuM* populations, respectively (Fig. 1). The mean F_{ST} values of divergence between clusters 1 and 2 from a hypothetical ancestral population were 0.016 and 0.024, respectively, with the populations of cluster 1 showing lower divergence from the hypothetical ancestral population. This indicates that the *ThM* populations are of more recent origin.

Results from the Bayesian analyses of population structure conducted with BAPS converged with STRUCTURE at $K = 2$ as the most likely number of genetic clusters. Also in agreement with STRUCTURE, *ThM* populations were assigned to cluster 2, whereas *SuM* populations were assigned to cluster 1 (Fig. 1). Clustering of *MsM* populations was also consistent in both analyses except for the VI population, which showed a higher proportion of membership to cluster 1 in STRUCTURE but was assigned to cluster 2 in BAPS (Fig. 1).

The maximum positive average values of the kinship coefficient were obtained at the shortest distance class that accounted for within-population comparisons (Fig. 2). At this distance class, siliceous populations had a higher kinship value than calcareous populations (0.0232 ± 0.004 vs. 0.0144 ± 0.0015). These values decreased progressively and reached zero at distances 18.79 and 23.91 km for individuals sampled, on calcareous and siliceous soils, respectively (Fig. 2). The autocorrelogram including pairwise kinship values between all sampled individuals followed this same trend, reaching zero at an intermediate value (22.23 km) between those obtained for individuals from calcareous and siliceous soils (Fig. 2).

Populations of *R. officinalis* showed significant isolation by distance as estimated by the correlation of pairwise geographical distances and pairwise linearized F_{ST} values ($r = 0.676$, $P = 0.001$, Fig. 3A). This same pattern was found for populations growing on calcareous ($r = 0.673$, $P = 0.002$) and siliceous ($r = 0.719$, $P = 0.001$) soils (Fig. 3B). For equivalent geographical distances, populations on siliceous soils showed a stronger IBD compared with calcareous populations (Fig. 3B). Accordingly, the comparison of F_{ST} values showed significantly higher differentiation of siliceous populations compared with calcareous populations (Table 2A). Generally, population differentiation increased with elevation; however, differences between bioclimatic belts were not significant (Table 2B). This result may be the consequence of the different genetic differentiation patterns of populations on calcareous and siliceous soils. In calcareous

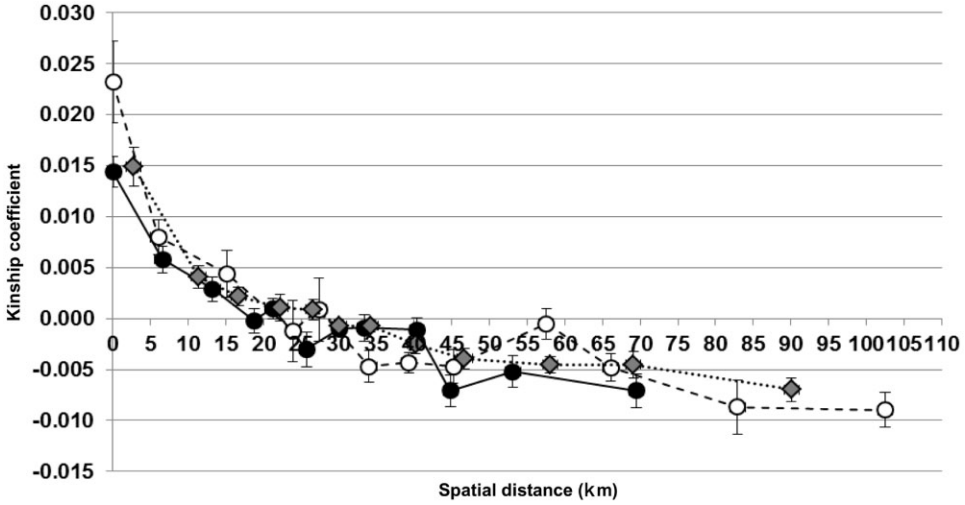

Figure 2. Spatial autocorrelogram based on kinship coefficients of Loiselle *et al*. (1995) (y-axis) for individual pairs of *Rosmarinus officinalis* at 12 distance classes (x-axis). Bars indicate standard errors estimated by jackknifing over loci. Grey diamonds: pairwise comparison for all sampled individuals; black circles, individuals on calcareous populations only; open circles, individuals on siliceous populations only.

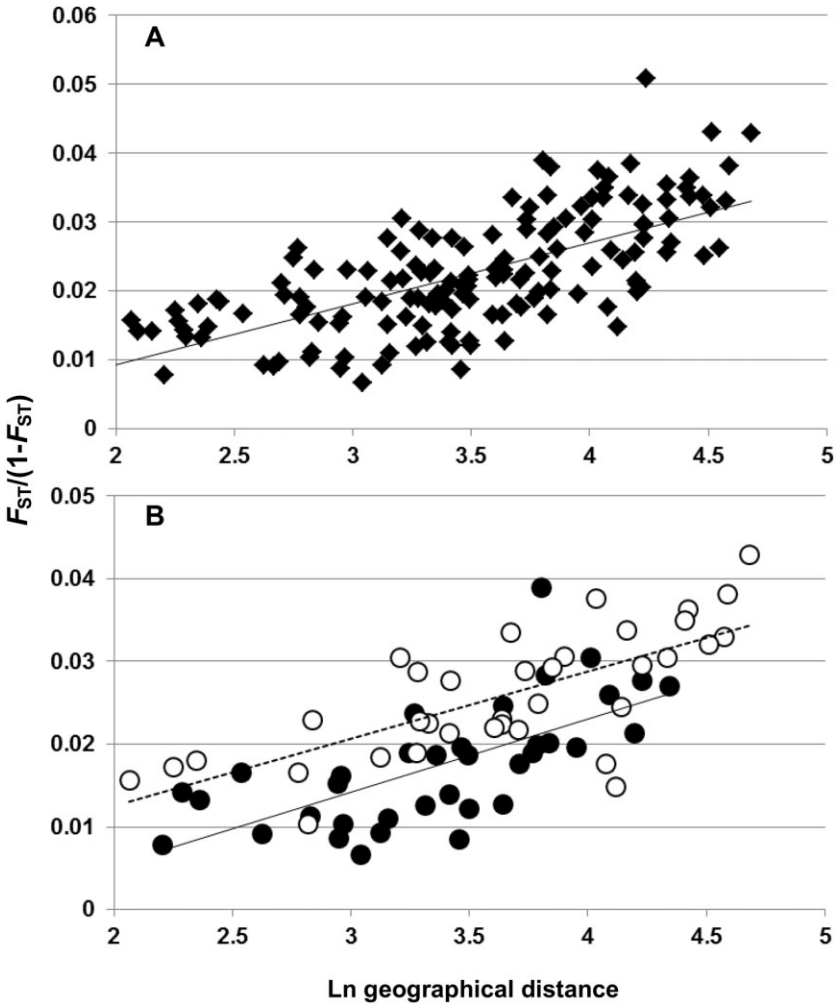

Figure 3. Isolation by distance analysis in *Rosmarinus officinalis*. Correlation between log-transformed pairwise geographical (*x*-axis) and linearized F_{ST} (Slatkin, 1995) pairwise values (*y*-axis). A, pairwise comparisons across all populations ($r = 0.676$, $P = 0.001$). B, pairwise comparisons of calcareous (black dots) and siliceous (white dots) populations. Correlation between matrices was $r = 0.673$, $P = 0.002$, and $r = 0.719$, $P = 0.001$ for calcareous and siliceous populations, respectively; *P* values are reported after 1000 random permutation Mantel tests.

populations genetic differentiation increased with elevation, with *ThM* and *MsM* showing considerably low F_{ST} values, whereas in siliceous populations genetic differentiation values were higher at the three bioclimatic belts and remained almost constant with increasing elevation (Table 2D).

DISCUSSION

Influence of biological and ecological factors on the genetic diversity of *R. officinalis* populations

Despite the overwhelming abundance of shrubs in plant communities around the Mediterranean basin, there is a generalized lack of knowledge of their population genetics compared with trees and perennial herbs (Conord, Gurevitch & Fady, 2012; but see Gil-López *et al*., 2014; García-Verdugo *et al*., 2015). Similarly, many more population genetic studies have been undertaken on Mediterranean narrow endemics than on widespread taxa, despite the key ecological roles the latter play in Mediterranean ecosystems.

Our study has revealed high levels of genetic diversity in *R. officinalis* (Table 1). Such high levels of genetic diversity may be favoured by a combination of life-history and reproductive traits in this Mediterranean shrub. On the one hand, the moderately short life-span of this non-sprouting species, which inhabits fire-prone ecosystems, would tend to shorten genera-

tion times and accelerate population turnover, thereby increasing genetic diversity within populations (Hamrick & Godt, 1996; Segarra-Moragues & Ojeda, 2010). Seed germination occurring during fire intervals (allowing for generation overlap) and massively from the fire-triggered soil seed bank (Moreira *et al*., 2010) would contribute to increase genetic diversity within populations (Hamrick & Godt, 1996; Segarra-Moragues & Ojeda, 2010). Notwithstanding this, the hermaphrodite flowers (allowing for geitonogamy) coupled with the massive flowering of *R. officinalis* could lead to high selfing rates. Accordingly, we found significant heterozygote deficiency in all studied populations (Table 1). It has been observed, however, that seed set in *R. officinalis* is highly sensitive to inbreeding depression, suggesting a strong post-zygotic purge of inbred embryos (Hidalgo-Fernández & Ubera-Jiménez, 2001). The eventual occurrence of gynodioecy in *R. officinalis* has been proposed as a mechanism to prevent selfing. However, the genetic determination of gynodioecy in *R. officinalis* is unclear and may include at least the implication of mitochondrial genes and environmental variables, as the proportion of gynodioecious versus hermaphrodite flowers in *R. officinalis* varies between individuals and time (Ubera-Jiménez & Hidalgo-Fernández, 1992; Hidalgo-Fernández *et al*., 1999; personal observation of the authors). This will not completely exclude geitonogamy or biparental inbreeding and thus cannot be considered a major driver of within-population genetic diversity levels.

We found an influence of the elevational gradient on genetic diversity levels across populations. Allelic richness and heterozygosity levels progressively increased with elevation (Table 2B, C). This is surprising for a thermophilous Mediterranean species with a lowland ecological optimum that would suggest higher genetic diversity in *ThM* populations compared with *SuM* ones. This may be the outcome of different evolutionary (i.e. higher mutation rates), mating (i.e. higher outcrossing rates) or historical (i.e. an earlier origin of high-elevation populations) processes to explain the increased genetic diversity with increasing elevation. The separate analysis of populations from elevational gradients revealed different patterns with respect to the soil characteristics (*calcareous* vs. *siliceous*). Populations on siliceous soils followed the trend in genetic diversity described above, whereas calcareous populations were homogeneous in their genetic diversity levels with respect to elevation (Table 2C). This indicates that our observed overall genetic patterns in *R. officinalis* populations were governed by siliceous populations and that populations growing on different soils were affected differently by the same or different evolutionary or demographic processes. Calcareous areas currently occupied by *R. officinalis* have been subject to intense agricultural practices and forest management during recent decades. Many of these populations occur in long abandoned cultivated areas or degraded oak and pine forests, especially at low (*ThM*) or intermediate (*MsM*) elevations. It is likely that a rapid spread of *R. officinalis* into these areas accounts for the lack of clinal variation of genetic diversity levels with elevation. In contrast, populations growing on siliceous soils are expected to have had more stable demographic histories with a lower impact of agricultural practices or other anthropogenic disturbances (García-Fayos, 1991). In the sampled range, siliceous outcrops are scattered and give rise to sloping, arid, stony, nutrient-poor soils (Rubio, Sánchez & Forteza, 1995). It is likely that selective pressures associated with soil characteristics differ between *Ca* and *Si* populations. An association between the amount and composition of volatile organic compounds and ecological conditions (including soil types) has been observed in *R. officinalis* (Zaouali *et al*., 2005; Zaouali & Boussaïd, 2008) and this association may be related to hydric stress (Figueiredo *et al*., 2008) or nutrient stress induced by the different physical and chemical properties of siliceous soils (Ormeño *et al*., 2008). Notably, the examination of loci potentially under selection revealed that three loci in *Si* populations were potentially affected by divergent selection whereas none was in *Ca* populations (Suporting Information Fig. S1B, C). Thus, selective sweeps at these loci could have contributed to the observed pattern. By contrast, our analysis of isolation by distance revealed a significant correlation of genetic and geographical distances (Fig. 3). A stepping-stone model, in which gene flow is a function of geographical distance, is likely to produce such a pattern if populations are at equilibrium. However, if populations have recently expanded into new areas, such a pattern may not have had time to emerge (Slatkin, 1993). The separate IBD analysis of calcareous and siliceous populations revealed, however, a stronger IBD in siliceous populations than in calcareous populations ($r = 0.719$, $P = 0.001$ vs. $r = 0.673$, $P = 0.002$, respectively; Fig. 3B) which is in agreement with a stronger geographical isolation of the siliceous outcrops in the area, thereby increasing kinship coefficients of individuals at short distances (Fig. 2), and a more stable demographic history.

Influence of historical factors on the elevational differentiation of *R. officinalis* populations

We found an overall weak genetic structure among populations of *R. officinalis*. This is consistent with life-history and reproductive traits such as the peren-

nial habit, the extended phenology and generalist insect pollination, favouring high levels of gene flow among populations (Hamrick & Godt, 1996) and thereby reducing differentiation among populations. Notwithstanding this, *ThM* and *SuM* populations grouped into two different genetic clusters and *MsM* populations showed varied admixture to one or the other genetic clusters in accordance with their intermediate-elevation location between both aforementioned groups (Fig. 1). STRUCTURE analysis revealed that *SuM* populations, those located at higher elevations in the sampled range, were less divergent from a hypothetical ancestral population as shown by the lower F_{ST} value of cluster 1 ($F_{ST} = 0.016$).

Historical factors, including Quaternary glaciations and the associated cyclical climatic changes, have helped to interpret the geographical patterns of distribution of genetic diversity in species of high and mid latitudes (Hewitt, 1996, 1999). However, unlike for alpine and high-elevation plants, the populations of *R. officinalis* studied here are not expected to have been exposed to the direct effect of ice sheets during the glacial periods. The effect of glaciers during the LGM did not reach significant continuous extensions further south of the Pyrenean range in the Iberian Peninsula (Ribera & Blasco-Zumeta, 1998). Nonetheless, Quaternary climatic oscillations probably affected lowland climatic regimes and lowered average temperatures during the glacial periods. Evidence of a LGM (22–18 cal. kyr BP) *SuM* vegetation occupying lowland areas under a current *ThM* climate has been obtained from the analysis of charcoal contained in archaeological sediments (Badal & Carrión, 2001; Badal-García *et al*., 2012). These charcoal remains provide evidence of the plant taxa used by human groups for fuel and reflect the palaeoecological conditions of the area under study, in particular the local flora and the characteristics of the local vegetation (Chabal, 1988). Thus, the presence of *R. officinalis* charcoal at a site can be an indicator of the local growth of the species. Besides, because wood-charcoal can be directly dated by accelerator mass spectrometry (AMS), it can aid in documenting the presence and expansion of a species in a region. Radiocarbon dates cited hereafter were newly calibrated for this study to 2 sigma using the software Calib 7.1 (Stuiver & Reimer, 1993) and the IntCal13 calibration data set (Reimer *et al*., 2013). An AMS radiocarbon date of 24 403–23 934 cal. a BP (Beta-295148) from a *R. officinalis* charcoal fragment from Cova de les Cendres (Moraira-Teulada, Alicante province, eastern Spain; Badal-García *et al*., 2012) proves its presence there during the last glacial cycle. This species was present within a conifer-dominated formation (*Pinus nigra* J.F.Arnold and a species of *Juniperus* L.) and in overlying layers dated between 20 991 and 13 513 cal. a BP (Badal & Carrión, 2001). *Pinus nigra* is a species characteristic of *SuM* vegetation that is currently absent from *ThM* and *MsM* areas and finding it in *ThM* charcoal remains with *R. officinalis* lends support to the existence of *R. officinalis* populations at least in lowland areas during the LGM. *Rosmarinus officinalis* has been also documented in other Pleistocene sequences from *ThM* areas in Iberia: Cova Bolumini in Alicante province (Badal & Carrión, 2001); and Cueva de Nerja, Hoyo de la Mina and Cueva Ambrosio in Andalusia (Badal, 1990; Rodríguez-Ariza, 2006; González-Sampériz *et al*., 2010). There is no information of vegetation from the current *MsM* and *SuM* areas during the LGM. They might have been under oro-Mediterranean conditions, as it is assumed that the bioclimatic belts descended about 1000 m in elevation, as a result of the drop in temperatures (Badal-García *et al*., 2012). Such conditions should have precluded the *in situ* persistence of *R. officinalis*; however, if the current *SuM* populations are the result of post-glacial recolonization, we could not find genetic evidence to support it. *SuM* populations showed higher genetic diversity than lowland *ThM* populations (Table 2B), whereas the contrary should be expected for populations in recolonized areas (Schönswetter *et al*., 2002; Segarra-Moragues *et al*., 2007). Bayesian analysis of population structure showed the ancestral state of these populations compared with *ThM* ones, indicating either *in situ* survival during the LGM of *SuM* populations or a rapid recolonization of *SuM* areas encompassing population sizes large enough to prevent strong genetic erosion by the effect of genetic drift.

Populations in *ThM* areas showed a higher proportion of membership to a different genetic cluster than *SuM* ones (Fig. 1) and, as indicated above, this genetic cluster appears to be of more recent divergence. This suggests the participation of different historical and evolutionary processes leading to their differentiation from populations of *SuM* areas. *ThM* areas have been subject of deep human landscape transformation including urbanization and intense agriculture expansion. These could have contributed to decreased population sizes and increased population fragmentation. Although the populations of *R. officinalis* may not be strongly sensitive to human perturbation, *ThM* populations showed lower allelic richness and significantly lower genetic diversity than *SuM* ones (Table 2B), which make them more prone to differentiation through the effect of genetic drift. This result was especially noticeable in populations on siliceous soils, where the effect of genetic drift is probably increased by the stronger natural patchiness of the habitat and by the lower fitness of the plants (Morales, 2010), compared with populations on calcareous soils (Table 2C, D).

Influence of biological factors on the elevational differentiation of *R. officinalis* populations

Reproductive biological factors could also have contributed to the observed patterns of population differentiation with increasing elevation. Indeed, Herrera (2005) showed that the size of the corolla in *R. officinalis* increased in size with increasing elevation, and we observed the same pattern in our study populations (data not shown). Herrera (2005) speculated that such clinal variation could be local adaptation to high-elevation pollinators with larger bodies, whereas more stressful ecological conditions accounted for the smaller corollas of lowland populations. In a common garden experiment that will be discussed elsewhere, we found that flower size in *R. officinalis* is highly plastic and responds quickly to environmental conditions, with drought causing strong corolla reductions on individual plants. It is therefore more likely that variation in flower size across elevation is the consequence of stressful conditions, as suggested by Herrera (2005), and not related to adaptation to different pollinator guilds. However, strong phenological differences between *ThM* and *SuM* populations could certainly contribute to the reproductive isolation of the two population groups and could thus add to historical factors in producing the genetic differentiation of *ThM* from *SuM* populations. Detailed studies of this possibility are underway.

CONCLUDING REMARKS

Our study revealed complex patterns of genetic diversity and population structure in *R. officinalis*, explained by a combination of biological, ecological and historical factors. Unexpectedly for a thermophilous Mediterranean species, we found that genetic diversity increased with elevation in the studied range. Historical factors emerged as the most influential factors in explaining this pattern as lowland populations were identified to be the result of more recent post-glacial colonization. Population genetic structure differed among populations of calcareous and siliceous soils. This was related to the coupled effects of higher spatial continuity among calcareous habitats and the more rapid expansion of populations across calcareous substrates, potentially combined to lower selective pressures during the colonization of calcareous soils. The high landscape heterogeneity in ecological conditions and the ability of *R. officinalis* to adapt successfully to such a variety of conditions is probably reflected in the high levels of genetic diversity found here and may be the basis for the extensive variation in morphology and chemical diversity of this Mediterranean plant.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Detection of microsatellite loci potentially affected by selection following Beaumont & Nichols (1996) implemented in LOSITAN software (Antao *et al*., 2008) including all populations and for predefined population groups according to soil types (*Ca*, *Si*) and bioclimatic altitudinal belts (*ThM*, *MsM*, *SuM*). A, all populations considered. B, populations from calcareous (*Ca*) soils. C, populations on siliceous (*Si*) soils. D, lowland populations from thermo-Mediterranean belt (*ThM*). E, intermediate altitude populations from meso-Mediterranean belt (*MsM*). F, high-altitude populations from supra-Mediterranean belt (*SuM*). For each locus the expected heterozygosity (H_E) and genetic differentiation F_{ST} are plotted on the *x*- and *y*-axes, respectively. The dashed lines represent the 95% confidence interval of the null model values of the statistics obtained after 100 000 simulations. The loci with significantly lower or higher F_{ST} values are indicated with their names.