Comparative genetic and epigenetic diversity in pairs of sympatric, closely-related plants with contrasting distribution ranges in southeastern Iberian mountains

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Abstract:

Genetic diversity defines the evolutionary potential of a species, yet mounting evidence suggests that epigenetic diversity could also contribute to adaptation. Elucidating the complex interplay between genetic and epigenetic variation in wild populations remains a challenge for evolutionary biologists, and the intriguing possibility that epigenetic diversity could compensate for the loss of genetic diversity is one aspect that remains basically unexplored in wild plants. This hypothesis is addressed in this paper by comparing the extent and patterns of genetic and epigenetic diversity of phylogenetically closely related but ecologically disparate species. Seven pairs of congeneric species from Cazorla mountains in southeastern Spain were studied, each pair consisting of one endemic, restricted-range species associated to stressful environments, and one widespread species occupying more favorable habitats. The prediction was tested that endemic species should have lower genetic diversity due to population fragmentation, and higher epigenetic diversity induced by environmental stress, than their widespread congeners. Genetic (DNA sequence variants) and epigenetic (DNA cytosine methylation variants) diversities and their possible covariation were assessed in three populations of each focal species using Amplified Fragment Length Polymorphism (AFLP) and Methyl Sensitive AFLP (MSAP). All species and populations exhibited moderate to high levels of genetic polymorphism irrespective of their ecological characteristics. Epigenetic diversity was greater than genetic diversity in all cases. Only in endemic species were the two variables positively related, but the difference between epigenetic and genetic diversity was greater at populations with low genetic polymorphism. Results revealed that the relationship between genetic and epigenetic diversity can be more complex than envisaged by the simple hypothesis addressed in this study, and highlight the need of additional research.
on the actual role of epigenetic variation as a source of phenotypic diversity before a realistic understanding of the evolutionary relevance of epigenetic phenomena in plant adaptation can be achieved.

Keywords: AFLP, DNA methylation, epigenetic diversity, endemism, genetic diversity, Mediterranean mountains, MSAP, population epigenetics
Introduction

The genetic diversity of species and populations has multiple implications for their ecology, evolution and survival. For example, reduced genetic diversity arising from inbreeding, fragmentation, bottlenecks or founder effects have been long known to pose threats on long-term survival of species and populations (Ellstrand and Elam 1993; Frankham 2005; Allendorf and Luikart 2007; Allendorf 2017). An increasing number of recent studies are showing, however, that genetic diversity (i.e., depending on variation in DNA nucleotide sequence) is not the only heritable genomic information that could influence the ecology, evolution or survival of populations. Epigenetic variations that depend on DNA methylation or chromatin states can influence phenotypic traits and are often inherited over generations in plant populations (Richards 2006; Verhoeven et al. 2010; Quadrana and Colot 2016; Herrera et al. 2018). One defining feature that set epigenetic variation apart from genetic variation is the capacity to exhibit modifications in response to environmental factors (see e.g., Dowen et al. 2012; Alonso et al. 2016).

There is now a growing consensus that natural epigenetic diversity could endow wild plant populations with an extra layer of heritable phenotypic variation that could complement genetically-based variation and contribute to local adaptation and survival (Grativol et al. 2012; Medrano et al. 2014; Schulz et al. 2014; Herrera et al. 2017; Gáspár et al. 2019; but see Herden et al. 2019). Compelling evidence supporting a role for epigenetic variation as an additional component of genomic variation has been provided by studies showing, for instance, that in wild plant populations epigenetic diversity is often greater than genetic variation (Herrera et al. 2016, 2017; and references therein) and can compensate for the complete or nearly complete loss of genetic variation in small colonizing populations, apomictic species or genetically homogeneous clones (Richards et al. 2012; Spens and Douhovnikoff 2016; Wilschut et
Interestingly, in experimental populations of *Arabidopsis thaliana* it has been recently shown that epigenetic variation is under selection and contributes to rapid phenotypic adaptive responses in absence of consistent genetic changes (Schmid et al. 2018). More studies with non-model plants growing in a real-world context are essential to understand the potential role of epigenetic variation in plant adaptation and in ecological and evolutionary processes.

Narrowly endemic plants provide an hitherto unexplored study system particularly well suited for assessing the hypothesis that epigenetic diversity could mitigate the loss of genetic diversity in wild plants. On one side, narrow endemics with restricted distributions often have small, discrete, isolated populations with lower levels of genetic diversity than close relatives with broader geographical distributions (Kruckeberg and Rabinowitz, 1985; Cole 2003). And on the other hand, at least in the Mediterranean, endemic plants often live in stressful environments, ecologically marginal habitats and/or highly specific habitat disturbance regimes (Lavergne et al. 2004; Thompson et al. 2005; Totté et al. 2015). Since biotic and abiotic stresses (e.g., herbivory, aridity, extreme temperatures) are able to induce heritable epigenetic modifications in plant genomes (Alonso et al. 2016; Quadrana and Colot 2016), the association of endemic plants with stressful environments could in itself promote epigenetic diversity via environmental induction of epigenetic variants. Recent models suggest that, when populations are small, epigenetic variation would particularly promote adaptation by rapidly restoring rare adaptive states that would otherwise be lost by genetic drift, or in divergent, peripheral environments, especially when epimutations are adaptively biased (Smithson et al. 2019).
This study examines comparative patterns of genetic and epigenetic diversity in wild populations of seven congeneric species pairs occurring sympatrically in the Sierra de Cazorla mountain range, one important glacial refuge and plant biodiversity hotspot in southeastern Spain (Médail and Quézel 1999; Médail and Diadema 2009), associated to various habitat types. Specifically, we test the expectation that endemic plants with restricted distributions should have lower genetic diversity but higher epigenetic diversity than widespread ones. The paired-species approach used here has been often applied in previous comparisons of ecological, biological or genetic features of endemic and widespread species, as it allows to control for possible phylogenetic effects on interspecific differences (e.g., Karron 1987; Lavergne et al. 2004). By analyzing pairs in seven different genera within the same region we also controlled for geographic effects. One novelty of our sampling design was the concurrent estimation of genetic and epigenetic diversity in replicated population samples of each species in each congeneric pair. In this way, robust statistical tests of hypothesized relationships between genetic and epigenetic diversity could be undertaken by applying linear mixed models to the data and treating plant species and populations as random effects. Linear mixed models allow drawing conclusions with reference to a broad inference space, which makes them particularly well suited to answer questions whose scope transcends the limits of the particular samples studied (Bolker et al. 2009; Bolker 2015). Specifically, we expected that (i) epigenetic diversity should be greater than genetic diversity at population level; (ii) after controlling by species relatedness and geographic location, endemic species with restricted distribution should have lower genetic diversity and higher epigenetic diversity than their widespread congeners. If so, then (iii) genetic and epigenetic diversities should have an inverse relationship particularly significant across populations of endemic plants.
Methods

Site and study species

Field sampling for this study was carried out in Sierras de Cazorla-Segura-Las Villas Natural Park (Jaén Province, Spain), one of several mountain units comprising the Baetic Ranges complex in southeastern Iberian Peninsula. This Natural Park is the largest protected space in Spain (Fig. 1) with a total area of 209920 ha and altitudes ranging from 590 to 2107 m above sea level. The region is characterized by rugged topography, large expanses of well-preserved habitats, a large proportion of endemic species, and outstanding floristic diversity (Médail and Quézel 1999; Mota et al. 2002; Melendo et al. 2003).

For the present study we selected seven congeneric species pairs. Each pair consisted of one narrow endemic species with a restricted geographic distribution and that is a specialist of stressing Mediterranean microhabitats or that occurs only in highly specific habitats, and one species with a widespread geographic distribution which utilizes a broad range of habitat types (see Table 1 for more details). The seven pairs of restricted and widespread selected species, mentioned in this order, were: Anthyllis ramburii Boiss and Anthyllis vulneraria L. (Fabaceae); Aquilegia pyrenaica DC. subsp. cazorlensis (Heywood) Galiano & Rivas Mart. and Aquilegia vulgaris L. subsp. vulgaris L. (Ranunculaceae); Convolvulus boissieri Steud. and Convolvulus arvensis L. (Convolvulaceae); Daphne oleoides Schreb. and Daphne laureola L. (Thymelaeaceae); Erodium cazorlanum Heywood and Erodium cicutarium (L.) L’Hér. (Geraniaceae); Teucrium rotundifolium Schreb. and Teucrium similatum T. Navarro & Rosúa (Lamiaceae); and Viola cazorlensis Gand. and Viola odorata L. (Violaceae). All except E. cicutarium are perennial plants. In addition, within the genera Anthyllis, Convolvulus, Erodium and Viola the restricted species has woody stocks whereas the
widespread congener is herbaceous. Data on species distribution and habitat requirements were obtained from Flora Iberica (Castroviejo et al. 1986-2012), Flora Vascular de Andalucía Oriental (Blanca et al. 2011) and Proyecto Anthos (http://www.anthos.es/; Aedo and Castroviejo 2005).

**Field sampling**

In order to assess intra and interspecific patterns of genetic and epigenetic diversity we sampled three populations per species and in each population 23–40 widely spaced flowering individuals (Table 1). A total of 1088 individuals were sampled for the 14 species included in this study. The sampling design aimed to fulfill the appropriate number of individuals analyzed to obtain right estimates of population diversities (Nybom 2004) and a balanced sample for the comparisons across species within a specific geographic location. Young leaves were collected from each plant, placed in paper envelopes and dried at ambient temperature in containers with silica gel. Field collections were carried out during the flowering season (April to June) of each studied taxa.

**Laboratory methods**

Total genomic DNA was extracted from dry leaf material of all plants sampled using ISOLATE II Plant DNA Kit (Bioline, London, UK) and the manufacturer protocol. Genetic and epigenetic analyses were conducted on the same DNA extracts.

Genetic fingerprints were obtained for each plant using amplified fragment length polymorphism markers (AFLP; Vos et al. 1995; Weising et al. 2005; Meudt and Clarke 2007). The AFLP analyses were performed using standard protocols involving the use of fluorescent dye-labeled selective primers (Weising et al. 2005). After testing different combinations of selective primer pairs, four different combinations of MseI + 3
/ PstI + 2 primer pairs that resolved more reproducible and easier to score bands were selected on each genus and AFLP analyses were conducted using them (see Supplementary Data Table S1 for a complete overview of all primers used for each genus with the AFLP protocol. Note that the comparisons between species within genera were done using the same four primer pairs.)

Plants were also characterized epigenetically using the methylation-sensitive amplified polymorphism (MSAP) technique (Reyna-López et al. 1997; Schulz et al. 2013; Fulneček and Kovařík 2014; Guevara et al. 2017). MSAP is a modification of the standard AFLP technique that uses the methylation-sensitive restriction enzymes HpaII and MspI in parallel runs in combination with another restriction enzyme (here the frequent cutter MseI is used). HpaII and MspI are two isoschizomers that recognize and cleave the same tetranucleotide sequence 5′-CCGG, but differ in their sensitivity to the methylation state of cytosine. HpaII can cut at sites that are either non-methylated or contain one methylated external cytosine, whereas MspI cuts non-methylated sites and those with one or two methylated internal cytosine (see Schulz et al. 2013; Fulneček and Kovařík 2014 for further details). Namely, both enzymes cut the DNA if the restriction site is not methylated, but they cut in a different way in the presence of cytosine methylation. After testing different combinations of selective primer pairs three or four MseI + 3 / HpaII-MspI + 2 primer combinations that resolved more reproducible and easier to score bands were selected on each genus and MSAP assays were conducted using them (see Supplementary Data Table S1 for a complete overview of the primers used in each genus for the MSAP protocol. Again, the same primer pairs were used for the two species within each genus).

Amplified products from both AFLP and MSAP protocols were analysed on an ABI PRISM 3130xl DNA sequencer, and fingerprint profiles were scored manually by
visualizing electrophoregrams with GeneMapper 5.0 software. All primer combinations used with each species were merged in a single binary data table to generate finally one genetic (AFLP) and two epigenetic (MSAP) raw data matrices (see below). We assessed the repeatability of banding patterns for each species by repeating the entire AFLP/MSAP protocol in a number of randomly selected samples. For each species an average of 11.2% (AFLP) and 25.8% (MSAP) of the samples were used as replicates (for further details see Supplementary Data Table S2). After elimination of inconsistent bands, overall error rates ranged from 1.05% to 3.48% and from 2.79% to 4.85%, respectively for AFLP and MSAP raw data sets (Supplementary Data Table S2). In the whole group of 1088 plants a total of 2918 AFLP markers were scored, with a mean of 208.4 markers scored per plant (range = 86 – 418).

Some of the greatest advantages of AFLP and MSAP techniques are their wide genome sampling, high reproducibility, and ability to generate many polymorphic bands per reaction without prior knowledge of genomic sequence of the organisms being assayed (Meudt and Clarke 2007; Paun and Schönswetter 2012). On the contrary, one of the most important disadvantages of these two techniques is that bands obtained are considered dominant, implying that polymorphism is scored only in terms of presence or absence, and thus it is not viable in any of them to distinguish between individuals being heterozygous or homozygous for the dominant allele (Paun and Schönswetter 2012). Although different attempts of codominant scoring using band intensities have been proposed (see for instance Gort and van Eeuwijk 2010; and Fall et al. 2010), various drawbacks, like high unreliable genotype assignments or important loss of information, advised against the regular use of those approaches to obtain codominant markers. For typical population diversity studies this problem can be at least partially mitigated by the high number of polymorphisms that are generated per reaction (Paun
and Schönswetter 2012) and by sampling a large number of individuals per population (Lynch and Milligan 1994), as we have done here. Another important limitation of these two techniques is that the sequence content of each AFLP or MSAP marker remains unknown throughout the whole process, i.e. they are anonymous markers, restricting their usefulness to the description of population patterns but obstructing it for further analyses. Although new developments in next-generation sequencing technologies are offering affordable ways to overcome marker anonymity, adopting this approach in a study like the present, with such a high number of individuals and populations from quite a few non-model species, is nowadays still unfeasible in terms of money and time.

A specific drawback of the MSAP technique is that it only detects differences in methylation that occur at the restriction sites of the cutter endonucleases, underestimating the overall level of methylation (Schrey et al. 2013; Fulneček and Kovařík 2014; Alonso et al. 2016). In spite of this limitation, MSAP markers have been validated as an alternative to whole genome bisulfite sequencing –WGBS– (Lauria et al. 2017) and proven useful to investigate variation in genome-wide patterns of cytosine methylation in non-model plants that lack sequenced genomes (Medrano et al. 2014; Foust et al. 2016; Herrera et al. 2016; Wilschut et al. 2016; Thiebaut et al. 2019). In particular, for comparative interspecific studies of non-model organisms with large sample sizes like this, the application of these two cost-effective techniques are probably the only affordable methods that currently can provide reliable, robust, and relatively simple genome-wide information simultaneously on both DNA polymorphisms as well as putative changes in DNA cytosine methylation.
Data analysis

DNA methylation analyses

The MSAP profiles were analyzed with the R script ‘MSAP_calc’ (Schulz et al. 2013) using the “Extract_MSAP_epigenotypes” function applied independently to each species with the following parameters: Epicode = “Mix1”, delete.monomorphic.loci = TRUE, and MinPoly = 2. This software analyses the MSAP binary matrix based on four types of methylation pattern according to the presence or absence of one or both fragments of MseI/HpaII and MseI/MspI: (1) fragments present in both profiles (1/1), indicating an unmethylated state; (2) fragments present only in MseI/HpaII profiles (1/0), indicating hemimethylated CHG-sites; (3) fragments present only for MseI/MspI (0/1), indicating hemi- or fully methylated CG-sites; and (4) absence of fragments in both profiles (0/0), representing an uninformative state caused either by different types of methylation, or due to restriction site polymorphism. Under the “Mix1” scoring scheme the MSAP profiles are transformed into two binary data sets: one dataset of methylated epiloci (hereafter M-MSAP) which scores conditions (2) and (3) as 1 and all other conditions as 0; and a dataset of un-methylated epiloci (U-MSAP) which scores condition (1) as 1 and conditions (2) and (3) as 0. All plants sampled were characterized epigenetically by presence–absence scores for U- and M-type MSAP markers. In total 1450 U-MSAP and 2197 M-MSAP markers were scored in the whole group of 1088 plants sampled, with a mean per individual plant of 103.6 (range = 54-181) and 156.9 (range = 95-213), respectively for U-MSAP and M-MSAP markers.

Diversity indices

Binary AFLP and MSAP raw data sets were analyzed following the same framework using a band based strategy which did not require calculating allele frequencies (Bonin et al. 2007). Genetic (AFLP) and epigenetic (MSAP) diversity within populations were
quantified using four different indices: i) proportion of polymorphic fragments (PPOL); ii) Shannon’s diversity index (SI); iii) proportion of private fragments (PPRIV), i.e. bands unique of each single population; and iv) the frequency-down-weighted marker value, in the following termed ‘rarity index’ (RI). SI was calculated for each locus within each population using the formula:

\[ SI = - \sum P_i \log_e (P_i) \]  

where \( P_i \) is the frequency of the presence or absence of the band. The mean SI per population is given by an average of the index values over individual loci (Pérez-Figueroa 2013). The rarity index for individual \( x \) (RI\(_x\)) was calculated according to Schönswetter and Tribsch (2005) using the formula:

\[ RI_x = \sum_{i=1}^{n} \frac{s_{ix}}{\sum_{j=1}^{k} s_{ij}} \]  

where \( n \) is the number of markers, \( s_{ix} \) is the binary state of the \( i \)-th marker in individual \( x \) (either 1 or 0), and \( k \) is the total number of individuals in the population data set (i.e., within the population sample). In the denominator the number of occurrences of the \( i \)-th marker in the total population data set is calculated. Population rarity index (RI) was estimated as the average of individual values. Calculations were carried out using ‘AFLPdat’ (function “Rarity — rarity I”, Version 20.10.2010; Ehrich 2006, available from http://www.nhm.uio.no/forskning-samlinger/forskning/forskningsgrupper/ncb/Online_publications/). Altogether we finally obtained 42 population data for each diversity index and marker type.

**Statistical analysis**

All statistical analyses were carried out using the R environment (R Development Core Team 2019). Differences in genetic and epigenetic diversity between restricted and
widespread species were analyzed using linear and generalized mixed effect models with each of the four population diversity indices (PPOL, PPRIV, SI, and RI) as response variables, “Markers” (with three levels: AFLP, U-MSAP, and M-MSAP) and “Distribution” (with two levels: restricted and widespread) and their interaction, as a fixed effects, and “Populations” nested within “species” as random effect to specify paired comparisons at population level. The “lmer” and “glmer” functions from the ‘lme4’ package were used to fit linear mixed and generalized linear mixed models, respectively (Bates et al. 2015). The number of loci was included as a weighing factor to account for variance across species and type of markers (Supplementary Data Table S3). In generalized linear models, proportions (PPOL, PPRIV) were modelled as binomial processes. Residuals were graphically inspected for linearity and homoscedasticity, and normal distribution of residuals was not rejected ($P > 0.05$) in any of the fitted models presented. Statistical significance of the two fixed effects and their interaction on response variables was determined in all cases with ordinary likelihood ratio tests using the “Anova” function from the R ‘stats’ library (Zuur et al. 2009). In each analysis, estimated marginal means (sensu Searle et al. 1980) and associated confidence intervals for the response variable at each factor level were obtained with the “emmeans” function of the ‘emmeans’ library (Lenth 2018). Post-hoc analysis was done by conducting multiple pairwise comparisons of the estimated marginal means with Tukey-adjustment. Marginal means from generalized linear models involving proportions were back-transformed to the original scale of measurement.

The relationship between epigenetic and genetic diversity of study populations was separately explored for restricted and widespread species using correlation analyses.
Results

Variation in genetic and epigenetic diversity in populations of restricted and widespread plants

Values of genetic (AFLP) and epigenetic (U-MSAP and M-MSAP) diversity indices obtained in each population for the group of restricted and widespread species included in our study are depicted in Figure 1 (see also Supplementary Data –Tables S3 and S4). Averaged values for each species are shown in Table 2. In general, all the genetic and epigenetic diversity indices varied widely and similarly in populations of restricted and widespread species, as denoted by their broad ranges of variation (Fig. 3). For AFLP markers PPOL ranged from 0.430 to 0.802 in populations of restricted species and from 0.351 to 0.789 in populations of widespread species (Fig. 3A), for U-MSAP markers PPOL varied from 0.406 to 0.890 and from 0.408 to 0.785, and for M-MSAP markers from 0.568 to 0.878 and 0.640 to 0.897, respectively for restricted and widespread species (Fig. 3B). PPRIV per population varied from 0.012 to 0.407 (note however that this is an outlier value) in restricted species and from 0.033 to 0.244 in widespread species for AFLP markers (Fig. 3C), and from 0.277 to 0.428 and from 0.237 to 0.446 for U-MSAP markers, and from 0 to 0.227 and from 0.014 to 0.131 for M-MSAP markers, respectively restricted and widespread species (Fig. 3E). The SI was less variable than PPOL in our dataset and, thus, less informative. It fluctuated from 0.261 to 0.430 in restricted species and from 0.280 to 0.466 in widespread ones for AFLP markers (Fig. 3C), and respectively from 0.277 to 0.428 and from 0.237 to 0.446 for U-MSAP markers, and from 0.278 to 0.405 and from 0.303 to 0.462 for M-MSAP markers (Fig. 3D). RI ranged from 0.66 to 4.55 in populations of restricted species and from 1.08 to 6.01 in populations of widespread ones for AFLP markers (Fig. 3G), and from 0.61 to 2.76 and from 0.50 to 2.31 for U-MSAP markers, and from 1.16 to 3.68 and
1.14 to 2.86 for M-MSAP markers, respectively in restricted and widespread species (Fig. 3H).

Results of the linear and generalized mixed models (Table 3) indicated a significant effect of the type of marker in all diversity indices considered, but that these differences among markers fluctuated between restricted and widespread species as revealed by the significant “Markers-by-Distribution” interaction term in all of them. Pairwise comparisons for each marker between restricted and widespread species of estimated marginal means (Fig. 4) revealed a complex pattern for most of the diversity indices. For instance, the proportion of polymorphic fragments was always significantly higher for M-MSAP than for AFLP or U-MSAP markers in both restricted (Z =12.09, P < 0.0001; and Z = 8.060, P < 0.0001) and widespread species (Z = 11.94, P < 0.0001; and Z = 12.35, P < 0.0001). However, opposing trends were found between restricted and widespread species when U-MSAP and AFLP markers were compared. Specifically, U-MSAP markers had higher values of polymorphic fragments than AFLP markers (Z = 2.75; P = 0.0164) in restricted species, but the opposite pattern occurred in widespread species (Z = 2.62; P = 0.0241; Fig. 4). Similarly, proportion of private fragments (PPRIV) were consistently higher for AFLP or U-MSAP than for M-MSAP markers in restricted (Z =9.38, P<0.0001; and Z =-8.06, P < 0.0001) and widespread species (Z = 8.79, P < 0.0001; and Z =12.35, P < 0.0001). However, U-MSAPs had more private markers than AFLPs only in widespread species (Z =2.62, P= 0.0241) but not in restricted species (Fig. 4). No significant difference in Shannon Index (SI) was noted among the three different types of markers in restricted species, and also when each type of marker was compared between restricted and widespread species. Only in widespread species SI had slightly lower values for U-MSAP markers than for AFLPs (t = 2.73, P = 0.0173) and M-MSAP markers (t = 3.02, P = 0.0071; Fig. 4). Similar
patterns of variation in Rarity Index (RI) were found among the three types of markers when comparing the two groups of species (Fig. 4). Actually, in both restricted and widespread species RI was always significantly higher in AFLPs than in U-MSAPs ($t = 4.64, P < 0.0001$ and $t = 8.50, P < 0.0001$) or in M-MSAPs ($t = 2.49, P = 0.0343$; and $t = 5.80, P < 0.0001$), and significantly lower in U-MSAPs than in M-MSAPs ($t = 2.34, P = 0.0506$ and $t = 8.50, P < 0.0001$). In summary, we were able to detect significant differences between restricted and widespread only in one group of epigenetic markers (U-MSAP), particularly for PPOL and PPRIV, but not in genetic markers or in epigenetic methylated markers (M-MSAP).

**Relationship between genetic and epigenetic diversity within populations**

In restricted species, populations that had more genetic diversity tended to have also more epigenetic diversity, as revealed by the statistically significant (or marginally significant) correlation between most of the diversity indices measured for AFLP markers, on one side, and on the other for the group of methylated (M-MSAP) markers ($r = 0.374, 0.653$ and $0.555$; and $P = 0.095, 0.0013$, and $0.0089$, for PPOL, SI and RI respectively), or for the group of un-methylated (U-MSAP) markers ($r = 0.372$ and $0.540$; $P = 0.096$ and $0.0113$, for SI and RI respectively; Fig. 5). However, in widespread species genetic and epigenetic diversity of study populations were largely independent of each other, as shown by statistically nonsignificant correlations between three of the four diversity indices measured for AFLP markers (PPOL, PPRIV, and SI), and M-MSAP ($r = 0.104, 0.118$, and $0.267$; and $P = 0.65, 0.61$, and $0.24$); or U-MSAP type of markers ($r = 0.044, 0.084$ and $0.181$; and $P = 0.848, 0.716$ and $0.431$, respectively for PPOL, PPRIV, and SI; Fig. 5). Just RI in widespread species had significant correlation between genetic and epigenetic markers ($r = 0.398$ and $P = 0.073$ for the correlation between AFLP and M-MSAP markers; $r = 0.563$ and $P = 0.007$ for
the correlation between AFLP and U-MSAP markers), concurring with the pattern found in restricted species (Fig. 5C).

**Discussion**

Genetic diversity of natural populations is a fundamental trait in their evolutionary trajectory that summarizes past events and defines the potential for future adaptation to environmental changes (Jump *et al.* 2009; Allendorf 2017). Epigenetic variation, in turn, results from genetic and environmental factors, as well as from stochastic epimutations (Richards 2006; Johannes and Schmitz 2019), and it can also shape the evolutionary trajectories of populations (e.g., Kronholm and Collins 2016; Smithson *et al.* 2019). Epigenetic diversity can enhance plant population response under environmental stress (e.g., Latzel *et al.* 2013) but its magnitude and association with genetic diversity in natural populations is still poorly understood (Herrera *et al.* 2016; Moler *et al.* 2018). The concurrent analysis of the genetic and epigenetic diversity of 14 species presented here illustrates the wide range of genetic and epigenetic diversities that plant populations may harbor within a relatively small area of well-preserved montane habitats. In the next sections we will discuss the patterns obtained and how the absence of global consistent relationships between estimates of genetic and epigenetic diversity might be interpreted as the variable outcome of multiple determinants of genetic diversity (Leimu *et al.* 2006; Stuessy *et al.* 2014; Ellegren and Galtier 2016) and possibly other factors that were not accounted for in our study design.

**Genetic diversity**

Endemic species account for the most original, phylogenetically distinctive component of local floras and a good portion of the rich biodiversity associated to hotspots (Médail and Quézel 1999; Melendo *et al.* 2003). Consequently, the magnitude of genetic
diversity of endemic and threatened plant species has been investigated for decades with the dual interest of understanding their evolution and contributing to their conservation (Allendorf 2017). At a global geographic scale, pioneer analyses based on allozyme markers found lower percentages of polymorphic loci in endemic plants compared to widespread congener (Karron 1987; Gitzendanner and Soltis 2000; Cole 2003). Likewise, López-Pujol et al. (2009) found a similar trend within a Mediterranean region, although without specifically controlling for relatedness due to scarcity of available congeneric comparisons. Later studies based on large numbers of anonymous DNA-markers (e.g., AFLP, RAPD) found only partial support for this trend and confirmed the need to control for phylogenetic relatedness (Reisch and Bernhardt-Römermann 2014; Ellegren and Galtier 2016, and references therein). In the Mediterranean region, many narrow endemic plants of Western European mountains bear moderate to high levels of genetic diversity (Jiménez-Mejías et al. 2015), suggesting that divergence in genetic diversity between endemic and widespread species could not occur in this region (Forrest et al. 2017, and references therein). We are not aware, however, of any previous analysis addressing comparatively the genetic diversity in several congeneric pairs of endemic and widespread species at a local scale (i.e., simultaneously accounting for species relatedness and geographic location) in the Mediterranean or elsewhere.

In this study, the average polymorphism in AFLP markers per population ranged between 52.9 % (Daphne laureola, widespread) and 75.7 % (Erodium cazorlanum, restricted). All species studied here thus exceeded the population average value (41.6 % ± 21.5) reported by Reisch and Bernhardt-Römermann (2014) for 75 AFLP studies worldwide. These data suggested that our study region might be particularly rich not only in species numbers but also in the genetic diversity they preserve, which provides
an extra value for future persistence (Jump et al. 2009). Previous studies in the Sierra de Cazorla region have likewise revealed consistently high levels of genetic diversity in plants and associated microfungi (Jordano and Godoy 2000; Medrano and Herrera 2008; Cánovas et al. 2015; Herrera et al. 2011, 2014), thus suggesting that this might be a distinctive feature of this biodiversity hotspot and highlighting the interest of conducting additional research in the framework of the “genetic hotspot” concept (Souto et al. 2015).

Genetic distinctiveness of populations is also a relevant parameter from a conservation perspective (López-Pujol et al. 2009; Jump et al. 2009; Allendorf 2017). Except for one outlier population of D. oleoides, the PPRIV was < 25% in all populations and <10.7% in half of them. We did not find significant differences between average PPRIV per population or RI of endemic and widespread plants at the local scale investigated here. Altogether our results supported the hypothesis that plant endemics from Western Mediterranean mountains do not necessarily harbor lower levels of genetic diversity (Forrest et al. 2017), although similar analyses in other geographic locations are required to verify the universality of this pattern. Furthermore, a wider geographic sampling should be conducted to conclude about population differentiation at the species level for conservation purposes. First, because genetic distinctiveness may appear only in a few populations that might be particularly valuable for the survival of endemic and widespread species (e.g., Hampe and Petit 2005; Forrest et al. 2017). And second, because in widespread species the few populations sampled in this study mostly occur at the rear-edge border of their distribution ranges, where genetic diversity could be particularly reduced or skewed and non-representative for the species (Hampe and Petit 2005; Eckert et al. 2008; Reisch and Bernhardt-Römermann 2014).
**Relationships between genetic and epigenetic diversity**

We investigated epigenetic diversity associated to changes in the methylation status of hundreds of MSAP markers and compared it to genetic diversity estimates for roughly similar numbers of AFLP markers in 42 populations and seven pairs of congeneric plant species occurring sympatrically in the same geographic region. To the best of our knowledge this is the first study that has used a multi-species framework and a population-level approach to compare epigenetic variation and its relation with genetic variation of wild plants in nature (but see Liu et al. 2015 for a study with animals). In recent years some advances have been made in the emerging field of population epigenetics (see Kilvitis et al. 2014 for a review), which addresses questions about the prevalence and importance of epigenetic variation in the natural world (Richards 2008), but for plants most of these advances came from microevolutionary studies performed on single-species and populations along ecological gradients, with unusual environmental exposures, or under invasive or range expansion scenarios (e.g., Herrera and Bazaga 2010; Lira-Medeiros et al. 2010; Richards et al. 2012; Schulz et al. 2014; Preite et al. 2015; Wilschut et al. 2016; Shi et al. 2019). Working in a multi-species framework we were searching for more general patterns so that our findings could provide valuable insight to fully understand the epigenetic phenomena under a broader evolutionary scale. In the group of our study species we have found that methylation polymorphism at cytosines that are frequently methylated (M-MSAP) ranged between 67.8 % and 86.4 % (in restricted *V. cazorlensis* and *E. cazorlanum*, respectively), and was higher than polymorphism in those cytosines that are most commonly unmethylated (U-MSAP), which varied between 52.0 % and 76.5 % (in restricted *T. rotundifolium* and *V. cazorlensis*, respectively). Epigenetic diversity was thus lower for U-MSAP than M-MSAP markers, and populations of endemic plants were more variable as regards the
two epigenetic markers. Contrary to our expectations, however, populations of endemic and widespread species did not differ in their average epigenetic diversity. Although our sampling design simultaneously accounted for species relatedness and geographic location we cannot discard however that other factors important for plant population evolutionary dynamics such as population size or isolation level, which were not taken into account in our study, must also be operating and could have influenced our epigenetic (as well as genetic) diversity estimates. Recent models suggest, for instance, that greater rates of spontaneous epimutations can increase adaptation to local conditions and can help maintain polymorphisms in small and peripheral populations (Smithson et al. 2019). However, direct evidence of the inheritance of environmentally induced changes in DNA methylation in wild populations is still scarce. Some experimental reports indicate that stress-induced methylation changes can be inherited for one or two generations in the form of a ‘stress memory’ even in the absence of the stressor, but are quickly reverted to the non-stressed state thereafter (Wibowo et al., 2016; Lämke and Bäurle, 2017). And, even under controlled experimental conditions, few studies have had the necessary experimental design to assess whether stress may also generate stable epimutations across multiple generations. In addition to this, recent studies have revealed that interspecific variation in plant epigenomic features is related also to other important intrinsic plant traits such as lifeform, as reflected by the fact that woody plants tend to have lower levels of global DNA methylation than perennial herbs (Alonso et al. 2019). Interestingly, three out of the four congeneric comparisons with strongest divergence in population epigenetic diversity involved pairs of herbaceous and woody congener (Anthyllis, Erodium, Viola), although the sign of divergence was not consistent. Such intriguing results suggest that a deeper analysis with more species within genera but differing in other traits such as habitat requirements, lifeform,
population size or isolation could be helpful to better understand the effect of species-specific plant traits in epigenetic diversity as previously suggested for genetic diversity (e.g., Gitzendanner and Soltis 2000).

Despite the heterogeneity of our study populations in the features mentioned above, we were still able to find significant relationships between genetic and epigenetic diversity, that were clearest for M-MSAP markers. Our results supported the expectation of higher epigenetic (M-MSAP) than genetic (AFLP) polymorphism in the whole sample, which was consistent with the idea that substantial epigenetic variation at shorter spatial distances might contribute significantly to population epigenetic diversity (see also Medrano et al. 2014; Herrera et al. 2016 and references therein). An excess of DNA methylation variation relative to genetic variation has been found in populations of the great majority of plant species examined to date and epigenetic variation has been linked to functional phenotypic variation (e.g., Gao et al. 2010; Herrera and Bazaga 2010; Lira-Medeiros et al. 2010; Richards et al. 2012; Medrano et al. 2014; Jueterbock et al. 2019; Shi et al. 2019; Wang et al. 2020), and also in most wild animals (for a recent review see Hu and Barret 2017). In particular, Wang et al. (2020) found that the introduced populations of the clonal herb Hydrocotyle vulgaris exhibited extremely low genetic diversity but variation in specific leaf area and other leaf traits were all positively related to intra-population polymorphism in MSAP markers. Also, in clonal meadows of Zostera marina methylation variation promotes variation in fitness-related traits of ecological relevance, specifically photosynthetic performance and heat stress resilience, and contributes to the long-term survival of its genetically depauperate populations by increasing mitotically heritable and ecologically relevant phenotypic variation (Jueterbock et al. 2019). Such findings suggest that extensive epigenotypic variations may support phenotypic variation and have a key role in the evolutionary
potential of wild populations, as predicted by theoretical models (e.g., Feinberg and Irizarry 2010; Furrow and Feldman 2014; Smithson et al. 2019). Moreover, for the group of endemic plants studied here the difference between epigenetic and genetic diversity was greater at populations with low genetic polymorphism, and the two variables were positively related, suggesting that at least in part epigenetic variation could be dependent on genetic variation. However, in widespread species, epigenetic polymorphism was consistently high regardless of the genetic polymorphism observed, and the two variables were not significantly related. Such contrasting patterns were partly consistent with our expectation that a higher epigenetic diversity could possibly alleviate the lower genetic diversity expected in populations of endemic plants. However, the relationship between the two variables remained positive and held at the population but not at the species level, suggesting that intraspecific variation was as relevant as interspecific variation in understanding the association between genetic and epigenetic diversities of wild plants (Herrera et al. 2016). A more complete understanding of the evolutionary relevance of epigenetic variation and the contribution of epigenetics to phenotypic differentiation and plant adaptation to environmental changes will require additional research on the extent to which epigenetically induced responses are also under genetic control and contribute to population and species persistence (Richards 2006).

Conclusions

We found that all populations studied exhibited moderate to high levels of genetic polymorphism. However, contrary to our expectations, populations of restricted-endemic and widespread species did not differ in average genetic or epigenetic diversity in our study region. The concurrent estimation of genetic and epigenetic diversity in
replicated population samples of each species confirmed that levels of polymorphism at frequently methylated cytosines (M-MSAP markers) were always higher than polymorphism at genetic (AFLP) markers in both restricted-endemic and widespread species. Importantly, correlation analyses showed that only in the group of species with restricted distribution did populations with higher epigenetic diversity tend to have also higher levels of genetic variation, although the difference between epigenetic and genetic diversity was greater at populations with low genetic polymorphism. This result is partly consistent with our expectation that a higher epigenetic diversity could possibly alleviate the lower genetic diversity expected in populations of endemic plants. Further work is needed to understand the effects that other intrinsic plant traits (e.g., lifeform) and population features (e.g., size, isolation) may have in comparative multi-species patterns of genetic and epigenetic covariation in this Mediterranean hotspot region or elsewhere.

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Contributions by the Authors

CA, CMH, and MM conceived the idea and designed the study; CA and MM gathered plant material from natural populations; EL and PB isolated all DNAs and performed all AFLP and MSAP analyses; MM performed data analysis and prepared all figures and tables. CA, CMH, and MM wrote the manuscript. All the authors read and approved the manuscript.

Conflict of Interest
None declared.

**Supplementary Data**

Supplementary data are available online and consist of the following:

Table S1. Primer combinations used for AFLP and MSAP protocols.

Table S2. Replicated samples and scoring error rates in the AFLP and MSAP protocols.

Table S3. Genetic and epigenetic diversity estimates obtained in each population for the 14 study species.

Table S4. Summary of the genetic and epigenetic diversity estimates for the group of restricted endemic and widespread species.

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Legends to figures

Figure 1. Geographical location of our study site, the Natural Park of Sierras de Cazorla Segura and Las Villas (red rectangle), in southeastern Iberian Peninsula (A). Maps showing the approximate location of the 21 populations from the seven restricted endemic species (left panel), and the 21 populations from the seven widespread congeners (right panel) included in this study, showing that they were similarly distributed across the study area (B).

Figure 2. Photographs showing the general aspect of flowering individuals from each the 14 study species (A-N), grouped by genders: the restricted species of each gender always on the left and the widespread on the right. Maps showing the exact location of the three populations sampled from each pair of study species (O-U). Anthyllis ramburii (A), Anthyllis vulneraria (B), Aquilegia p. cazorlensis (C), Aquilegia v. vulgaris (D), Convolvulus boissieri (E), Convolvulus arvensis (F), Daphne oleoides (G), Daphne laureola (H), Erodium cazorlanum (I), Erodium cicutarium (J), Teucrium rotundifolium (K), Teucrium similatum (L), Viola cazorlensis (M), Viola odorata (N). Population names refer to Table 1. In each map purple points represent populations of restricted species while blue crosses populations of its widespread congener.

Figure 3. Variation in genetic (AFLP) and epigenetic (U-MSAP and M-MSAP) diversity estimates between populations of restricted and widespread species included in this study. Values of the four diversity indices: proportion of polymorphic fragments (PPOL), Shannon’s diversity index (SI), proportion of private fragments (PPRIV), and Rarity Index (RI) are depicted. In each figure each dot denotes a population, the lower and upper boundaries of the boxplot indicate the 25th and 75th percentiles, the horizontal line within the box marks the median and the whiskers indicate data range.

Figure 4. Comparisons of model-estimated marginal means (EMMs) in restricted and widespread species between genetic (AFLP) and epigenetic (U-MSAP and M-MSAP) markers obtained for the four diversity indices included in this study: proportion of polymorphic fragments (PPOL), Shannon’s diversity index (SI), proportion of private fragments (PPRIV), and Rarity Index (RI). EMMs are
represented by horizontal lines. The vertical bars are confidence intervals for the EMMs, and the red arrows are for the comparisons among them. Statistically significant differences (P < 0.05) are indicated by non-overlapping arrows.

**Figure 5.** Pairwise relationships within populations between genetic (AFLP) and epigenetic (U-MSAP and M-MSAP) diversity indices for restricted and widespread species. Note that the relationship is illustrated only for three of the four indices that we have studied, in which at least one statistical significant correlation was found: (A) proportion of polymorphic fragments (PPOL); (B) Shannon’s diversity index (SI); and (C) Rarity Index (RI). Only statistically significant linear regressions (solid lines) and their 95% confidence intervals (colored area) were depicted. In all the figures the identity line (x = y) is also shown as a reference (dashed lines), and to emphasize that all points above these lines represent a greater epigenetic value compared to the same genetic value, whereas points below dashed lines indicate the reverse pattern.
| Plant Family | Restricted | Widespread |
|-------------|------------|------------|
| Anthyllis   | ![Image](https://example.com/image1) | ![Image](https://example.com/image2) |
| Aquilegia   | ![Image](https://example.com/image3) | ![Image](https://example.com/image4) |
| Convolvulus | ![Image](https://example.com/image5) | ![Image](https://example.com/image6) |
| Daphne      | ![Image](https://example.com/image7) | ![Image](https://example.com/image8) |
| Erodium     | ![Image](https://example.com/image9) | ![Image](https://example.com/image10) |
| Teucrium    | ![Image](https://example.com/image11) | ![Image](https://example.com/image12) |
| Viola       | ![Image](https://example.com/image13) | ![Image](https://example.com/image14) |
Table 1. Description of the habitat type and geographic distribution for the 14 species included in this study, and details of the location (longitude and latitude), altitude, and sample sizes (N) for each of the three populations sampled per species. Longitudinal and latitudinal coordinates are given as decimal coordinates (WGS84).

| Taxa                  | Habitat type                                      | Distribution                  | Population | Longitude | Latitude  | Altitude (m asl) | N  |
|-----------------------|--------------------------------------------------|-------------------------------|------------|-----------|------------|-----------------|----|
| *Anthyllis ramburii*  | High mountain dwarf procumbent scrubs, sandy dolomitic soils | Baetic Ranges                 | anra1      | -2.84164 | 37.91791  | 1460            | 25 |
|                       |                                                  |                               | anra2      | -2.86832 | 37.92836  | 1636            | 25 |
|                       |                                                  |                               | anra3      | -2.93244 | 37.86575  | 1473            | 25 |
| *Anthyllis vulneraria*| Dry grasslands and rocky environments with calcareous soils, broad altitudinal range | European, Mediterranean basin east to the Caucasus | anvu1      | -2.97559 | 37.83554  | 1402            | 25 |
|                       |                                                  |                               | anvu2      | -2.86850 | 37.92843  | 1645            | 25 |
|                       |                                                  |                               | anvu3      | -2.83235 | 37.94364  | 1332            | 25 |
| *Aquilegia p. cazorlensis* | Riffs of limestone outcrops, sandy soils in shady, damp sites at cliff bases | Baetic Ranges                 | aqca1      | -2.96022 | 37.78849  | 1495            | 25 |
|                       |                                                  |                               | aqca2      | -2.95573 | 37.80753  | 1901            | 25 |
|                       |                                                  |                               | aqca3      | -2.93202 | 37.86538  | 1475            | 25 |
| *Aquilegia v. vulgaris* | Stream margins, poorly drained open meadows around springs, broad altitudinal range | European Temperate element and Mediterranean | aqvu1      | -2.83231 | 37.94395  | 1356            | 25 |
|                       |                                                  |                               | aqvu2      | -2.85351 | 37.96322  | 1186            | 25 |
|                       |                                                  |                               | aqvu3      | -2.78665 | 37.92610  | 1713            | 25 |
| *Convolvulus boissieri* | High mountain dwarf procumbent scrubs, sandy dolomitic soils | Baetic Ranges                 | cboi1      | -2.95572 | 37.80722  | 1919            | 25 |
|                       |                                                  |                               | cboi2      | -2.74480 | 37.99704  | 1711            | 28 |
|                       |                                                  |                               | cboi3      | -2.97231 | 37.90047  | 1535            | 25 |
| *Convolvulus arvensis* | Cultivated areas, wasteland, roadsides, grassy slopes, broad altitudinal range | Temperate and tropical regions, except Australia | carv1      | -2.84134 | 37.92845  | 1511            | 25 |
|                       |                                                  |                               | carv2      | -2.86664 | 37.92967  | 1630            | 25 |
|                       |                                                  |                               | carv3      | -2.92134 | 37.95132  | 818             | 25 |
| *Daphne oleoides*     | Calcareous xerophytic woodlands and scrublands, rocky slopes | North Africa, southern Europe, Asia Minor | dole1      | -2.74612 | 37.99791  | 1725            | 25 |
Daphne laureola<sup>W</sup>  
Sclerophyllous and semi-deciduous forests, understory of montane forests, mostly in basic soils  
Sclerophyllous and semi-deciduous forests, understory of montane forests, mostly in basic soils  
Sclerophyllous and semi-deciduous forests, understory of montane forests, mostly in basic soils  

Erodium cazorlanum<sup>R</sup>  
High mountain dry grasslands and rocky environments, sandy dolomitic soils  
High mountain dry grasslands and rocky environments, sandy dolomitic soils  
High mountain dry grasslands and rocky environments, sandy dolomitic soils  

Erodium cicutarium<sup>W</sup>  
Meadows, flood plains, gravel areas, roadsides and disturbed areas, broad altitudinal range  
Meadows, flood plains, gravel areas, roadsides and disturbed areas, broad altitudinal range  
Meadows, flood plains, gravel areas, roadsides and disturbed areas, broad altitudinal range  

Teucrium rotundifolium<sup>R</sup>  
Rocky outcrops  
Rocky outcrops  
Rocky outcrops  

Teucrium similatum<sup>W</sup>  
Sclerophyllous and semi-deciduous forests, calcareous scrublands and grasslands, rocky mountain slopes, broad altitudinal range  
Sclerophyllous and semi-deciduous forests, calcareous scrublands and grasslands, rocky mountain slopes, broad altitudinal range  
Sclerophyllous and semi-deciduous forests, calcareous scrublands and grasslands, rocky mountain slopes, broad altitudinal range  

Viola cazorlensis<sup>R</sup>  
Rocky outcrops, cliffs, sandy dolomitic soils  
Rocky outcrops, cliffs, sandy dolomitic soils  
Rocky outcrops, cliffs, sandy dolomitic soils  

Viola odorata<sup>W</sup>  
Open woodlands, hedge banks and scrublands, edges of forests and clearings, broad altitudinal range  
Open woodlands, hedge banks and scrublands, edges of forests and clearings, broad altitudinal range  
Open woodlands, hedge banks and scrublands, edges of forests and clearings, broad altitudinal range  

<sup>R</sup> restricted distribution; <sup>W</sup> widespread distribution
Table 2. Summary of the genetic (AFLP) and epigenetic (U-MSAP and M-MSAP) diversity estimates obtained per species. Means and standard errors (in parenthesis) are shown for proportion of polymorphic fragments (PPOL), Shannon’s diversity index (SI), proportion of private fragments (PPRIV), and Rarity Index (RI).

| Species                  | PPOL     | SI     | PPRIV | RI     |
|--------------------------|----------|--------|-------|--------|
|                          | AFLP     | U-MSAP | M-MSAP| AFLP   | U-MSAP | M-MSAP | AFLP | U-MSAP | M-MSAP |
| **Anthyllis ramburii**   | 0.707    | 0.593  | 0.769 | 0.104  | 1.96   | (0.023) | (0.020) | (0.010) | (0.013) | (0.019) | (0.020) | (0.019) | (0.024) |
| **Anthyllis vulneraria** | 0.647    | 0.604  | 0.855 | 0.118  | 3.12   | (0.043) | (0.000) | (0.006) | (0.012) | (0.011) | (0.007) | (0.025) | (0.006) | (0.004) |
| **Aquilegia p. cazorlensis** | 0.595 | 0.556  | 0.759 | 0.127  | 2.17   | (0.068) | (0.076) | (0.056) | (0.019) | (0.012) | (0.019) | (0.053) | (0.067) | (0.018) |
| **Aquilegia v. vulgaris** | 0.614    | 0.550  | 0.680 | 0.169  | 2.01   | (0.035) | (0.008) | (0.019) | (0.009) | (0.016) | (0.013) | (0.041) | (0.032) | (0.023) |
| **Convolvulus boissieri** | 0.656    | 0.678  | 0.762 | 0.099  | 2.97   | (0.090) | (0.073) | (0.039) | (0.034) | (0.033) | (0.018) | (0.032) | (0.025) | (0.010) |
| **Convolvulus arvensis** | 0.616    | 0.739  | 0.770 | 0.085  | 2.38   | (0.066) | (0.027) | (0.064) | (0.012) | (0.014) | (0.011) | (0.027) | (0.010) | (0.020) |
| **Daphne oleoides**      | 0.0529   | 0.627  | 0.784 | 0.132  | 1.52   | (0.099) | (0.059) | (0.024) | (0.029) | (0.015) | (0.015) | (0.037) | (0.034) | (0.005) |
| **Daphne laureola**      | 0.554    | 0.747  | 0.772 | 0.129  | 1.15   | (0.101) | (0.022) | (0.035) | (0.028) | (0.009) | (0.007) | (0.125) | (0.012) | (0.006) |
| **Erodium cazorlanum**   | 0.0757   | 0.724  | 0.864 | 0.064  | 1.56   | (0.039) | (0.026) | (0.010) | (0.011) | (0.007) | (0.007) | (0.027) | (0.009) | (0.004) |
| **Erodium cicutarium**   | 0.629    | 0.0639 | 0.707 | 0.091  | 1.60   | (0.039) | (0.026) | (0.010) | (0.011) | (0.007) | (0.007) | (0.027) | (0.009) | (0.004) |
| Species          | Distribution | Population 1 | Population 2 | Population 3 | Population 4 | Population 5 | Population 6 | Population 7 | Population 8 |
|------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Teucrium rotundifolium | R | 0.675 | 0.541 | 0.762 | 0.327 | 0.241 | 0.306 | 0.111 | 0.187 | 0.056 | 5.57 | 1.73 | 2.84 |
| Teucrium similatum  | W | 0.636 | 0.520 | 0.794 | 0.317 | 0.288 | 0.351 | 0.132 | 0.217 | 0.047 | 4.25 | 1.33 | 2.48 |
| Viola cazorlensis   | R | 0.596 | 0.764 | 0.678 | 0.292 | 0.357 | 0.293 | 0.112 | 0.048 | 0.110 | 3.48 | 2.41 | 2.47 |
| Viola odorata       | W | 0.657 | 0.627 | 0.776 | 0.378 | 0.341 | 0.355 | 0.126 | 0.115 | 0.065 | 4.26 | 1.25 | 1.44 |

R: restricted distribution; W: widespread distribution
Table 3. Results of the linear and generalized mixed models examining the effect of the fixed predictors: type of Markers (AFLP, U-MSAP and M-MSAP), Distribution (restricted and widespread), and their interaction, on each of the four response variables, the diversity indices: proportion of polymorphic fragments, PPOL; Shannon’s diversity index, SI; proportion of private fragments, PPRIV; and Rarity Index, RI.

| Response variable | Predictors         | $\chi^2$ | $P$     |
|-------------------|--------------------|----------|---------|
| PPOL              | Markers            | 325.41   | <2.2e-16*** |
|                   | Distribution       | 1.03     | 0.30958 |
|                   | Markers*Distribution| 15.79    | 0.00077*** |
| SI                | Markers            | 5.53     | 0.06284* |
|                   | Distribution       | 0.20     | 0.65303 |
|                   | Markers*Distribution| 4.99     | 0.08268* |
| PPRIV             | Markers            | 207.66   | <2.0e-16*** |
|                   | Distribution       | 0.33     | 0.56618 |
|                   | Markers*Distribution| 7.61     | 0.02222* |
| RI                | Markers            | 93.68    | <2.2e-16*** |
|                   | Distribution       | 0.36     | 0.54758 |
|                   | Markers*Distribution| 9.57     | 0.00834** |

Signif. codes: ’***’ $P < 0.001$; ’**’ $P < 0.01$; ’*’ $P < 0.05$; ’.’ $P < 0.1$