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## Research article

### Assessing the links between pollinators and the genetic and epigenetic features of plant species with contrasting distribution ranges

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In flowering plants, pollinators contribute to gene flow while they also respond to variation in plant traits together determined by genetic, epigenetic and environmental sources of variation. Consequently, a correlation between abundance and diversity of pollinators and the genetic and epigenetic characteristics of plant populations such as diversity or distinctiveness is expected. However, no study has explored these long-term dimensions of plant–pollinator interactions. Mediterranean narrow endemics often exhibit unexpectedly high levels of population genetic and epigenetic diversity. We hypothesize that pollinators may contribute to explain this pattern. Specifically, given the higher sensitivity of small, isolated population to gene flow, we expect a stronger association of pollinators with population genetic and epigenetic variability in narrow endemics than in widely distributed congeners. We studied five pairs of congeneric plant species, consisting of one narrow endemic with a restricted distribution and one widespread congener, found in the Sierra de Cazorla mountains (SE Spain). We characterized the pollinators in up to three populations per species to estimate their diversity and visitation rates. Additionally, we calculated the genetic and epigenetic diversity and distinctiveness of each population using AFLP markers and methylation-sensitive AFLP markers (MSAP), respectively. We assessed the relationship between pollinator diversity and visitation rates. The diversity of pollinators did not vary according to the plant's distribution range, but visitation rate was higher in widespread species. As predicted, only narrow endemics showed a significant association between pollinators and their population genetic and epigenetic characteristics. Specifically, higher pollinator diversity and visitation rates entailed higher population genetic diversity and lower epigenetic distinctiveness. This work shows the importance of investigating the relationship between pollinator diversity and population genetics and epigenetics to better understand the evolution of plant rarity.

Keywords: DNA methylation, endemism, epigenetic diversity, genetic diversity, pollinator diversity, population epigenetics



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## Introduction

Genetic diversity of animal-pollinated plant populations depends to a great extent on pollinators (Ellstrand et al. 1989, Ellstrand 1992, 2003, Wessinger 2021). In addition to the well documented effects of pollinator flight distance (Breed et al. 2015, Brunet et al. 2019, Gamba and Muchhala 2020, 2022, Wessinger 2021), other pollinator-specific characteristics such as pollen carryover (Morris et al. 1994, Holmquist et al. 2012, Mitchell et al. 2013) or foraging strategies (i.e. number of flowers visited per plant individual; Lloyd 1992, Eckert 2000) together determine the provenance of pollen carried by pollinators. In fact, several studies demonstrate that pollinators with differences in the former attributes contribute with contrasting mixtures of pollen donors to the final mating portfolio of a plant individual (Castilla et al. 2017, Valverde et al. 2019). Thus, the combined effect of multiple pollinators can increase polyandry (Devaux et al. 2014, Barrett and Harder 2017), which, in the long term could shape population genetic diversity (Pannell and Labouche 2013). On the other hand, pollinators preference towards different floral traits or plant microenvironments have been documented (Herrera 1995, Benitez-Vieyra et al. 2006, Gómez et al. 2006, 2008, Norgate et al. 2010) suggesting that increased genetic diversity underlying large floral phenotypic variation or environmental heterogeneity could in part determine the diversity of pollinators visiting a plant population. Despite these findings, there is a lack of studies exploring the correlation between the genetic diversity of plant populations and the diversity of their pollinator assemblages (Burgin and Hopkins, 2022, Feigs et al. 2022).

In addition to the genetic variation, epigenetic regulation – which involves DNA modifications that do not affect the DNA sequence – provides an additional source of potentially heritable variation. In plants, variation in epigenetic DNA methylations can predict a substantial amount of the phenotypic variance observed within natural populations (Herrera and Bazaga, 2010, Medrano et al. 2014, 2020, Schulz et al. 2014, Albaladejo et al. 2019). Significant variation in DNA methylation originates either stochastically or as a rapid response to environmental changes, and some of these epimutations can be inherited across many generations (Richards 2006, Turner 2009, Verhoeven et al. 2010, Meyer 2015, Johannes and Schmitz, 2019). These attributes confer to environmentally responsive epigenetic marks a distinct role in natural selection (Richards 2006, Jablonka and Raz 2009, Balao et al. 2018) and points to a potential correlation with pollinators (Alonso et al. 2019) that, as with genetic variability, may be bidirectional or simply correlative. On the one hand, due to its heritability, epigenetic variation can be expected to be subject to the same dispersal processes mediated by pollinators. On the other hand, due to its ability to influence phenotype, it could influence pollinator attraction in a similar way as genetic variation. However, the versatility of epigenetic fingerprints and the challenge of predicting the impact of gene flow on population epigenetic variability (Smithson et al. 2019, Greenspoon et al. 2022) indicate that the relationship with pollinator diversity must be complex

and, to some extent, different from association to genetic diversity. Moreover, the lack of knowledge on such relationships in natural populations makes it difficult to formulate specific hypotheses. Therefore, exploring the correlations between pollinator diversity and genetic and epigenetic variation in wild plant populations represents a promising avenue for understanding the ecological mechanisms underlying plant adaptation and evolution (Alonso et al. 2019).

The interrelationship between population genetic and epigenetic features and pollinator diversity may be important in explaining the adaptation of narrowly distributed plants. In theory, small and isolated populations should have reduced allelic variability due to the effects of genetic drift and inbreeding (Barrett and Kohn 1991, Ellstrand and Ellam 1993, Gitzendanner and Soltis 2000, Boyd et al. 2022), but also, genetic variability in small inbred populations can be more sensitive to gene flow (Richards 2000, Willi et al. 2007). In western Mediterranean mountains, many plant endemics show restricted geographical distributions consisting of patchy, small and isolated populations inhabiting harsh environments such as rocky outcrops, dolomitic soils or cliffs (Lavergne et al. 2004, Thompson et al. 2005, Molina-Venegas et al. 2015, Giménez-Benavides et al. 2018). Several studies in these species have found higher genetic diversity than expected from the detrimental effects of drift and inbreeding (Fernández-Mazuecos et al. 2014, Jiménez-Mejías et al. 2015, Forrest et al. 2017, Medrano et al. 2020). Likewise, other studies in the same region have shown high levels of epigenetic diversity in natural plant populations (Herrera and Bazaga 2010, Medrano et al. 2014, but Avramidou et al. 2015) that are comparable to those found in widespread congeneric species (Medrano et al. 2020). These findings indicate that other, as yet unexplored factors may have historically influenced gene flow, counteracting the expected genetic and epigenetic impoverishment in populations of Mediterranean endemic species.

Considering that 1) pollinator diversity can influence and be influenced by genetic (and potentially epigenetic) diversity, 2) the genetic (and potentially epigenetic) diversity of small, isolated populations is more sensitive to variations in gene flow, and 3) the Mediterranean harbours a high diversity of pollinator species (Herrera 1988a, b, 2019, Petanidou and Ellis 1993, Bosch et al. 1997, Ropars et al. 2020), we hypothesize that genetic and epigenetic variation may correlate more strongly to variation in pollination diversity in Mediterranean narrow endemics than in widely distributed congeners. To test this hypothesis, we selected five pairs of congeneric species found in the Sierras de Cazorla mountain range (southern Spain), each pair consisting of one narrow endemic and one widespread species, and studied up to three populations per species. Populations of these geographically restricted species have similar or even higher genetic and epigenetic diversities than their respective congeners (Medrano et al. 2020). By characterising the pollinator fauna and the genetic and epigenetic diversity of these populations we aim to explore: 1) whether visitation rate and pollinator diversity within populations depend on the plant distribution range; and 2) if these pollinator descriptors associate more strongly to variation in

genetic and epigenetic diversity in narrow endemics. To our knowledge, this is the first study explicitly addressing the relationships that link pollinator diversity with genetic and epigenetic features of plant populations.

## Material and methods

### Site and study system

Field work was carried out in the Sierras de Cazorla, Segura y Las Villas Natural Park (Jaén Province, Spain), a mountain system which is part of the Baetic Ranges in southeastern Iberian Peninsula. This area is characterised by a complex topography and heterogeneous environmental conditions that makes it one of the richest and most diverse natural habitats in Europe (Médail and Quézel 1999) particularly rich in plants (Mota et al. 2002, Melendo et al. 2003), and bee fauna (Ortiz-Sánchez et al. 2023).

Five pairs of congeneric plant species (Table 1, Supporting information) from five different families were considered, each pair consisting of one narrow endemic with a restricted distribution (R, hereafter) and a congeneric species with a widespread distribution (W, hereafter). These pairs inhabit the same geographical area and correspond to a subset of those analysed by Medrano et al. (2020): *Anthyllis ramburii* (R) and *A. vulneraria* (W); *Convolvulus boissieri* (R) and *C. arvensis* (W); *Erodium cazorlanum* (R) and *E. cicutarium* (W); *Teucrium rotundifolium*

(R) and *T. similatum* (W); and *Viola cazorlensis* (R) and *V. odorata* (W). All species are entomophilous. Experimental essays and pollen/ovule ratios indicate that most of the species are allogamous, with the exception of *E. cicutarium*, that can self-pollinate (studies reported in the Supporting information).

In the conceptual framework proposed by Rabinowitz (1981), a restricted species has a small geographic range with small sparse populations, and a narrow habitat specificity. The geographically restricted species considered here are narrow endemics from the Baetic mountain range that inhabit dolomitic rocky habitats with a complex topography and with poor sandy or rocky soils. These habitats usually have a great proportion of bare ground and a high diversity of endemic and rare plants (Mota et al. 2002). In contrast, widespread congeners are more common, growing on a wider spectrum of soils, usually in grasslands, shrublands or anthropised ecosystems (see the Supporting information and Medrano et al. 2020 for a detailed descriptions).

## Sampling and data acquisition

### Pollinator data

We initially selected three populations per species (Supporting information, Medrano et al. 2020). However, herbivory damage, insufficient flowering individuals or

Table 1. Pollinator sampling effort. For each population of the narrow endemic (R) and widespread (W) plant congeners, the number of censuses performed and the average and standard deviation number of flowers per sampling patch are provided. The number and probability of visits to a flower during three minutes of observation are also provided. Sampling coverage is an estimate of the percentage of species sampled using the visitation to a flower as sampling unit.

Species	Population	No. censuses	No. observed flowers per census	No. flower visits	Flower visitation prob.	Sampling coverage
<i>Anthyllis ramburii</i> (R)	anra1	71	141.2 ± 94.5	90	0.01	0.97
	anra2	62	222.8 ± 207.6	901	0.05	1.00
	anra3	65	60.2 ± 39.7	36	0.02	1.00
<i>Anthyllis vulneraria</i> (W)	anvu1	62	200.3 ± 93.7	500	0.05	0.99
	anvu3	92	317.7 ± 199.5	505	0.02	0.99
<i>Convolvulus boissieri</i> (R)	cboi2	77	19.4 ± 21.5	125	0.10	0.99
	cboi3	72	23.1 ± 9.9	137	0.12	0.96
<i>Convolvulus arvensis</i> (W)	carv1	69	5.1 ± 1.5	82	0.24	0.92
	carv2	68	9.0 ± 3.1	149	0.40	0.94
<i>Erodium cazorlanum</i> (R)	ecazF	77	5.4 ± 3.8	29	0.08	0.76
	ecazL	72	11.9 ± 5.3	60	0.08	0.90
	ecazT	37	2.6 ± 1.0	21	0.19	0.91
<i>Erodium cicutarium</i> (W)	ecicC	101	21.1 ± 13.6	115	0.06	0.92
	ecicF	86	8.8 ± 4.8	59	0.06	0.95
	ecicT	87	3.3 ± 1.4	42	0.15	0.79
<i>Teucrium rotundifolium</i> (R)	trot1	71	57.8 ± 35.5	104	0.03	1.00
	trot2	66	38.8 ± 20.4	176	0.07	0.99
	trot3	62	58.1 ± 42.7	1706	0.54	1.00
<i>Teucrium similatum</i> (W)	tpol1	66	252.6 ± 118.1	1028	0.06	1.00
	tpol2	60	122.0 ± 79.0	574	0.06	1.00
	tpol3	60	232.6 ± 111.0	2947	0.21	1.00
<i>Viola cazorlensis</i> (R)	vcaz1	63	21.7 ± 13.4	38	0.05	0.98
	vcaz2	65	49.1 ± 34.4	81	0.02	1.00
	vcaz3	70	22.9 ± 15.1	20	0.02	1.00
<i>Viola odorata</i> (W)	vodo1	107	22.0 ± 12.7	25	0.01	1.00
	vodo2	74	32.3 ± 23.7	35	0.01	1.00

logistic limitations limited our study to 26 populations. During March–June 2021 the pollinator assemblage of each population was characterised by conducting a minimum of 60 pollinator censuses (mean =  $73 \pm 15$  censuses per population). Here, a pollinator census is the sampling unit and consisted of 3 minutes of observation of a flowering patch, i.e. a delimited area with a number of flowers that are easily accessible to visual inspection. At each census, the identity of each pollinator and the number of flowers visited, as well as the number of open flowers in the patch, were recorded. Censuses were carried out in randomly selected patches, always in direct sunlight and on sunny days and were distributed at the beginning and at the peak of flowering to capture a fair representation of the pollinator fauna (Valverde et al. 2016). This sampling method and observation effort have demonstrated to give reliable estimates of pollinator diversity in a wide range of plant species (Herrera 2019, 2020).

In this study, a pollinator is considered to be any taxon that at any time contacted the reproductive organs of the plant. For a precise taxonomical identification of pollinators, each pollinator–flower interaction was photographed. Difficult to identify specimens were collected and contrasted against our own reference pollinator checklist (Ortiz-Sánchez et al. 2023) or sent to a number of experts listed in the acknowledgements for identification. Because of the unavoidable uncertainty in the identification of certain taxonomical groups, some specimens were clumped. For example, *Lasioglossum pauperatum* also includes *L. transitorium*, Nitidulidae species were all considered *Brassicogethes aeneus* as this species represents 75% of all specimens in most of these plant species (Herrera and Otero 2021). In other cases, we used morphospecies, e.g. for specimens from the Tachinidae or Anthicidae families or from the *Empis* or *Hilara* genus (Empididae) (see the Supporting information resumes all observed pollinators). As a result, up to 92% of pollinator individuals were characterised at the species or morphospecies level.

It is important to highlight that the availability of pollinator data from previous years of some of our study populations (Herrera 2019) allowed us to compare our census data with data collected 5 to 16 years before using this same methodology (Supporting information for a detailed description and results of these analyses). This comparison showed a high correlation in species richness between years ( $\beta = 0.80$ ,  $t = 4.15$ ,  $df = 10$ ,  $p = 0.002$ ). This supports the interannual stability of pollinator diversity in our study system as previously shown in Herrera (2019) for a much larger number of species in the study area, and allows us to feel confident when comparing contemporary pollinator data with historically inherited genetic and epigenetic data of plants.

## Genetic and epigenetic data

The genetic and epigenetic data are a subset of those published in Medrano et al. (2020). Specifically, from the populations for which pollination data were obtained, an average

of 26 random individuals per population were genotyped (range 23–40). Genetic profiles of sampled individuals were obtained by amplified fragment length polymorphism (AFLP; Meudt and Clarke 2007) and epigenetic profiles by amplified methylation-sensitive polymorphism (MSAP; Reyna-López et al. 1997, Fulneček and Kovařík 2014, Schulz et al. 2014), a technique useful to identify genome-wide methylation profiles in ecological epigenetics studies of species without detailed genomic information (Schrey et al. 2013). As a result, for the AFLP markers we obtained a binary matrix depicting the presence or absence of each loci. For the MSAP markers, we followed Schulz et al. (2014) to obtain two binary matrices: one with hemi- or fully methylated epiloci (hereafter M-MSAP), and one data matrix of unmethylated epiloci (U-MSAP). We used on average 11.2% (AFLP) and 25.8% (MSAP) of the samples to eliminate inconsistent loci based on their repeatability (8.6–17.6% of samples for AFLP; 13.3–29.3% of samples for MSAP). We also discarded non-informative monomorphic loci by selecting those in which the proportion of presence or absence of the fragment exceeded 5%. This yielded 140 to 316 AFLP, 84 to 181 U-MSAP and 100 to 213 M-MSAP loci per species. For a detailed description of these methodologies we refer to the Supporting information or to Medrano et al. (2020).

Despite the drawback of being dominant markers, using AFLP and MSAP has the important advantage of allowing reliable detection of mutually comparable genetic and epigenetic variants in several non-model species with large sample sizes (Schrey et al. 2013, Medrano et al. 2014, 2020, Herrera and Bazaga 2016, Wilschut et al. 2016).

## Data analyses

### Pollinator descriptors

For each population we evaluated the visitation rate and pollinator diversity. We calculated the visitation rate using two indices: the patch visitation probability (number of visits to a patch  $\times 3$  min census<sup>-1</sup>), which is irrespective of the number of flowers visited, and the flower visitation probability (number of visited flowers  $\times$  number of open flowers<sup>-1</sup>  $\times 3$  min census<sup>-1</sup>), which takes into account the number of flowers visited.

We estimated the sample coverage of each population (proportion of the total estimated number of species; Chao and Jost 2012) based on the number of visited flowers and using the R package ‘iNEXT’ (www.r-project.org, Hsieh et al. 2016). Following, we calculated the diversity of pollinators on each population using Hill numbers (<sup>q</sup>D; Hill 1973) at an equal sample coverage of 0.8. At this value, the extrapolation of diversity values of the two populations showing lower sampling coverages (e<sub>cazF</sub>, 0.76 and e<sub>cicT</sub>, 0.79; Table 1) is reliable (Chao et al. 2014).

Hill numbers are a unified family of diversity indexes that equals the number of equally abundant taxa needed to obtain a given diversity, allowing for easier comparisons among assemblages (Jost 2007). These are defined as:



$${}^qD = \left( \sum_{i=1}^S p_i^q \right)^{(1/1-q)}$$

Where  $S$  is the total number of species,  $p_i$  is the relative frequency of species  $i$ , and  $q$  is a parameter that determines the sensitivity of the index to the relative frequencies. By varying  $q$  between 0 and 2, Hill numbers range between species richness ( $q=0$ ), the logarithm of the Shannon information index ( $q \approx 1$ , Shannon's diversity herein) which considers true relative abundances of species, and Simpson's diversity ( $q=2$ ) which weights for dominant species (Hill 1973, Jost 2006, Chao et al. 2014). To obtain a fine characterisation of the pollinator diversity of each population, we constructed the diversity profiles by calculating the Hill numbers from  $q=0$  to  $q=2$  by increments of 0.1, allowing us to visually explore the diversity along a gradient of relative importance of pollinator abundances (Jost 2007).

### Genetic and epigenetic descriptors

Each population was characterised by its genetic and epigenetic diversity and distinctiveness. We calculated the diversity for the genetic and epigenetic markers by means of two indices: the 'proportion of polymorphic loci' and the per-locus average of the 'Shannon diversity index' (King and Schaal 1989, R package 'MSAP' Pérez-Figueroa 2013, Supporting information). To calculate the genetic and epigenetic distinctiveness, we used two indexes: the 'proportion of private alleles' and a 'rarity index' (Schönswetter and Tribsch 2005, R package 'AFLPdat', Ehrlich 2006, Supporting information). Values from the rarity index are expected to be high at isolated populations with an historical accumulation of rare markers.

### Statistical analysis

Differences in pollinator diversity between narrow endemics and widespread congeners were analysed using species richness, Shannon's and Simpson's indexes (Hill numbers of  $q$  equal to 0, 1 and 2, respectively). For each diversity index, we calculated the difference between all possible pairs of congeneric populations. We used non-overlapping 95% confidence intervals as criteria to assess the significance of each comparison.

Differences in pollinator visits between narrow endemics and widespread species were assessed using generalised mixed effects models (R package 'lme4', Bates et al. 2015). We modeled the global effect of species distribution range across all congeneric pairs of species using a binomial error distribution and a log-link function. In these models, population was nested within genus as random effects. Further, we assessed the effect of species distribution range at each congeneric pair through additional models that included population as random effects and genus as a fixed term. Such models were applied using both the patch and the flower visitation probabilities as response variables. For the patch visitation probability we included the scaled number of flowers per species as

a covariate to control for the potential effect of flower display in attracting pollinators. For the flower visitation probability, an observation-level random effect was included to deal with overdispersion in our data (Harrison 2014). Differences between narrow endemics and widespread congeners were assessed through post hoc pairwise contrasts on the estimated marginal means and using Bonferroni correction in models considering multiple comparisons (R package 'emmeans' ver. 1.5.4, Lenth 2021).

Finally, we assessed the relationship between population estimators of pollinator diversity and genetic and epigenetic descriptors of plant populations. First, we used Pearson correlations to explore general trends in populations from narrow endemics and widespread species separately. Correlations with Hill diversities of intermediate  $q$  values were included in this exploration to visualize such relationships along a gradient of relative importance of pollinator abundances. Afterwards, a more exhaustive analysis was performed using generalised mixed effects models on those relationships showing a significant correlation. By including species as a random term, these models allowed to control for variation among species. Family and linkage functions were chosen in each model according to data structure and goodness-of-fit. For all relationships, the genetic and epigenetic descriptors were used as dependent variables and plant genus was included as random factor. In all cases we used random intercept models because of our limited data per species and because these outperformed in all cases the random slopes models (Supporting information).

## Results

### Pollinator composition

We recorded 1214 pollinator individuals from 179 different taxa. The relative frequency of pollinator orders varied considerably across sample populations and plant species (Fig. 1). Hymenoptera performed 84.1% of all visits, but were not the most frequent pollinators in all studied species (Fig. 1 and Supporting information). At two genera *Anthyllis* and *Teucrium* congeneric species showed similarities in the main pollinator order. In *Anthyllis* species Hymenoptera were the main pollinators (*A. vulneraria* (W)=85–94% of all flower visits; *A. ramburii* (R)=71–100%), although they differed in the identity of the main pollinator family: Apidae (79–85%)—mainly Anthophorini—in *A. vulneraria* and Andrenidae (44–72%) and Apidae (53%) in *A. ramburii*. In *Teucrium*, Hymenoptera were the main pollinators in all populations from the widespread *T. similitum* (W; 78–98%) and in two populations of *T. rotundifolium* (R; 73–94%), while Diptera were the most frequent pollinators in the third population (43%). On the other hand, contrasting pollinator assemblages were found between congeneric species in *Convolvulus*, *Erodium* and *Viola* genera. In *Convolvulus*, Coleoptera were the main pollinators in *C. arvensis* (W; 51–59%) while Hymenoptera dominated in *C. boissieri* (R; 52–67%). In *Erodium*, Diptera visited most flowers in *E. cicutarium* (W; 50–83%), while in *E. cazorlanum*

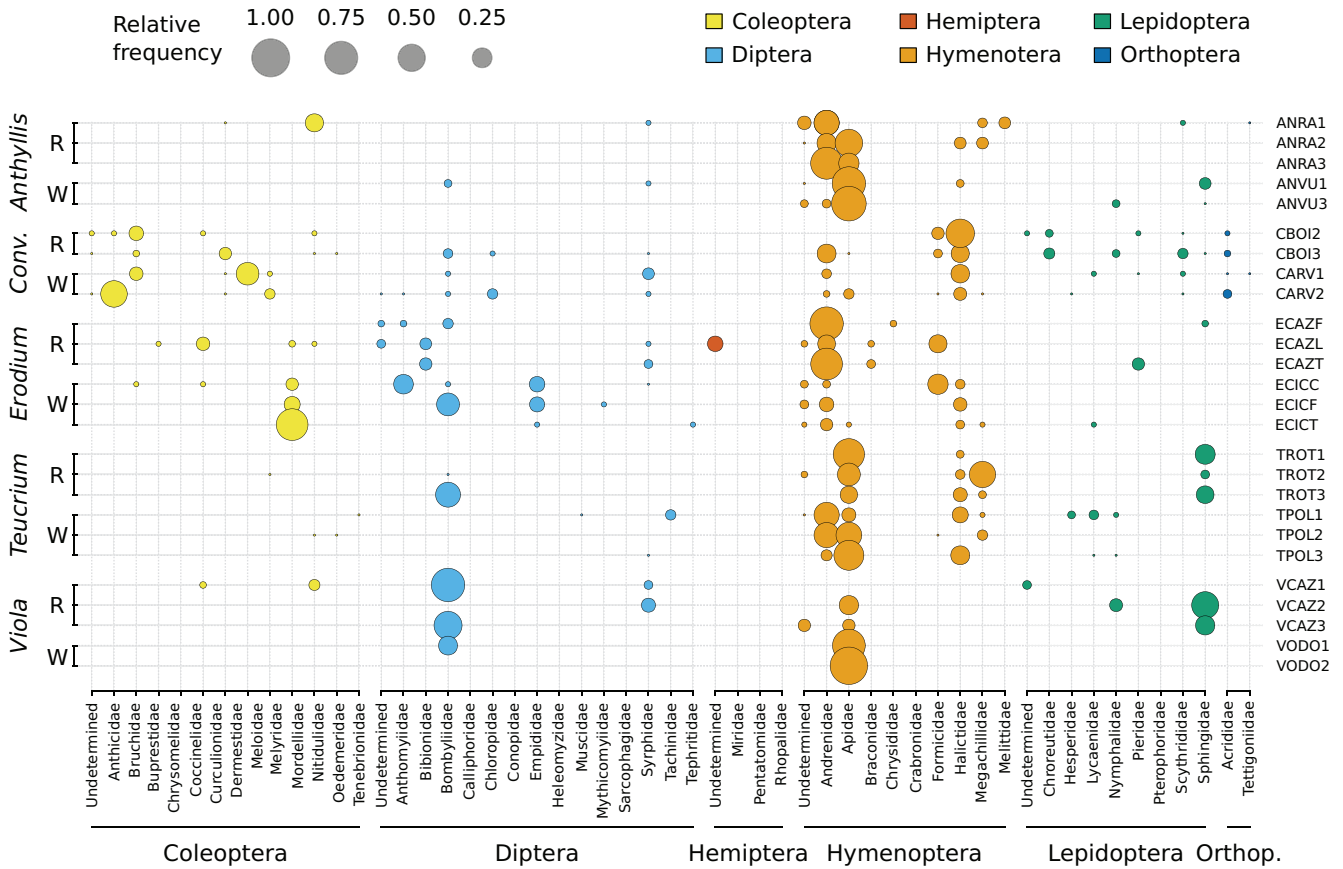


Figure 1. Pollinator assemblages observed at the studied populations. Circle sizes are relative to the proportion of flower visits made by each insect family in a given population. R and W stand for narrow endemic and widespread species respectively. *Conv.* denotes *Convolvulus*, and *Orthop.* Orthoptera.

(R) Hymenoptera were the main flower visitors in two populations (44–53%) and Coleoptera in the other (71%). Finally, in *Viola odorata* (W), Hymenoptera – mainly *Anthophora dispar* – were the main pollinators (76–100%), while in *V. cazorlensis* (R) Diptera – mainly Bombyliidae – were the main pollinators in two populations (55–84%) and Lepidoptera – mostly *Macroglossum stellatarum* (Sphingidae) – in the other (62%).

### Variation in pollinator diversity and visitation rate

The diversity profiles did not suggest any consistent trend in the comparison of pollinator diversities between populations of narrow endemics and widespread congeneric species (Supporting information). Only two genera showed consistent differences in pollinator diversity (Fig. 2). In *Anthyllis*, flowers of the widespread *A. vulneraria* were visited by a more diverse pollinator assemblage than flowers from its narrow endemic congener, with non-overlapping confidence intervals in most comparisons of the pollinator richness, Shannon and Simpson diversity (Fig. 2). In contrast, the narrow endemic *Viola cazorlensis* showed significantly higher pollinator diversity in most comparisons. The rest of congeneric pairs did not show any clear association between pollinator diversity and plant distribution range.

On the contrary, the type of distribution range had an effect on the visitation rate. The patch visitation probability varied with the distribution range (estimate = -0.73,  $z = -2.09$ ,  $p = 0.037$ ; Supporting information), being higher for widespread species (Fig. 3). Flower display showed a positive significant effect on the patch visitation probability (estimate = 0.33,  $z = 5.37$ ,  $p < 0.001$ ). The higher patch visitation probability in the widespread species was particularly noticeable in the *Teucrium* pair ( $z_{\text{ratio}} = 4.28$ ,  $p < 0.001$ ; Fig. 3, Supporting information). The flower visitation probability was also influenced by the distribution range (estimate = -0.74,  $z = -1.95$ ,  $p = 0.05$ ; Supporting information), with flowers from the widespread species having higher visitation probabilities (Fig. 3). This global pattern reflected the significantly higher probabilities found for the widespread species in the genera *Anthyllis*, *Convolvulus* and *Teucrium* ( $z_{\text{ratio}} = 1.97$ – $6.08$ ,  $p < 0.05$ ; Fig. 3, Supporting information).

### Relationship between pollinators and plant population genetic and epigenetic descriptors

Only narrow endemics showed significant correlations between pollinator and the genetic or epigenetic descriptors of plant populations (Supporting information). The

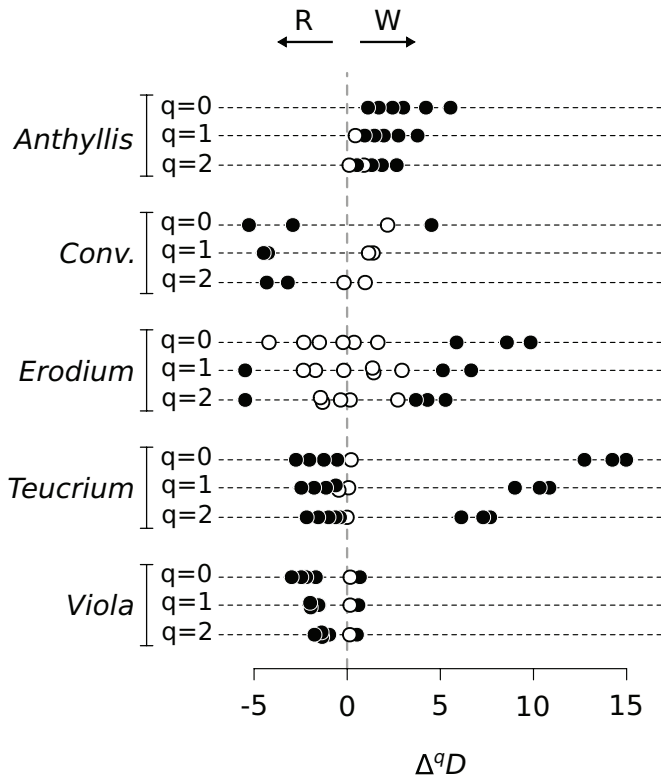


Figure 2. Differences in pollinator diversity between congeneric populations. Each point represents the difference in diversity between a population from a widespread plant species and a population from the corresponding narrow endemic congener ( $\Delta^qD$ ). Thus, negative values indicate higher diversity in the population from the narrow endemic (R) while positive values indicate higher diversity in the population from the widespread congener (W). Filled symbols denote comparisons with non-overlapping confidence intervals. *Conv.* stands for *Convolvulus*.

Shannon diversity of AFLP genetic markers showed positive significant correlations with patch and flower visitation probabilities ( $\beta=0.59$ ,  $t=2.53$ ,  $df=12$ ,  $p=0.026$  and  $\beta=0.54$ ,  $t=2.19$ ,  $df=12$ ,  $p=0.049$ , respectively) and with pollinator richness ( $\beta=0.60$ ,  $t=2.58$ ,  $df=12$ ,  $p=0.024$ ). Similarly, the Shannon diversity of U-MSAP epigenetic markers showed a positive significant correlation with the patch visitation probability ( $\beta=0.59$ ,  $t=2.51$ ,  $df=12$ ,  $p=0.027$ ) and with pollinator richness ( $\beta=0.57$ ,  $t=2.40$ ,  $df=12$ ,  $p=0.034$ ) and pollinator Shannon's diversity ( $\beta=0.53$ ,  $t=2.19$ ,  $df=12$ ,  $p=0.044$ ). After controlling for interspecies variability, the mixed-effects models showed a significant relationship between AFLP Shannon diversity and patch visitation probability (estimate=0.08,  $t=2.59$ ,  $p=0.027$ ; Table 2), while a decrease in significance was observed for the rest of the previous significant correlations ( $p > 0.105$ ).

As for the genetic and epigenetic distinctiveness, the rarity index of AFLP genetic markers showed a negative significant correlation with the patch visitation probability ( $\beta=-0.54$ ,  $t=-2.20$ ,  $df=12$ ,  $p=0.048$ ), while the proportion of private M-MSAP epigenetic alleles had a negative

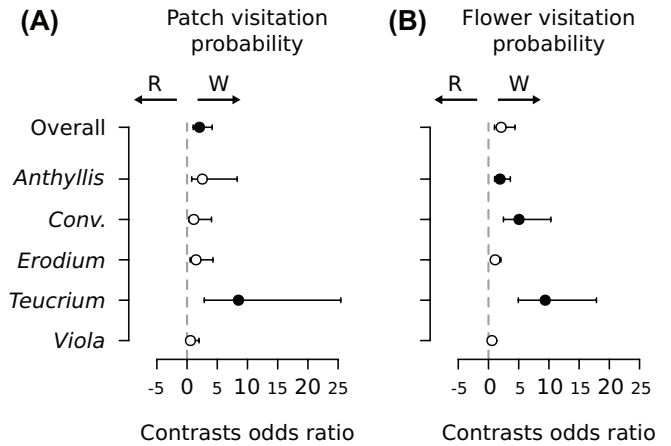


Figure 3. Differences in visitation rates between populations of congeneric species. Panels show post hoc paired comparisons of flower (A) and patch (B) visitation probabilities. Estimated odds ratios and the corresponding 95% confidence interval are shown for each comparison. Negative and positive values indicate higher visitation rates in the narrow endemics (R) and widespread species (W) respectively. Filled symbols denote significant differences. *Conv.* stands for *Convolvulus*.

significant correlation with all the range of pollinator diversities ( $\beta=-0.57$  to  $-0.55$ ,  $t=-2.28$ – $-2.41$ ,  $df=12$ ,  $p < 0.042$ ; Fig. 4 and Supporting information). Mixed effect models showed a significant negative relationship of the proportion of private M-MSAP with any measurement of pollinator diversity using a log-link function ( $\beta=-0.47$ – $-0.76$ ,  $t=-6.42$  to  $-6.94$ ,  $p < 0.001$ ; Table 2, Fig. 5).

## Discussion

This study explores whether population genetic and epigenetic variation show a stronger correlation with pollinator diversity in Mediterranean plants with a restricted distribution range than in widely distributed congeners. We used a multi-species approach consisting of a narrow endemic and a widespread species, and replicate populations at a local scale. Our findings show a positive relationship between the diversity of pollinators and the diversity of plant genetic and epigenetic markers, and a negative relationship between pollinator diversity and epigenetic distinctiveness only in populations of narrow endemics. This study represents a novel effort, explicitly addressing the links between pollinator diversity and genetic and epigenetic features of plant populations.

The data collected in this study operate at two temporal levels. Firstly, the genetic and epigenetic data reflect the historical accumulation of evolutionary processes in the studied populations. These were assumed representative provided we did not record recent substantial disturbances in any of them. Secondly, pollinator data represent a contemporary picture of plant–pollinator interactions. While plant populations may undergo interannual turnover in their pollinators (e.g. *Lavandula latifolia*, Herrera 1988a, b, *Erysimum mediohispanicum*, Valverde et al. 2016), the plant species in this study

Table 2. Summary of the significant relationships observed between pollinator and genetic and epigenetic descriptors in narrow endemics. The table shows the correlations and mixed model results of those relationships that are significantly correlated (Fig. 4, Supporting information). Note that for the proportion of private M-MSAP epigenetic alleles the coefficients from the mixed effects models correspond to estimates using the log-link function. p-values in bold denote significance at  $\alpha=0.05$ . Models use the following syntax in the 'lme4' R package:  $lmer(Y \sim X + (1 | genus))$ . For the proportion of private M-MSAP epigenetic alleles, the syntax is as follows:  $glmer(Y \sim X + (1 | genus), family = gaussian(link = 'log'))$ . In these models Y and X denote the response and predictor variables respectively.

Response	Predictor	Pearson's correlation			Linear mixed effects model		
		Correlation	t	p	Estimate	t	p
Descriptors of genetic and epigenetic diversity							
Genetic AFLP: Shannon index	Patch visitation probability	0.59	2.53	<b>0.026</b>	0.08	2.59	<b>0.027</b>
	Flower visitation probability	0.54	2.19	<b>0.049</b>	-0.04	-0.21	0.837
	Pollinator richness	0.60	2.58	<b>0.024</b>	0.00	1.27	0.234
Epigenetic U-MSAP: Shannon index	Patch visitation probability	0.59	2.51	<b>0.027</b>	0.08	1.76	0.109
	Pollinator richness	0.57	2.39	<b>0.034</b>	0.00	1.78	0.105
	Pollinator Shannon diversity	0.53	2.19	<b>0.048</b>	0.00	1.56	0.149
Descriptors of genetic and epigenetic distinctiveness							
Genetic AFLP: rarity index	Patch visitation probability	-0.54	-2.20	<b>0.048</b>	-1.01	-1.44	0.180
Epigenetic M-MSAP: proportion of private alleles (log-link)	Pollinator richness	-0.55	-2.31	<b>0.039</b>	-0.47	-6.42	<b>&lt;0.001</b>
	Pollinator Shannon diversity	-0.57	-2.39	<b>0.034</b>	-0.59	-6.89	<b>&lt;0.001</b>
	Pollinator Simpson diversity	-0.55	-2.28	<b>0.041</b>	-0.76	-6.93	<b>&lt;0.001</b>

show temporal stability between years in both visitation rate (Herrera 2019) and pollinator richness in a subset of the studied populations (analysis in the Supporting information). This, indicates that the current pollinator data are sufficiently representative of past situations, providing some confidence for comparison with population genetic data. Thus, although interpreted with caution, our results provide insights on the relationships between pollinators and genetic and epigenetic descriptors of plant populations which are relevant for a better understanding of the ecology of narrow endemic species.

In the following sections, we first provide a naturalistic overview of the observed pollinator composition. We then

discuss the lack of association found between pollinator diversity and plant distribution range and the higher visitation rates measured in widespread species. Finally, we discuss potential explanations about the correlative findings between pollinators and plant genetic and epigenetic features found in populations of narrow endemics.

### Pollinator composition

The number of pollinator taxa recorded is in consonance with previous studies reporting the high diversity of pollinator species found in these mountains (Herrera 2019, 2021,

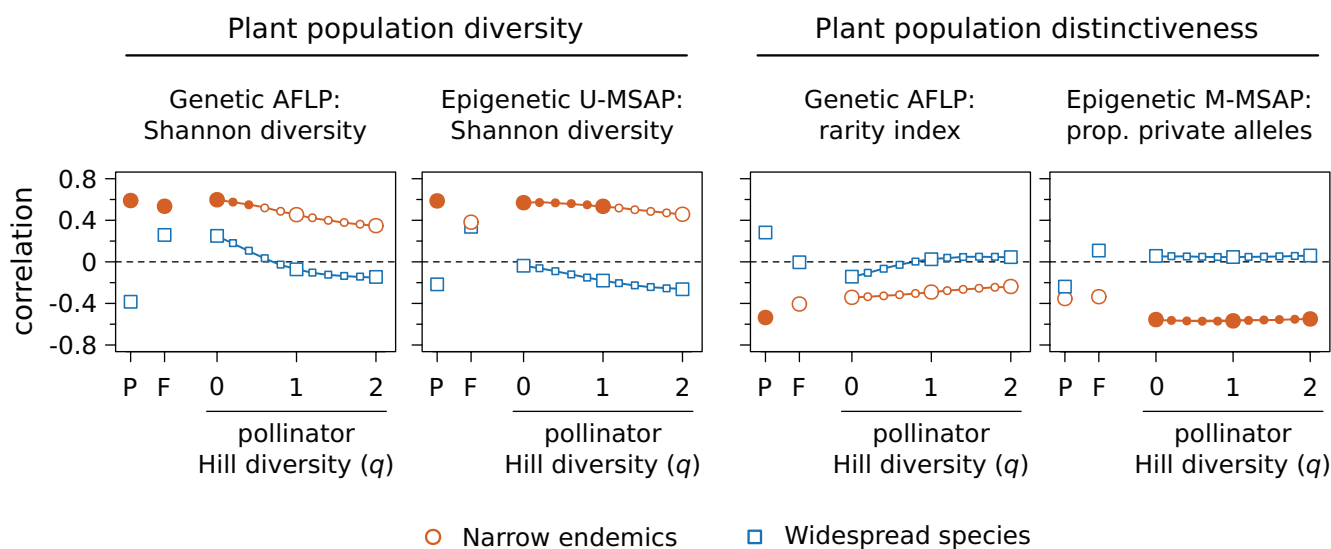


Figure 4. Correlations between estimators of pollinator and genetic and epigenetic descriptors. The panels show the correlations for the diversity and distinctiveness indices for which any significant correlations were found (for all correlations see the Supporting information). Orange circles depict narrow endemics and blue squares widespread species. Filled symbols indicate significant correlations at  $\alpha=0.05$ . Correlation values correspond to those for the patch (P) and flower (F) visitation probabilities and for Hill diversities from  $q=0$  to  $q=2$  by increments of 0.2. Increased size symbols highlight correlations at pollinator diversity measures corresponding to species richness, Shannon and Simpson indices ( $q$  equal to 0, 1 and 2 respectively). See the Material and methods for an accurate description of these indices.



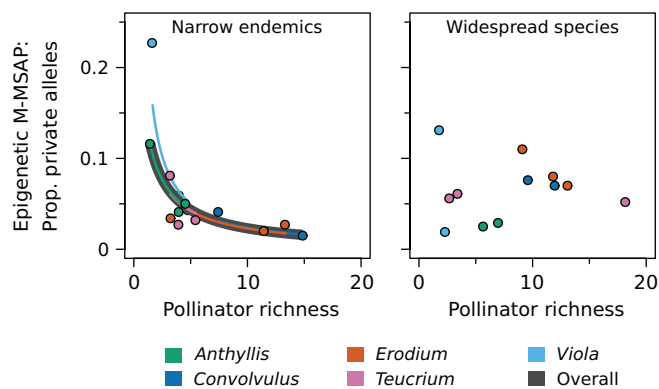


Figure 5. Relationship between the proportion of private M-MSAP epigenetic alleles and pollinator richness. Model fit for the narrow endemics is shown in the left panel: predicted relationships for all populations (fixed effect level; thick grey line) and for each plant species separately (random effect levels; thin coloured lines) are depicted.

Ortiz-Sánchez et al. 2023) and adds to previous evidences that the Mediterranean region is a hotspot of pollinator diversity (Herrera 1988a, b, 2019, Petanidou and Ellis 1993, Bosch et al. 1997, Ropars et al. 2020). Hymenoptera were the most important visitors in most plant species and populations (84% of flower visits), but the variability in ranked frequency of pollinator visits evidences the quantitative relevance of other non-hymenoptera species (e.g. Lepidoptera in *V. cazorlensis*, Coleoptera in *C. arvensis*; Supporting information). These historically underestimated taxonomic groups may also be important and efficient pollinators (Jauker and Wolters, 2008, Orford et al. 2015, Valverde et al. 2019) and thus should be considered when endorsing functional meaning to pollinator diversity.

Pollinators have been posed as key in the evolution of rarity in flowering plants (Orians 1997), yet the few studies that analyse this prediction show discrepancies in their results, making it difficult to draw general conclusions. This is probably due to ambiguity in classifying plant species as rare (Rabinowitz 1981) and the way studies are conducted – e.g. single species studies (Jabis et al. 2011, Fernández et al. 2015) versus comparative approaches (Mehrhoff 1983, Karron 1987, Purdy et al. 1994) –, but also to the diverse features of the studied species (Lavergne et al. 2004, Giatzouzaki et al. 2022). Aiming to overcome these problems, our study considers several pairs of geographically restricted and widely distributed congeneric species (according to Rabinowitz 1981) from different plant families. As a consequence, we studied species with very contrasting phenologies – from the earliest *V. odorata*, (March–April) to the latest *Convolvulus* species (June) –, and with contrasting floral features – i.e. specialised flowers *Anthyllis*, *Teucrium*, *Viola* versus open accessible flowers *Convolvulus*, *Erodium* –, that certainly may explain part of the differences in pollinator composition recorded. But, by considering them all in a multispecies population framework allowed us to explore more general patterns about pollinator diversity and plant population features, as discussed below.

## Variation in pollinator diversity and visitation rate

Our results support previous findings on the differences between plants with contrasting distribution ranges in the diversity and visitation rates of pollinators. First, in line with Karron (1987), we did not find a clear effect of plant distribution range on pollinator diversity. Three of the studied congeneric pairs showed no clear pattern in diversity differences, while the other two pairs showed clear but contrasting differences. In *Anthyllis*, the widespread *A. vulneraria* showed higher species diversity than its narrow endemic congener in most comparisons, although the latter was visited by a higher number of insect families and orders, indicating a more diverse functional diversity. On the contrary, in the *Viola* pair, the narrow endemic *V. cazorlensis* showed higher diversity than its widespread congener in most comparisons. Very likely, this result reflects the contrasting flowering periods of these *Viola* species. Specifically, the earlier flowering of the widespread congener could be a limiting factor for the availability of pollinator species.

Second, we observed a tendency towards higher patch and flower visitation probabilities in widespread species, a result that is consistent with previous findings on this issue (Mehrhoff 1983, Karron 1987, Purdy et al. 1994, Rymer et al. 2005, Powell et al. 2011, but see Banks 1980 and Ruilova and Marques 2016). This pattern was particularly evident in *Anthyllis*, *Convolvulus* and *Teucrium* pairs (Supporting information). The widespread species from two of these pairs with more specialised corollas *A. vulneraria* and *T. simlatum* displayed considerably more flowers per patch than their respective narrow endemic congeners (Table 1) suggesting a positive effect of flower display in attracting more pollinator visits (Willson and Price 1977, Thomson 1988, Klinkhamer et al. 1989, reviewed by Ohashi and Yahara 2001). The observed positive and significant effect of the number of flowers on the patch visitation probability would also corroborate this idea (Supporting information). Other variables than floral display, such local soil substrate and its effect on pollinator abundance (Potts and Willmer 1997, Potts et al. 2005, Sardiñas and Kremen 2014) may be responsible of the lower visitation rates obtained in narrow endemics. This hypothesis should further be tested in studies that consider a fine characterisation of soil characteristics and its spatial heterogeneity.

## Relationship between pollinators and plant population genetic and epigenetic diversities

Our results found support for a positive association between pollinators and plant population genetic and epigenetic diversities only in narrow endemics. In particular, higher visitation rates and pollinator diversity related to higher genetic and epigenetic diversities. Such correlation was similar for the three studied markers, but significant for the genetic (AFLP) and the unmethylated epigenetic markers (U-MSAP), i.e. those that increased methylation in a few individuals. Interestingly, epigenetic U-MSAP and M-MSAP markers did not respond equally, suggesting that the use of more informative markers

capable of giving genomic location and functional significance to genetic and epigenetic variants (Paun et al. 2019) and other techniques suitable to estimate transgenerational transmission of such markers (Johannes and Schmitz 2019) could provide additional insight.

The stronger association between pollinators and population genetic and epigenetic diversities found in narrow endemics could be related to the generally smaller and more isolated populations of plant species with a restricted distribution range. In small populations, correlated paternity – the proportion of full siblings within maternal progeny arrays – is higher (Hardy et al. 2004, DiLeo et al. 2018), thus promoting inbreeding and the consequent reduction in genetic diversity (Wright 1969, Ellstrand and Elam 1993). Under these circumstances, higher pollinator diversities could counteract these expected outcomes by the deposition of more genetically diverse pollen loads on stigmas that result from the combination of contrasting pollen transportation efficiencies (Karron et al. 2006, Castilla et al. 2017, Rhodes et al. 2017, Valverde et al. 2019). This rationale might be specially relevant in habitats with high pollinator diversity such as those found in the Mediterranean ecosystems. In addition, higher visitation rates in a set of populations not particularly attractive to pollinators (in comparison to those of widespread species) would further reinforce pollen deposition and gene flow, and promote genetic diversity in these plant populations. Remains unclear to what extent the contribution of the most efficient pollinator(s) could illustrate the same or alternative associations, given that for instance, some frequent pollinators such as *Macroglossum stellatarum* (Sphingidae) in *V. cazorlensis* have long flight distances and could efficiently transport pollen within and between populations (Herrera and Bazaga 2008, Cánovas et al. 2015, Hennessy et al. 2021).

Our results have to be interpreted correlatively. Clarifying the causal hypothesis mentioned above as well as other alternative causalities is challenging and will require future studies with more populations per species and covering a wide population size range (Campbell and Husband 2007). Conversely, experimental studies focussing on certain species might add evidence on this little explored relationship. For instance, studies limiting access to certain pollinators that allow addressing their pollination effectiveness and demographic consequences (Herrera 2000) or their contribution to the genetic diversity of the progeny of individual plants (Valverde et al. 2019) can be key to understand the links between genetic and epigenetic diversities and pollinator diversity.

### Relationship between pollinators and plant population genetics and epigenetic distinctiveness

Only narrow endemics showed a negative association between pollinators and genetic and epigenetic population distinctiveness. This association was generalised for most pollinator descriptors but significant for the relationship between genetic distinctiveness and pollinator visits and between methylated epigenetic (M-MSAP) distinctiveness and pollinator diversity.

Again, we can argue that this result could be related to the higher sensitivity of small populations to gene flow. Several studies have experimentally demonstrated that the proportion of immigrant pollen flow can be high in small populations (Klinger et al. 1992, Goodell et al. 1997). More important, immigrant rare alleles can spread more easily in smaller populations, especially if the inbreeding level of a population is high (reviewed by Tallmon et al. 2004 and Whiteley et al. 2015). In this sense, populations of geographically restricted plant species with lower pollinator activity than their widespread congeners could retain more rare alleles, explaining the observed negative relationship of genetic and epigenetic distinctiveness and patch visitation probability.

The relationship between pollinators and the genetic cohesion of plant populations has been studied mainly by comparing plant species or populations whose main pollinator differs widely in its flight capabilities (Castellanos et al. 2003, Breed et al. 2015, Gamba and Muchhala 2020). We can speculate that relevance of the flying behaviour of the main pollinator could be more informative to understand population distinctiveness. However, several studies have observed that even small insects with presumably low flight distances can generate pollen flow between populations (Dick et al. 2003, Byrne et al. 2008), which would support the hypothesis that pollinator diversity is a major determinant of allele flow between and within small plant populations. Altogether, our results should serve as a correlative evidence for future studies to consider pollinator diversity as a potential factor affecting genetic and epigenetic cohesion between populations. On top of this, the significant pattern exhibited by distinctiveness of M-MSAP markers (Fig. 5) support the interest of analysing more informative epigenetic markers to better understand the behaviour of epigenetic variation and the causality of such association.

### Conclusions

This study contributes to highlight the importance of pollination studies in understanding the evolution of plant rarity. First, the results corroborate a lower pollinator visitation rate in geographically restricted plant species, and, more unexpectedly, that diversity of pollinators was not reduced in comparison to that in widespread congeners. Second, this study is the first in assessing the relationships between pollinator visitation rate and diversity and estimators of genetic and epigenetic diversity and distinctiveness in natural plant populations. Notably, the positive relationship of pollinators with genetic and epigenetic diversity, and negative with epigenetic distinctiveness of populations of geographically restricted Mediterranean species, points to the importance of pollinators to counterbalance genetic erosion in small populations inhabiting harsh environments. Future studies should include more informative markers capable of providing functional significance to genetic and epigenetic variation, and shed light on the causal direction of the relationships found here.

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## Author contributions

**Javier Valverde:** Data curation (equal) Formal analysis (lead) Investigation (equal) Methodology (equal) Writing – original draft (lead) Writing – review and editing (lead). **Mónica Medrano:** Conceptualization (equal) Data curation (equal) Formal analysis (equal) Investigation (equal) Methodology (equal) Writing – review and editing (equal). **Carlos M. Herrera:** Conceptualization (equal) Investigation (equal) Methodology (equal) Supervision (equal) Validation (equal) Writing – review and editing (equal). **Conchita Alonso:** Conceptualization (equal) Formal analysis (equal) Funding acquisition (lead) Investigation (equal) Methodology (equal) Supervision (lead) Writing – review and editing (equal).

## Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.dbrv15f9d> (Valverde et al. 2024).

## Supporting information

The Supporting information associated with this article is available with the online version.

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