# Evolution of pollination niches and floral divergence in the generalist plant *Erysimum mediohispanicum*

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• *Background and Aims* How generalist plants diverge in response to pollinator selection without becoming specialized is still unknown. This study explores this question, focusing on the evolution of the pollination system in the pollination generalist *Erysimum mediohispanicum* (Brassicaceae).
• *Methods* Pollinator assemblages were surveyed from 2001 to 2010 in 48 geo-referenced populations covering the entire geographic distribution of *E. mediohispanicum*. Bipartite modularity, a complex network tool, was used to find the pollination niche of each population. Evolution of the pollination niches and the correlated evolution of floral traits and pollination niches were explored using within-species comparative analyses.
• *Key Results* Despite being generalists, the *E. mediohispanicum* populations studied can be classified into five pollination niches. The boundaries between niches were not sharp, the niches differing among them in the relative frequencies of the floral visitor functional groups. The absence of spatial autocorrelation and phylogenetic signal indicates that the niches were distributed in a phylogeographic mosaic. The ancestral *E. mediohispanicum* populations presumably belonged to the niche defined by a high number of beetle and ant visits. A correlated evolution was found between pollination niches and some floral traits, suggesting the existence of generalist pollination ecotypes.
• *Conclusions* It is conjectured that the geographic variation in pollination niches has contributed to the observed floral divergence in *E. mediohispanicum*. The process mediating this floral divergence presumably has been adaptive wandering, but the adaptation to the local pollinator faunas has been not universal. The outcome is a landscape where a few populations locally adapted to their pollination environment (generalist pollination ecotypes) coexist with many populations where this local adaptation has failed and where the plant phenotype is not primarily shaped by pollinators.

**Key words:** Adaptive wandering, generalist pollination ecotype, pollination niches, floral divergence, *Erysimum mediohispanicum*, Brassicaceae, speciation.

## INTRODUCTION

The evolution of floral diversity has been considered mostly as the adaptive outcome of specialized interactions between flowers and their major pollinators (Darwin, 1862; Harder and Johnson, 2009; Willmer, 2011). As a consequence of adaptation to the same functional group of pollinators, plants exhibit a similar suite of covarying floral traits that heighten the attraction and pollen transfer of that specific group of pollinators, the so-called pollination syndrome (Faegri and van der Pijl, 1979; Fenster *et al.*, 2004; Willmer, 2011). Within this theoretical framework, the origin of floral diversity is mediated by the divergent evolution that occurs when a plant species pollinated by one group of pollinators shifts to a different group of pollinators, i.e. when there is a transition between pollination syndromes (Castellanos *et al.*, 2003; Thomson and Wilson, 2008; Harder and Johnson, 2009). In contrast to specialist plants, generalist plants are simultaneously visited by a diverse assemblage of floral visitors with different morphologies, foraging behaviour, preference patterns and per-visit effectiveness (Gómez and Zamora, 1999). As different types of floral visitors show distinct preferences for floral traits and contrasting morphological fitting with floral traits, thereby imposing conflicting selections, the floral traits of most generalist plants are either not adapted to any particular pollinator or reflect adaptation to multiple pollinator groups (Herrera, 1996; Wilson *et al.*, 2004; Sahli and Conner, 2011). For this reason, generalist plants do not belong to any pollinator syndrome, and most pollination biologists question the role of pollinators as evolutionary drivers in generalist systems (Waser, 2001; Kay and Sargent, 2009).

Pollination systems may be considered as ecological niches (Armbruster *et al.*, 1994; Johnson, 2010; Pauw, 2013). Pollination niches occur in both generalist and specialist plant species, the only difference between them being the breadth of the niches: narrow in specialist plants but wide in generalist plants. Whereas the boundaries between pollination niches in specialist plants are sharp, generalist plant species differ from each other in the relative frequency of different types of pollinators visiting their flowers (Grant and Grant, 1965; Armbruster, 1988, 1993; Dilley *et al.*, 2000; Thomson and Wilson, 2008). Differences among generalist plants in pollination niches are quantitative rather than qualitative. Although pollination niches have been studied mostly to investigate the structure of plant communities and the possibility of plant coexistence under interspecific competition (Parrish and Bazzaz, 1978,

1979; Armbruster *et al.*, 1994; Abe, 2006; Ollerton *et al.*, 2007; Geib and Galen, 2012), they can also be useful to understand floral divergence. In fact, because of the tight equivalence between pollination niche (the array of animals visiting the flowers) and syndrome (the suite of floral traits displayed by the plant to attract those animals) in specialist plants, pollinator-driven floral diversification occurs in these plants when there is a transition between pollination niches. A similar process might also occur in generalist plants, where floral divergence could be driven by shifts between different generalist pollination niches. This possibility for pollinator-mediated divergence in floral morphology, however, has scarcely been explored in generalist clades (Dilley *et al.*, 2000; Sargent and Otto, 2006; Medel *et al.*, 2007; Cooley *et al.*, 2008; Smith *et al.*, 2008).

Scaling down from species to populations, pollinator identity and abundance also vary between conspecific populations in most generalist plants (Aigner, 2005; Moeller, 2005; Price *et al.*, 2005; and references therein). This quantitative variation in pollinator fauna creates concomitant spatial variation in the selection undergone by plants (Galen and Cuba, 2001; Thomson and Wilson, 2008) and could ultimately cause phenotypic divergence, mostly in geographically isolated populations where gene flow is disrupted (Waser, 2001; Kay and Sargent, 2009). A potential consequence of this between-population variation in pollination niche is the evolution of pollination ecotypes, floral variants adapted to the local pollinator fauna (Grant and Grant, 1965; Armbruster, 1993; Johnson, 2006, 2010; Pérez-Barrales *et al.*, 2007; Andersson *et al.*, 2009; Armbruster and Muchhala, 2009). Pollination ecotypes may be interpreted as a path to the specialization in a sub-set of functionally similar pollinators (Fenster *et al.*, 2004; Johnson, 2010; van der Niet *et al.*, 2014). Whether generalist plants diverge in response to changes in pollination niches and develop different pollination ecotypes without becoming specialized is still an open question. Thomson and Wilson (2008; see also Dilley *et al.*, 2000) have envisaged a potential process, called adaptive wandering, to explain the pollinator-mediated phenotypic divergence of generalist flowers. They posit a geographical divergence in plant phenotype as a consequence of local adaptation to slightly different selection regimes, as imposed by local pollinator communities. Plants adapt to new local pollinators without excluding other pollinators. This would eventually result in new plant species adapting to slightly different but still diverse pollinator fauna, i.e. adaptive wandering (Thomson and Wilson, 2008). Since adaptive wandering does not involve a shift towards a new functional group of pollinators, floral traits should vary in generalist clades not in absolute terms but gradually and continuously (Armbruster, 1993; Armbruster and Muchhala, 2009).

The main aim of this study is to investigate the role played by pollinators in the phenotypic divergence of a generalist plant species, *Erysimum mediohispanicum* (Brassicaceae). The main hypotheses we will check in this study are as follows. (1) Although pollinators vary geographically, they are not distributed randomly across plant populations; consequently, there exists intraspecific, between-population variation in pollination niches. (2) These pollination niches are generalist, depending exclusively on the spatial variation in the relative abundance of some pollinators rather than in the specialization of some plant populations to sub-sets of pollinators. (3) There is geographic and phylogeographic structure in pollination niche variation,

with nearby and genetically similar populations belonging to the same pollination niches. (4) Populations belonging to the same pollination niche display similar floral traits and can be considered generalist pollination ecotypes. We have tested these hypotheses by means as follows. Using tools from the network theory, we first identify the pollination niches of this plant species, based on the relative abundance of different types of flower visitors. We then determine the spatial, between-population variation of these pollination niches throughout the entire geographic distribution of the plant. We subsequently infer how these pollination niches have evolved over the course of the plant's evolutionary history. Finally, we quantify the phylogeographic association between the pollination niches and the floral phenotype of the plants using within-species comparative analyses. Collectively, this information will provide a framework for future investigations on pollinator-mediated phenotypic evolution and divergence of generalist plants.

## MATERIALS AND METHODS

### Plant natural history

*Erysimum mediohispanicum* (Brassicaceae) is a mostly biennial, monocarpic herb endemic to the Iberian Peninsula, where it occupies two separate regions, one in the north and the other in the south-east of Spain (Nieto-Feliner, 1993). Individual plants grow for 2–3 years as vegetative rosettes, and then die after producing 1–8 reproductive stalks bearing up to several hundred hermaphroditic, bright-yellow flowers containing 30–40 ovules (Gómez *et al.*, 2009*a*). *Erysimum mediohispanicum* is partially self-compatible but requires pollen vectors for full seed set. The pollination system of this species is extremely generalist (Gómez *et al.*, 2007), its flowers being visited by several hundred insect species over its entire distribution area (Gómez *et al.*, 2007, 2009*a*). Despite this generalization, pollinators act as effective selective pressures on several floral traits (Gómez *et al.*, 2008*a*, 2009*a*). Previous studies have demonstrated that spatial variation in pollinators causes concomitant geographical variation in the selection acting on *E. mediohispanicum* floral traits (Gómez *et al.*, 2008*a*, 2009*a*), which can result in patterns of local adaptation to pollinator fauna (Gómez *et al.*, 2009*b*).

### Sampling design

During the years 2001–2010, we studied 48 geo-referenced populations spanning the entire distribution of *E. mediohispanicum*. To do so 14 populations were sampled in two areas from the northern region, one in the Iberian Plateau including localities in Guadalajara, Soria and Zaragoza provinces, and the other in the Pre-Pyrenees, including localities in the Sierra del Montsec (Lleida province) and Sierra de Guara (Huesca; Supplementary Data Table S1). We also sampled 34 populations in two areas from the southern region: the Prebaetic System, including localities in Sierras de Cazorla, Segura y Las Villas (Jaén), Sierra de la Guillimona (Granada) and Sierra de Alcaraz (Albacete); and the Penibaetic System, including localities in Sierra Nevada (Granada and Almería), Sierra de Cogollos (Granada), Sierra de Lújar (Granada), Sierra de Gádor (Almería) and Sierra de Espuña (Murcia) (Fig. 1; Supplementary Data Table S1). The genetic relationship of these populations was inferred from the
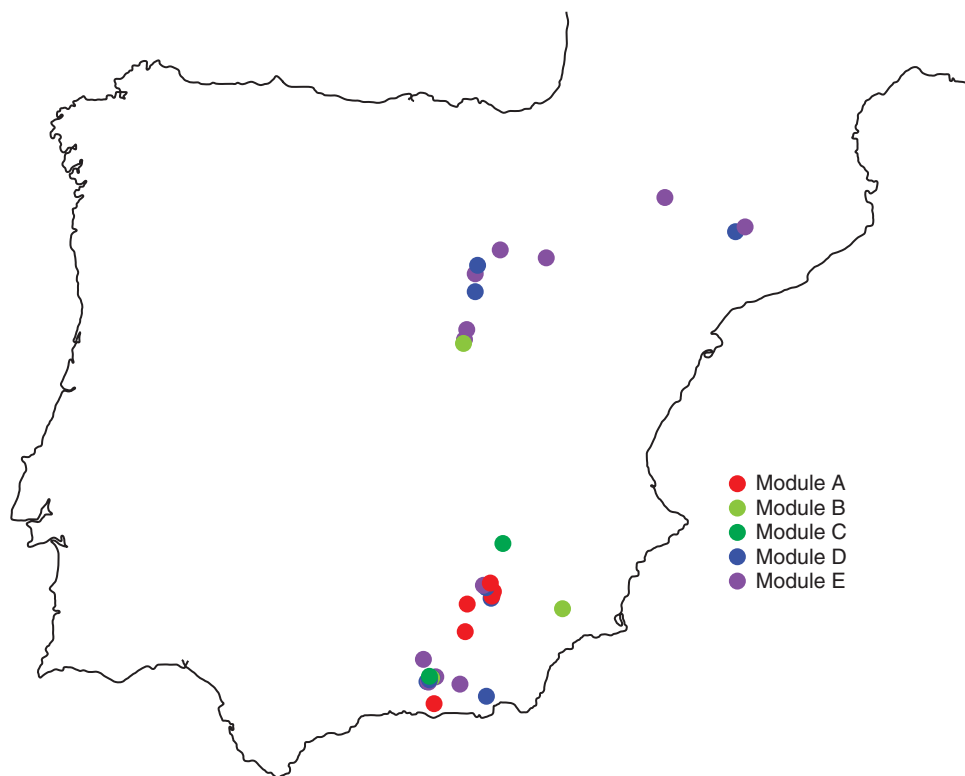
Fig. 1. Geographic distribution of the 48 populations of *Erysimum mediohispanicum* studied in the Iberian Peninsula. Each colour represents a different module (see key).

trnL-trnF intergenic spacer (IGS) chloroplast DNA (cpDNA) region using a new method developed by Muñoz-Pajares (2013) that combines the information from indels and substitutions (see Supplementary Data Methods for a full description of the methodology).

*Plant phenotypic traits*

The phenotype of at least 30 individual plants per population and year (4316 plants in total; Supplementary Data Table S1) was characterized, quantifying the following phenotypic traits: (1) stalk height, i.e. the height in centimetres of the tallest flowering stalk; (2) flower number, i.e. the total number of flowers and floral buds produced by each plant; (3) corolla diameter, i.e. the distance in millimetres between the apical edges of two opposite petals; (4) corolla tube length, i.e. the distance in millimetres between the corolla tube aperture and the base of the sepals; and (5) corolla shape, i.e. a multidimensional trait estimated by geometric morphometric tools using a landmark-based methodology (Zelditch *et al.*, 2004). For each individual, we selected one flower at anthesis and took a digital photo of the front view and planar position. We defined 32 co-planar landmarks covering the corolla shape and using midrib, primary and secondary veins, and petal edges and connections (see Gómez and Perfectti, 2010 for a full description of the methodology applied to *E. mediohispanicum*). Using the two-dimensional co-ordinates of landmarks, the generalized orthogonal least-squares Procrustes average was computed using the Generalized Procrustes Analysis (GPA) superposition method. To visualize

shape differences, we computed the relative warps (RWs), which are the principal components of the covariance matrix of the partial warp scores and uniform components (Adams *et al.*, 2004). Although our set of 32 landmarks generated 60 RWs, we used the first four RWs in all subsequent analyses because they were the only ones that separately explained >5 % of the total variation in shape (Gómez and Perfectti, 2010). RW1 is associated with changes in petal parallelism, RW2 with dorsoventral asymmetry, RW3 with left and right petal asymmetry and RW4 with changes in corolla roundness (Supplementary Data Fig. S1).

*Pollinator surveys*

Pollinator counts were conducted for at least 2 years in most populations (Supplementary Data Table S1). We visited each population during bloom peak and recorded the insects visiting the flowers, recording only those insects that made contact with the anthers or stigma at least during part of their visit to the flowers (i.e. they could act as pollinators). Previous studies in *E. mediohispanicum* (Gómez *et al.*, 2009a) indicate that 130–150 insects per population provided an accurate estimate of the local pollinator assemblage. For this reason, each population was visited several times to ensure this sampling effort (Supplementary Data Table S1). Unfortunately, it was impossible to reach this amount of floral visitors in a few populations where insects were especially scarce. However, we kept these populations in our study because it was observed that their removal did not change our main outcomes. Pollinators were

identified in the field, and some specimens were captured for further identification in the laboratory. Some rare pollinators could not be captured and we could thus identify them only to genus or family (see Gómez *et al.*, 2007, for details).

We grouped the insects visiting *E. mediohispanicum* flowers into functional groups (Fenster *et al.*, 2004). A 'functional group' is defined here as those insects that interact with the flowers in a similar manner. Basically, we used criteria of similarity in body length, proboscis length, foraging behaviour and feeding habits. Thus, taxonomically related species were sometimes placed in different functional groups. Sixteen functional groups were established. (1) Long-tongued large bees: mostly nectar-collecting females ≥10 mm in body length belonging to the families Anthophoridae (mostly *Anthophora* spp.) and Apidae (*Apis mellifera* and several *Bombus* spp.). (2) Short-tongued large bees: mostly pollen- and nectar-collecting females >10 mm belonging primarily to the families Halictidae (*Lasioglossum* spp., *Halictus* spp.), Megachilidae (*Osmia* spp.), Colletidae (*Colletes* spp.) and Andrenidae (*Andrena* spp.). (3) Short-tongued medium-sized bees: mostly pollen- and nectar-collecting females between 5 and 10 mm also belonging to the families Halictidae (*Lasioglossum* spp., *Halictus* spp.) and Andrenidae (*Andrena* spp.). (4) Short-tongued small bees: mostly pollen- and nectar-collecting females <5 mm. Although they were pollinators, they could act as nectar thieves and belonged primarily to the families Halictidae (*Lasioglossum* spp.), Colletidae (*Hyleaus* spp.), Andrenidae (*Andrena* spp.), Apidae Xylocopinae (*Ceratina* spp.) and Apidae Nomidinae (*Nomada* spp.). (5) Ants: both orthodox pollinators and nectar thieves belonging mostly to the genera *Formica*, *Camponotus*, *Proformica*, *Plagiolepis* and *Leptothorax*. (6) Large wasps: large aculeate wasps, parasitic wasps and kleptoparasitic bees collecting only nectar (mostly *Polistes* spp.). (7) Small wasps: small parasitic wasps belonging to Chalcidoidea and Ichneumonoidea, collecting only nectar, and acting both as pollinators and nectar thieves. (8) Beeflies: long-tongued nectar-collecting flies belonging to the families Bombyliidae (mostly *Bombylius* spp.) and Nemestrinidae. (9) Hoverflies: nectar- and pollen-collecting Syrphidae and short-tongued Bombyliidae. (10) Large flies: nectar-collecting flies >5 mm, mostly belonging to the families Muscidae, Calliphoridae, Tabanidae, Scatophagidae and Anthomyiidae. (11) Small flies: nectar-collecting flies <5 mm mostly belonging to families Muscidae, Anthomyiidae, Micetophyliidae, Empididae, Bibionidae, Drosophilidae and Stratiomyidae (these flies, although pollinating the flowers, may also act also as nectar thieves). (12) Beetles: including species collecting nectar and/or pollen mostly belonging to the families Melyridae (Malachidae and Dasytidae), Cleridae, Oedemeridae, Nitidulidae, Elateridae, Bruchidae, Buprestidae, Phalacridae and Chrysomelidae. (13) Butterflies: mostly Rhopalocera belonging to the families Nymphalidae and Pieridae plus some diurnal moths belonging to the family Sphingidae, all nectar collectors. (14) Moths: small nectar-collecting Lepidoptera mostly belonging to the families Adelidae and Incurvariidae. (15) Bugs: nectar-collecting Hemiptera belonging mostly to the family Lygaeidae and Pentatomidae (outstanding *Eyridema* spp.) (these insects also act as sap suckers). (16) Others: some species of grasshoppers, snakeflies, thrips and earwigs that visit the flowers to collect both pollen and nectar.

*Determination of pollination niches*

The occurrence of different pollination niches in our study populations was determined using bipartite modularity, a complex network metric. An ecological network is modular when it can be sub-divided into groups (= modules) sharing most of their interactions with members in the same group and a few interactions with members from other groups (Guimerà *et al.*, 2007). Network modularity is a measure of the extent to which species have more links within their modules than would be expected if the linkage were random.

A weighted bipartite network was constructed that included all the populations and the frequency of visits of each pollinator functional group per population. In this network, we pooled the data from the whole study period (2001–2010) and did not consider the time difference involved in sampling across different populations. We subsequently determined the modularity level in this weighted bipartite network using the QuaBiMod algorithm (Dormann and Strauss, 2013). This method used a recurrent Markov chain Monte Carlo (MCMC) algorithm to find the best division of populations into modules. A total of $10^6$ MCMC steps were used with a tolerance level of $10^{-10}$. As random networks may also have strong modularity, we explored whether our network was significantly more modular than random networks by running the same algorithm in 100 random networks, with the same linkage density as the empirical one (Guimerà and Amaral, 2005). Modularity significance was tested by comparing the empirical vs. the random modularity indices (Guimerà and Amaral, 2005). After testing the modularity of our network, we determined the number of modules using the approach proposed by Newman (2004). We subsequently identified the floral visitor functional groups, defining each module and the populations that were ascribed to each module. To check for uncertainty in module identity due to method accuracy, we repeated the analysis 50 times and recorded the distribution of populations across modules each time. Thus, for each population, we weighed the probability of belonging to each module. Furthermore, we tested for temporal consistency in module identity by performing a separate modularity analysis for every year in which we had sampled >10 populations (2007, 2008, 2009 and 2010). All modularity analyses were performed using R package bipartite 1·18 (Dormann and Gruber, 2012).

*Spatial structure of pollination niches*

We tested whether the pollinator fauna of *E. mediohispanicum* was spatially structured by testing variation between regions (south vs. north) and areas (Prebaetic System, Penibaetic System, Iberian Plateau and Pre-Pyrenees) in module identity by means of a nested generalized linear mixed model (glmm). The dependent variable (module identity) was fitted to a multinomial, and area was considered a random factor nested onto region. This analysis was performed using the R package lme4 0·99-0 (Bates, 2005).

*Comparative analysis across* Erysimum mediohispanicum *populations*

In this study, we test whether pollination niches depend on the genetic relatedness of the populations (phylogenetic signal),

inferring the characteristics of the most likely pollination niche in *E. mediohispanicum* ancestral populations, and exploring the correlated evolution between pollination niches and floral traits (pollination ecotypes). Population traits are not statistically independent because they include a component inherited from ancestral populations plus a component owing to gene flow with other populations (Stone *et al.*, 2011). Across-population analyses need to cope with these sources of non-independence. However, as opposed to cross-species comparative analyses, the within-species comparative analysis is not yet fully developed (Stone *et al.*, 2011; Mardulyn, 2012). We tried to solve this problem by using the between-population genetic distance matrix established with the trnL-trnF IGS cpDNA region to control for across-population non-independence when possible.

*Phylogenetic signal.* Phylogenetic signal in pollinators was tested by means of the Mantel test (Hardy and Pavoine, 2012; Pavoine and Ricotta, 2013). We correlated the genetic distance matrix with a matrix containing the between-population pollinator distances determined using the Bray–Curtis dissimilarity index (Pavoine and Ricotta, 2013). In addition, we included the geographic distance matrix to control for spatial location of the populations.

*Ancestral reconstruction of pollination niches.* Unfortunately, methods to estimate ancestral states are not available for network topologies and this requires the use of phylogenetic trees (Nunn, 2011). For this reason, the matrix of between-population relatedness was converted into a phylogenetic tree (see Honnay *et al.*, 2009 for a similar approach with *Erysimum cheiranthoides*). Nevertheless, we exercised caution when interpreting the outcomes from this analysis, since we were assuming no between-population gene flow.

The genetic relationships between *E. mediohispanicum* populations were represented by means of the Neighbor–Joining (NJ) algorithm. Ancestral states were estimated using an updated version of the 'ancThresh' command implemented in the R package phytool 0·2-14 (Revell, 2012). This function uses Bayesian MCMC to estimate ancestral states and the threshold model to establish the thresholds for a discrete character to change between states (Felsenstein, 2012). Under the threshold model, the evolving discrete trait is considered to have a continuous, underlying liability (Felsenstein, 2012). When the liability exceeds a threshold value, the discretely valued state of the observable character trait changes. Moreover, this method makes it possible to find the ancestral states, either by considering the state of the tips as a fixed value or by considering uncertainty in character state by assigning to each tip a prior probability of belonging to any of the states conforming the character (Revell, 2012). We followed both of these approaches. Thus, we first reconstructed the ancestral module identity by considering the module identity of the *E. mediohispanicum* populations as a fixed value. We then reconstructed the ancestral state in module identity by considering the uncertainty in ascribing the populations to different modules. To do this, we reran the ancestral reconstruction analysis but included the possibility that some populations belonged to different modules (see above). Finally, we also reconstructed ancestral state in module identity, taking into account the uncertainty in ascribing the populations to different modules as a consequence of temporal changes in pollinator fauna. Again, we reran the ancestral reconstruction analysis but included the possibility of some populations of changing

module identity between years (see above). In all cases, we ran four chains of $10^6$ generations each. The posterior probabilities for each character state at each internal node were determined with the 'ace' command in R packages ape 3·0-6 (Paradis *et al.*, 2004) and phytool 0·2-14 (Revell, 2012).

*Correlated evolution of pollination niches and phenotypic traits.* The correlated evolution of pollination niche and plant phenotype was explored using MCMCglmm (Hadfield and Nakagawa, 2010; Stone *et al.*, 2011). MCMCglmm can incorporate the between-population relatedness due to migration and population history as a correlated random effect (Hadfield, 2013). In the models, we included as a random variance structure both the genetic distance matrix and the geographic distance matrix across populations. The dependent variable, module identity, was considered a non-ordered multinomial variable. We used $10^5$ MCMC steps with a burn-in of 50 000 and a thinning interval of 10. All the analyses were performed using the R package MCMCglmm 2·17 (Hadfield, 2013).

## RESULTS

### Determining pollination niches

A total of 6442 flower visits from 545 insect morphospecies belonging to 99 families and seven orders were recorded. The average number of insect species ($\pm$ s.e.) recorded per population was $28·6 \pm 1·46$. All the populations studied were generalists. The most frequent floral visitors belonged to the orders Coleoptera (40 % of the visits), Hymenoptera (28 %), Diptera (17 %) and Lepidoptera (9 %). The rest of the insect orders accounted for $< 5$ % of the visits. However, the most frequent functional groups were beetles, long-tongued large bees, short-tongued large bees, short-tongued medium-sized bees, short-tongued small bees, beeflies and butterflies (Supplementary Data Table S2).

The network between *E. mediohispanicum* populations and the pollinator functional groups was significantly modular [empirical modularity $\pm$ s.e. = $0·311 \pm 0·001$; random modularity $\pm$ s.e. (95 % confidence interval) = $0·0301 \pm 0·0003$ ($0·0300–0·0302$); $n = 100$ replicates]. This analysis detected five modules, depending on the frequency of the different groups of floral visitors per population (Fig. 2): module A, defined by medium-sized bees and, to a lesser extent, small flies and moths; module B, defined by long-tongued large bees and, to a lesser extent, large wasps, hoverflies and large flies; module C, defined by butterflies and, to a lesser extent, small wasps; module D, defined mostly by beeflies, short-tongued small and large bees, and bugs; and module E, defined by beetles and ants. The average number of functional groups found per population was $8·7 \pm 0·5$. Overall, the number of populations belonging to each modules was heterogeneous. Whereas modules A, B and C were composed of five, seven and three populations, respectively, module D was composed of 13 populations, and module E was composed of 20 populations (Table 1, Fig. 2).

The module identity of the populations was very consistent. Thus, only four populations (Em10, Em27, Em42 and Em52) changed considerably between modules when the analysis was repeated 50 times, whereas the remaining 44 populations were consistently ascribed to a single module (Table 1). Furthermore, temporal consistency in module identity was also
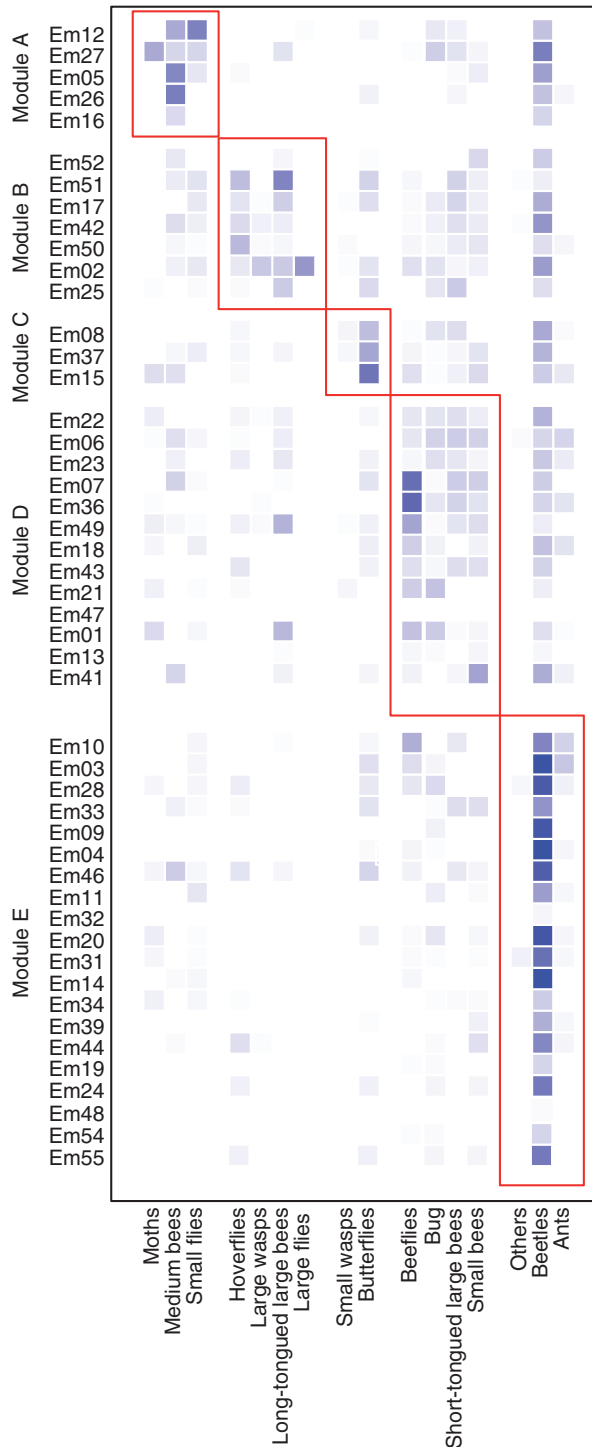
FIG. 2. Plot showing the average classification (*n* = 50 replicates) of the study populations into different pollinator modules, according to the analysis of bipartite modularity. The intensity of the colours indicates the relative abundance of each flower visitor's functional group per population.

TABLE 1. *Frequency of each Erysimum mediohispanicum population belonging to each module when the modularity analysis is repeated 50 times*

| Population | Frequency of belonging to each module | | | | | Module ascription |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | |
| Em01 | 0 | 0·12 | 0 | 0·88 | 0 | D |
| Em02 | 0 | 0·96 | 0 | 0·04 | 0 | B |
| Em03 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em04 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em05 | 1·00 | 0 | 0 | 0 | 0 | A |
| Em06 | 0 | 0·14 | 0 | 0·86 | 0 | D |
| Em07 | 0 | 0·02 | 0 | 0·98 | 0 | D |
| Em08 | 0 | 0 | 1·00 | 0 | 0 | C |
| Em09 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em10 | 0 | 0 | 0 | 0·36 | 0·64 | E |
| Em11 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em12 | 1·00 | 0 | 0 | 0 | 0 | A |
| Em13 | 0 | 0·02 | 0 | 0·98 | 0 | D |
| Em14 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em15 | 0 | 0 | 1·00 | 0 | 0 | C |
| Em16 | 1·00 | 0 | 0 | 0 | 0 | A |
| Em17 | 0 | 1·00 | 0 | 0 | 0 | B |
| Em18 | 0 | 0·02 | 0 | 0·98 | 0 | D |
| Em19 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em20 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em21 | 0 | 0·12 | 0 | 0·88 | 0 | D |
| Em22 | 0 | 0·18 | 0 | 0·82 | 0 | D |
| Em23 | 0 | 0·18 | 0 | 0·82 | 0 | D |
| Em24 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em25 | 0 | 1·00 | 0 | 0 | 0 | B |
| Em26 | 1·00 | 0 | 0 | 0 | 0 | A |
| Em27 | 0·60 | 0·26 | 0 | 0·14 | 0 | A |
| Em28 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em31 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em32 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em33 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em34 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em36 | 0 | 0·02 | 0 | 0·98 | 0 | D |
| Em37 | 0 | 0 | 1·00 | 0 | 0 | C |
| Em39 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em41 | 0 | 0·04 | 0 | 0·96 | 0 | D |
| Em42 | 0·38 | 0·62 | 0 | 0 | 0 | B |
| Em43 | 0 | 0·08 | 0 | 0·92 | 0 | D |
| Em44 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em46 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em47 | 0 | 0·02 | 0 | 0·98 | 0 | D |
| Em48 | 0 | 0 | 0 | 0 | 1·00 | E |
| Em49 | 0 | 0·08 | 0 | 0·92 | 0 | D |
| Em50 | 0 | 1·00 | 0 | 0 | 0 | B |
| Em51 | 0 | 1·00 | 0 | 0 | 0 | B |
| Em52 | 0·28 | 0·44 | 0 | 0·28 | 0 | B |
| Em54 | 0 | 0·02 | 0 | 0·16 | 0·82 | E |
| Em55 | 0 | 0 | 0 | 0 | 1·00 | E |

The module to which each population was ascribed is also shown.

high. Only five populations changed between modules, depending on the year. In fact, yearly modularity indices were as high as the overall index (2007, M = 0·321; 2008, M = 0·261; 2009, M = 0·345; 2010, M = 0·334).

*Spatial structure and phylogenetic signal of pollination niches*

Neither the region ($\chi^2 = 7·47$, $P = 0·113$) nor the area ($\chi^2 = 10·32$, $P = 0·243$; generalized linear model) affected module identity. Populations belonging to all modules except modules A and C were found in both geographic regions occupied by the species and in the four study areas (Fig. 1). There was no phylogenetic signal in pollinator functional groups ($r = -0·04$, $P = 0·98$; partial Mantel test). As shown in Fig. 3, there were populations belonging to most of the modules in all of the
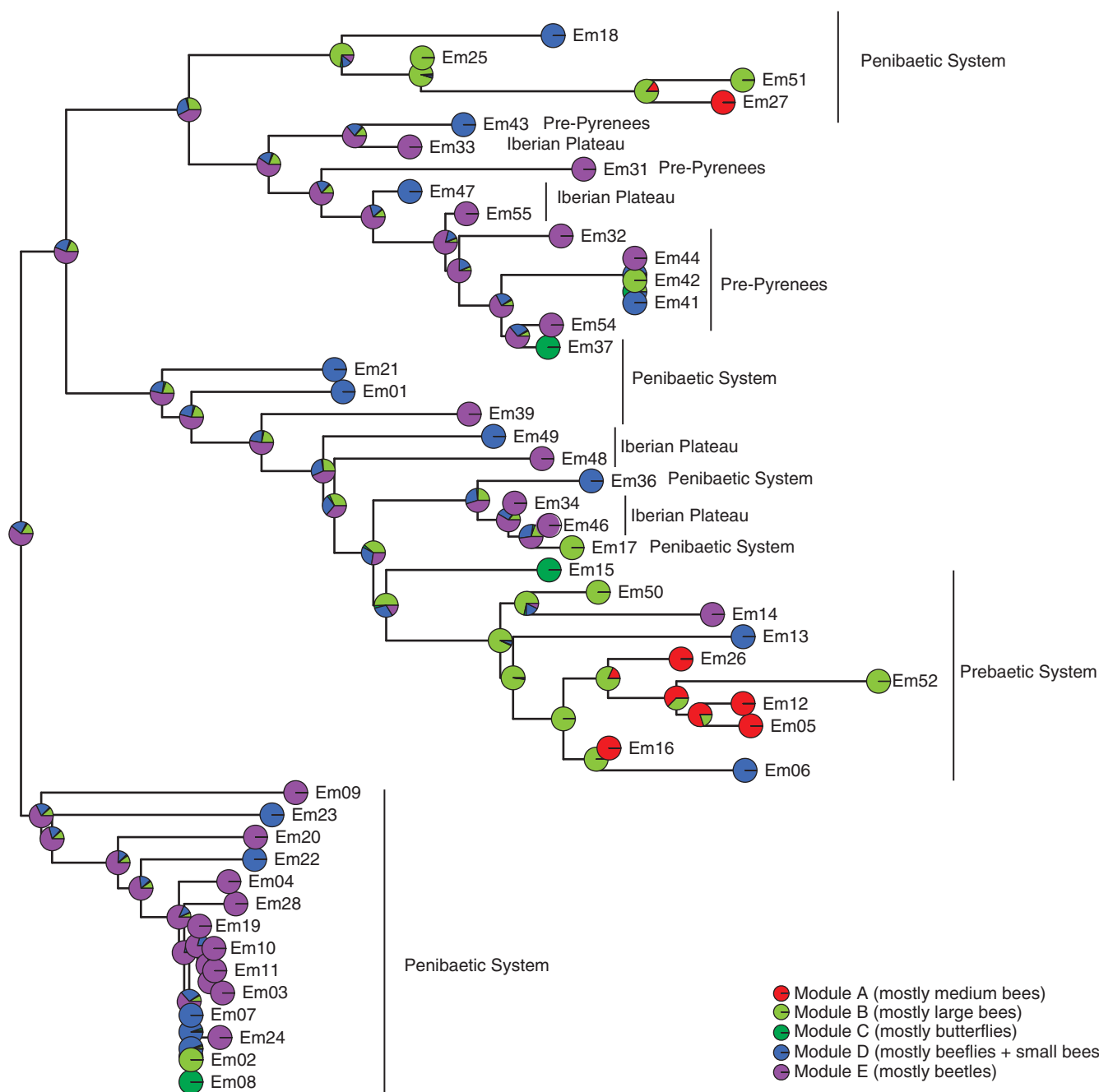
FIG. 3. Ancestral reconstruction of *Erysimum mediohispanicum* pollination niches according to the NJ tree describing the population genealogical relationship. The module identity of the terminal nodes (present-day populations) was considered as a fixed state (see Fig. 2). Different colours in the pie graphs of the internal nodes represent their distribution of posterior probability of belonging to each of the five modules.

three main *E. mediohispanicum* lineages (one including populations from the Penibaetic System, mostly Sierra Nevada, another including populations from the Penibaetic System and Pre-Pyrenees, and a third including a few populations from the Penibaetic System and all the populations from the Iberian Plateau and Prebaetic System; Supplementary Data Table S1), although populations from modules D and E appeared to be more frequent in the lineage from the Penibaetic System (Fig. 3). Populations from module A also appeared to be restricted mostly to the Prebaetic System (Fig. 3).

*Ancestral reconstruction of pollination niches and transition rates*

When the module identity of each *E. mediohispanicum* population was considered to be a fixed state, the reconstruction method suggested that the ancestral populations were visited mostly by beetles and ants (module E) or by beeflies and small bees (module D), since the posterior probabilities of the most ancestral node of belonging to these two modules ranged between 0·57 and 0·71 and between 0·17 and 0·35, respectively (Table 2). Module B (long-tongued large bees) was also reconstructed as the potential ancestral module in some chains (Table 2).

TABLE 2. *Probability of the ancestral node in the* E. mediohispanicum *NJ tree belonging to each of the modules when (A) considering the module ascription of the populations as a fixed state; (B) including the uncertainty in module identity originated by repeating the modularity analysis 50 times; and (C) including the uncertainty in module identity originated by performing the modularity analysis separately each year*

| Population | Probability of belonging to each module | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| (A) Module identity of populations as a fixed state | | | | | |
| Chain 1 | 0 | 0·11 | 0·01 | 0·17 | 0·71 |
| Chain 2 | 0 | 0·08 | 0·04 | 0·30 | 0·57 |
| Chain 3 | 0·01 | 0·04 | 0·02 | 0·25 | 0·68 |
| Chain 4 | 0 | 0·09 | 0·01 | 0·19 | 0·71 |
| (B) Uncertainty in the module identity of the populations | | | | | |
| Chain 1 | 0 | 0·09 | 0·04 | 0·18 | 0·69 |
| Chain 2 | 0 | 0·12 | 0·04 | 0·15 | 0·69 |
| Chain 3 | 0 | 0·08 | 0·04 | 0·15 | 0·73 |
| Chain 4 | 0 | 0·10 | 0·05 | 0·20 | 0·65 |
| (C) Temporal uncertainty in module identity of the populations | | | | | |
| Chain 1 | 0 | 0·13 | 0·02 | 0·18 | 0·67 |
| Chain 2 | 0 | 0·15 | 0·06 | 0·29 | 0·50 |
| Chain 3 | 0 | 0·15 | 0·06 | 0·31 | 0·48 |
| Chain 4 | 0 | 0·20 | 0·08 | 0·31 | 0·42 |

Populations visited mostly by butterflies (module C) and by medium-sized bees (module A) seem to have appeared later during the evolutionary history of *E. mediohispanicum*. This is especially true for module A. Moreover, it seems that on both occasions populations belonging to module A have appeared, presumably having evolved from populations belonging to module B (Fig. 3). Most other modules have appeared many times during the evolutionary history of *E. mediohispanicum* (Fig. 3; see Supplementary Data Table S3).

The ancestral reconstruction of modules was very similar when both computational and temporal uncertainty were included in the module identity of *E. mediohispanicum* populations (Table 2). The most ancestral *E. mediohispanicum* populations were again reconstructed as belonging to the modules visited by beetles/ants or by beeflies/small bees (Table 1), although the probability of belonging to module B was also considerable (Table 2; see Supplementary Data Tables S4 and S5). Again, modules A and C seemed to have appeared later in the evolutionary history of *E. mediohispanicum* (Supplementary Data Figs S2 and S3).

### Correlated evolution of pollination niche and phenotypic traits

The number of flowers, corolla diameter, and the RW1 and RW2 shape components were correlated with the identity of the module when controlling both for across-population genetic relationship and for spatial distance (Table 3). Populations from module B had plants with more flowers than the rest of the modules (Fig. 4). Moreover, populations from modules B, D and E had larger corollas than did plants from modules A and C (Fig. 4). Finally, populations from module A had plants with open petals (positive RW1 shape component), whereas populations from modules B and D had plants with zygomorphic flowers (positive RW2 shape component; Fig. 4).

TABLE 3. *Outcome of the MCMC generalized linear mixed models controlling for genetic relatedness and spatial location of the populations*

| Traits | Posterior mean | 95 % confidence interval | MCMC *P*-value |
|---|---|---|---|
| Flower number | 65·9957 | 42·49 to 87·81 | 0·0001 |
| Stalk height | −22·6974 | −51·52 to 10·14 | 0·166 |
| Corolla diameter | 17·3994 | 0·42 to 32·76 | 0·049 |
| Corolla tube length | 11·6836 | −7·92 to 29·41 | 0·258 |
| RW1 | −322·2784 | −593·25 to −97·01) | 0·0001 |
| RW2 | −743·8254 | −1087·48 to −361·84) | 0·0001 |
| RW3 | 328·452 | −237·03 to 1015·57 | 0·333 |
| RW4 | −136·9831 | −677·01 to 334·14 | 0·716 |

Deviance information criterion (DIC) = 114·30.

## DISCUSSION

### The existence of different pollination niches in a generalist plant

The flower visitors of *E. mediohispanicum* vary between populations in both diversity and identity (Gómez *et al.*, 2007, 2009*a*). Between-population variation in pollinator assemblages is common in most generalist plants (Aigner, 2005; Moeller, 2005; Price *et al.*, 2005; Burkle and Alarcon, 2011; Rader *et al.*, 2012; and references therein). However, our network analysis indicates that the spatial variation in *E. mediohispanicum* pollinators was not random but rather was organized across populations. The 48 *E. mediohispanicum* populations surveyed in this study, despite being generalists, can be assembled into five modules according to their quantitative similarity in the type of insects visiting their flowers. It is possible to envisage a multidimensional space determined by orthogonal axes defined by changes in the abundance of each flower visitor type (Pauw, 2013). Each module would occupy a specific region within this niche space. Seen from this perspective, and if it is taken into account that flower visitors are resources for plants (Pauw, 2013), each module can be considered a different Eltonian pollination niche of *E. mediohispanicum* (Soberon, 2007). Our study suggests the occurrence of intraspecific, between-population variation in pollination niches in *E. mediohispanicum*. As a consequence of belonging to the same species, the boundaries between niches become fuzzy, and the degree of overlap between these five pollination niches is high (Wiens and Graham, 2005). Indeed, most populations were visited by functional insect groups associated with more than one pollination niche (Fig. 2; see also Supplementary Data Table S2). Furthermore, most flower visitors – such as beeflies, small bees, bugs and, above all, beetles – appeared in almost all populations, with only some functional groups (such as moths, butterflies, hoverflies or large wasps) restricted to a few populations (Fig. 2). However, despite this background of between-population variation in pollinators, we found that the ascription of each population to a given pollination niche was very robust.

It is tempting to consider populations from different pollination niches as belonging to different pollination syndromes. In fact, network modularity can reveal pollination syndromes in some systems (Dick *et al.*, 2002; Danieli-Silva *et al.*, 2012). However, we are not advocating the idea of generalist pollination
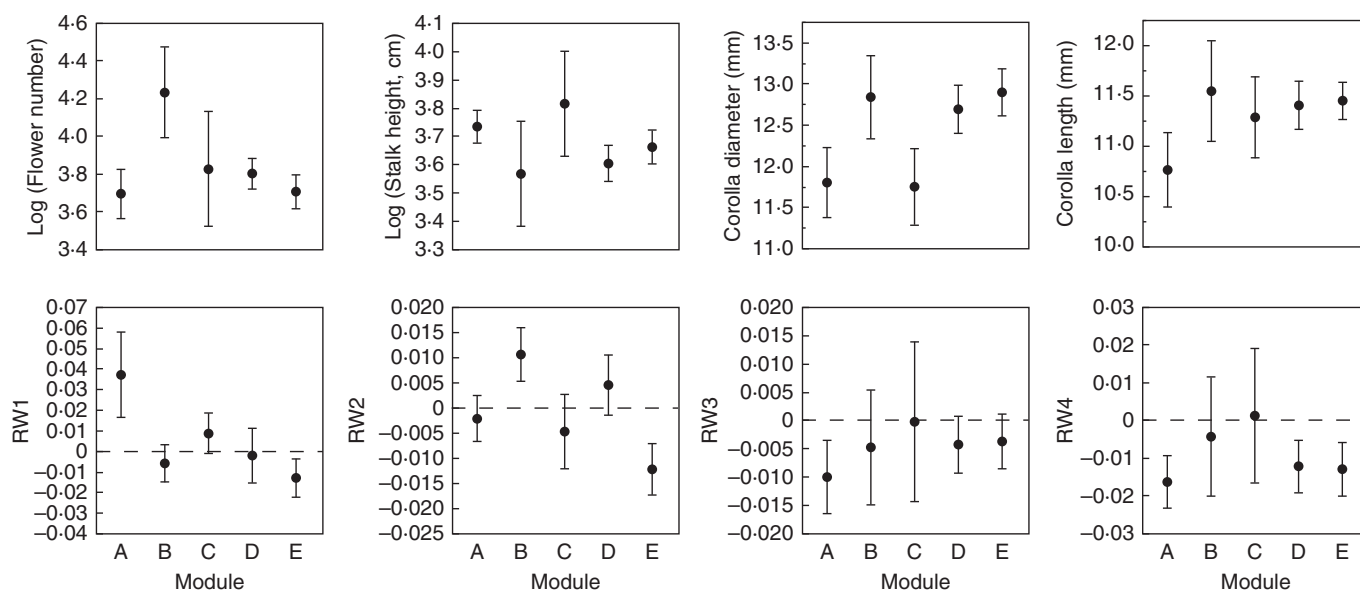
FIG. 4. Differences among pollination niches in the floral traits of *Erysimum mediohispanicum*. See Table 3 for statistical results using MCMC generalized linear mixed models. RW = relative warp (see Methods).

syndromes. Unlike true syndromes, the pollination niches detected in this study were not caused by the turnover of major functional groups of pollinators but by changes in the relative abundance of their floral visitors. Also, all the populations studied remained generalist. We did not detect any evolution towards increased specialization.

The floral visitors of *E. mediohispanicum* differed in their efficiency as pollinators. For example, although beetles are the main pollinators of *E. mediohispanicum* in some populations (Gómez, 2003) and ants can also act as pollinators of several coexisting crucifers (Gómez and Zamora, 1999), these insects are low-efficiency pollinators and behave more frequently as nectar thieves, pollen feeders and florivores than as legitimate pollinators (Leavitt and Robertson, 2006; Rico-Gray and Oliveira, 2007; Cardel and Koptur, 2010). The importance of these insects as pollinators strongly depends on how frequently they visit flowers rather than their per-visit effectiveness (Gómez and Zamora, 1999; Gómez et al., 2010). Other insects showing very low efficiency as pollinators of *Erysimum* spp. and other Brassicaceae are small and large flies, moths, bugs and small wasps (Gómez et al., 2009a, 2010; Lay et al., 2011; Fernández et al., 2012; Rader et al., 2012). Most of these insects frequently act as nectar thieves, entering the flowers from the bottom part of the corolla tube without making contact with the sexual organs (Gómez et al., 2009a). Short-tongued small, medium-sized and large bees, hoverflies and beeflies, unlike all the above-mentioned insects, can be efficient pollinators of *E. mediohispanicum* (Gómez et al., 2009a, 2010), other *Erysimum* species (Lay et al., 2011; Fernández et al., 2012) and many other crucifers (Rush et al., 1995; Cruden et al., 1996; Sahli and Conner, 2007; Rader et al., 2009, 2013; Robertson and Leavitt, 2011; Jauker et al., 2012). Although they sometimes act as pollen and nectar thieves, they act as legitimate pollinators most of the time. However, the most efficient pollinators of *Erysimum*, including *E. mediohispanicum*, seem to be long-tongued large bees. These insects move very fast,

visiting many flowers per time unit but few flowers within the same individual plant, and deliver many pollen grains per visit (Ollerton et al., 2007; Gómez et al., 2010; Lay et al., 2011). In fact, the *E. mediohispanicum* pollinators can be ranked in descending order according to their per-visit effectiveness (quantified as seed production), from long-tongued large bees, to short-tongued large bees, large wasps, medium-sized bees, beeflies, small bees, beetles, hoverflies, butterflies and flies (J. Valverde, Universidad de Granada, Spain, unpubl. data). Consequently, spatial variation in these types of insect causes geographical variations in the pollen limitation intensity and reproductive output, not only in *E. mediohispanicum* (Gomez et al., 2009a, 2010) but also in other congeneric species (*E. popovii*, Fernández et al., 2012; *E. capitatum*, Lay et al., 2011). Accordingly, belonging to a given pollination niche would have important consequences for the reproductive output of the plants.

### The evolutionary history of E. mediohispanicum *pollination niches*

Variation in pollinator niches was not spatially structured but instead occurred at multiple geographic scales: between nearby populations, between areas within a geographic region and between regions. This finding suggests that, rather than varying as a cline (Anderson et al., 2009; Santos-Gally et al., 2013), the pollinator assemblages of *E. mediohispanicum* form a mosaic (Grant and Grant, 1965). The presence of a geographic mosaic in pollination environments seems to be frequent in generalist plants (Grant and Grant, 1965; Dilley et al., 2000). Furthermore, there was no phylogenetic signal in the evolution of the pollination niches. In fact, most pollination niches were scattered through both the geographic range and evolutionary history of *E. mediohispanicum* populations. This was especially true of the three scarce pollination niches (A, B and C), since populations from any of the three lineages were assigned to these niches. This pattern was also found, however, in the most common pollination niches (D and E). A few populations from

the Prebaetic System belonged to these two modules, although most of the remaining populations in that area (and clade) were ascribed to modules A and B. Altogether, these outcomes suggest that the evolution of the *E. mediohispanicum* pollination niches is labile. Many evolutionary and ecological factors may cause low phylogenetic conservatism and high lability in niche evolution (Losos, 2008; Cooper *et al.*, 2010; Crisp and Cook, 2012), such as the occurrence of non-stabilizing selection – whether convergent or divergent – and local adaptation, low linkage of co-adapted traits, high genetic variation in niche-associated traits and a strong opportunity for the dispersal of species and populations (Crisp and Cook, 2012). More information is needed to disentangle these potential causes, although previous studies have indicated that divergent selection and local adaptation to pollinators is indeed occurring in some parts of the distribution area of *E. mediohispanicum* (Gómez *et al.*, 2009*a*, *b*).

It appears that the ancestral *E. mediohispanicum* populations belonged to pollination niches E and, to a lesser extent, D. Thus, beetles, ants, beeflies, and short-tongued large and small bees were the most likely flower visitors of ancestral populations. It bears noting that the Penibaetic System lineage, the most ancient *E. mediohispanicum* lineage according to a phylogeographic study (Muñoz-Pajares, 2013), is composed almost entirely of populations belonging to these ancestral niches. Many other populations from other lineages and geographic areas also belong to these ancestral niches. Furthermore, we have even found several changes to these niches over the course of the evolutionary history of *E. mediohispanicum*, despite their being associated with pollinators with low or intermediate efficiency. Our analysis also suggests that there have been numerous shifts to other pollination niches during the evolution of *E. mediohispanicum*. In particular, the pollination niches B and C seem to have appeared several times, whereas niche C, being presumably of low efficiency, appeared three times. Altogether, the frequency of populations belonging to high-efficiency niches seems not to have increased during the evolution of *E. mediohispanicum*.

### Pollinator-mediated floral divergence in Erysimum mediohispanicum

Phylogenetic changes in pollinators have been explored for many systems (e.g. *Disa*, Johnson *et al.*, 1998; *Schizanthus*, Peres *et al.*, 2006; *Aquilegia*, Whittall and Hodges, 2007; Bastida *et al.*, 2010; *Iochroma*, Smith *et al.*, 2008; *Ruellia*, Tripp and Manos, 2008). These studies have shown that shifts between pollination systems normally entail concomitant changes in floral phenotype. However, most of these studies have focused on the transitions between pollination syndromes found in various specialized plant species with divergent phenotypes. In contrast, it is widely assumed that pollinators play a minor role in the evolution and diversification of generalist plant clades because they do not provide strong reproductive isolation between diverging plant populations or pollination races in sympatry (Waser, 1998, 2001; Waser and Campbell, 2004; Kay and Sargent, 2009). Our study, however, suggests that a similar distinctive evolution may also occur during the evolutionary history of a single generalist plant species. In fact, we found a significant association between pollination niches and some *E. mediohispanicum* phenotypic traits. Plants from the

pollination niches D and E were very similar, having few and large flowers (Fig. 4), whereas plants from pollination niches A and C had smaller flowers, and plants from pollination niche B had many and large, high-rewarding flowers with highly zygomorphic (positive RW2) corollas. It is difficult to know whether these changes in floral size and shape are adaptations to local pollinators and can be considered pollination ecotypes, or on the contrary are due to other extrinsic factors. Biotic and abiotic agents, such as herbivores or altitude, also trigger phenotypic changes in *E. mediohispanicum* (Gómez, 2003). We presume, however, that pollinators may be at least partially responsible for some of the observed phenotypic differences. In fact, corolla size and the RW2 shape component are associated in this plant species not only with attractiveness but also with reward, i.e. pollen and nectar production (Gómez *et al.*, 2008*b*). Furthermore, in a sub-set of eight populations from Sierra Nevada (south-east Spain) studied during 2005 and 2006, we found a geographic mosaic of selection produced by the quantitative spatial variation in pollinators with opposing preferences for *E. mediohispanicum* phenotypes (Gómez *et al.*, 2008*a*, *b*, 2009*a*). Due to this geographic mosaic of selection, we detected divergent selection across some populations (Gómez *et al.*, 2009*a*). A reciprocal translocation experiment even showed that this spatial variation in pollinator fauna causes the adaptation of plants to local pollinators in some localities (Gómez *et al.*, 2009*b*). That is, there is experimental verification that changes in some *E. mediohispanicum* floral traits may be the consequence of local adaptation to contrasting pollinator faunas. For these reasons, we conjecture that geographic variation in pollination niches has contributed to the observed floral divergence in *E. mediohispanicum*.

It is important to establish how this floral variation has occurred. We presume that the process mediating this floral divergence has been similar to adaptive wandering (Thomson and Wilson, 2008). Assuming that our ancestral reconstruction analysis is correct, the *E. mediohispanicum* ancestral flowers (most probably belonging to pollination niche E) were large and symmetric. When *E. mediohispanicum* colonized new areas in the Iberian Peninsula, it found new, slightly different, although still generalist, pollination environments. Consequently, the plant faced contrasting pollinator-mediated selective scenarios. In some scarce localities where the pollinator fauna exerted strong and consistent selective pressures and where no other factors arrested this selection (selective hotspots; Gómez *et al.*, 2009*a*), plants locally adapted to these newly found pollination niches (Gómez *et al.* 2009*b*), and generalist pollination ecotypes emerged. However, because the boundaries between generalist pollination niches are not sharp, the pollination ecotypes would differ, not abruptly, but gradually in a subtle way. The evolution of plants with many zygomorphic flowers in populations from pollination niche B would be an example of this type of pollination ecotype in *E. mediohispanicum*. In a previous experiment, we found that *E. mediohispanicum* was locally adapted to large bees through changes in, among other traits, the number of flowers and corolla zygomorphy (Gómez *et al.*, 2009*b*). In addition, we presume that pollination ecotypes have not arisen universally across the plant's distribution area. In most localities, where pollination niches were sub-optimal, of low efficiency and unable to exert strong selection (selective coldspots; Gómez *et al.*, 2009*a*), plants have not adapted locally to their pollinator fauna. The outcome is a geographic mosaic with a few populations

locally adapted to their pollination environment, where pollinator-driven floral divergence is occurring, coexisting with many populations where this local adaptation has failed and where the plant phenotype is not primarily shaped by pollinators. It is vital to gather further intra- and interspecific evidence to ascertain whether the scenario found in this study is idiosyncratic of some species or is a general pattern for pollinator-mediated floral evolution in generalist plants.

## SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford-journals.org and consist of the following. Methods: determining the phylogenetic relationship between *E. mediohispanicum* populations. Figure S1: summary of the geometric morphometric analysis. Figure S2: ancestral reconstruction of pollination niches including computational uncertainty of belonging to each module according to 50 replicates. Figure S3: ancestral reconstruction of pollination niches including temporal uncertainty of belonging to each module according to 50 replicates. Table S1: details of study populations. Table S2: frequency of each flower visitor functional group in each *Erysimum mediohispanicum* population. Table S3: posterior probability of internal nodes calculated using the 'ace' command in the R package phytool for both MCMC chains. Table S4: posterior probability of internal nodes calculated using the 'ace' command in the R package phytool for both MCMC chains when including a prior probability of each character state (= module) for each tip (= *E. mediohispanicum* population) resulting from running 50 networks. Table S5: posterior probability of internal nodes calculated using the 'ace' command in the R package phytool for both MCMC chains when including a prior probability of each character state (= module) for each tip (= *E. mediohispanicum* population) resulting from running a different network each year (2007–2010).

## ACKNOWLEDGEMENTS

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