

The role of pollinators in floral diversification in a clade of generalist flowers

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Pollinator-mediated evolutionary divergence has seldom been explored in generalist clades because it is assumed that pollinators in those clades exert weak and conflicting selection. We investigate whether pollinators shape floral diversification in a pollination generalist plant genus, *Erysimum*. Species from this genus have flowers that appeal to broad assemblages of pollinators. Nevertheless, we recently reported that it is possible to sort plant species into pollination niches varying in the quantitative composition of pollinators. We test here whether floral characters of *Erysimum* have evolved as a consequence of shifts among pollination niches. For this, we quantified the evolutionary lability of the floral traits and their phylogenetic association with pollination niches. As with pollination niches, *Erysimum* floral traits show weak phylogenetic signal. Moreover, floral shape and color are phylogenetically associated with pollination niche. In particular, plants belonging to a pollination niche dominated by long-tongued large bees have lilac corollas with parallel petals. Further analyses suggest, however, that changes in color preceded changes in pollination niche. Pollinators seem to have driven the evolution of corolla shape, whereas the association between pollination niche and corolla color has probably arisen by lilac-flowered *Erysimum* moving toward certain pollination niches for other adaptive reasons.

KEY WORDS: Adaptation, coevolution, macroevolution, morphological evolution, plant–insect interaction, selection—natural.

Floral traits are often considered the adaptive outcome of specialized interactions with efficient pollinators (Darwin 1862; Harder and Johnson 2009; Willmer 2011). Pollinators, by exerting selection on traits, may play a role as agents of plant adaptation and of phenotypic diversification. When belonging to the same pollination niche, and thereby interacting with the same type of pollinators, plants will undergo identical selective pressures. This scenario would prompt convergent evolution, causing plants pollinated by the same functional group of pollinators to exhibit a similar suite of covarying floral traits, the so-called pollination syndrome (Faegri and van der Pijl 1979; Fenster et al. 2004). In principle, the effectiveness and abundance of pollinators defining a given syndrome would be maximal in plants belonging to that

pollination syndrome (Fenster et al. 2004; Rosas-Guerrero et al. 2014). That is the case for specialized systems in which there is a tight association between pollination niche and floral phenotype, plants from a pollination syndrome being adapted to their major pollinators (Fenster et al. 2004).

In this theoretical framework, pollinator-mediated floral diversification occurs when there are transitions between pollination niches, with plant species pollinated by a group of pollinators shifting to other groups of pollinators (Grant and Grant 1965; Gómez et al. 2014a). Because pollinator types differ in preference, behavior and morphological match with the flowers (Fenster et al. 2004), variation in pollination niche creates concomitant spatial variation in the pollinator-mediated selection undergone by

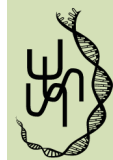




Figure 1. Corolla shape and color of the species studied.

Row 1: *E. baeticum*, *E. bastetanum*, *E. bicolor*, *E. bonannianum*, *E. cazorlense*, *E. cheiranthoides*, *E. cheiri*, *E. collisparsum*. Row 2: *E. crassistylum*, *E. crepidifolium*, *E. duriaei*, *E. etnense*, *E. fitzii*, *E. geisleri*, *E. gomezcampoi*, *E. gorbeanum*. Row 3: *E. incanum*, *E. jugicola*, *E. lagascae*, *E. linifolium*, *E. mediohispanicum*, *E. metlesicci*, *E. merxmulleri*, *E. myriophyllum*. Row 4: *E. nervosum*, *E. nevadense*, *E. odoratum*, *E. penyalarens*, *E. popovii*, *E. pseudorhaeticum*, *E. riphaeum*, *E. rondae*. Row 5: *E. ruscinnense*, *E. seipkae*, *E. scoparium*, *E. semperflorens*, *E. sylvestre*, *E. wilczekianum*, *E. virgatum*, *E. rhaeticum*.

plants (Thomson and Wilson 2008; Johnson 2010). Divergent selection may ultimately cause phenotypic divergence, mostly in geographically isolated populations in which gene flow is disrupted (Waser 2001; Kay and Sargent 2009). This process would promote the evolution of pollination ecotypes, floral variants adapted to their local pollinator fauna (Grant and Grant 1965; Armbruster 1993; Perez-Barrales et al. 2007; Armbruster and Muchhala 2009; Anderson et al. 2009; Johnson 2006, 2010). Several studies have shown that changes between pollination niches normally entail concomitant changes in floral phenotype (Johnson et al. 1998; Whittall and Hodges 2007; Smith et al. 2008; Tripp and Manos 2008; Sakai et al. 2013). Pollinator-driven speciation and floral divergence is complete when reproductive isolation between pollination ecotypes arises (Nosil 2012; Van der Niet et al. 2014). The outcome of this process is a shift between pollination syndromes (Wilson and Thomson 1996).

It is widely assumed that pollinators play just an ever-changing role in the adaptive evolution and diversification of generalist plant clades (Waser, 2001; Kay and Sargent, 2009). Pollinator-mediated floral diversification is thought to be more probable in plants with specialized pollination niches because their pollinators exert strong and consistent selection. In contrast, generalist plants are simultaneously visited by many different

floral visitors. Because different types of floral visitors show distinct preference patterns, per-visit effectiveness, foraging behavior, and contrasting morphological fitting with floral traits, they are thought to impose weak and conflicting selection (Gómez et al. 2009a; Sahli and Conner 2011). Consequently, the overall and diverse array of pollinators visiting generalist plants could not cause evolutionary divergence or provide strong reproductive isolation between diverging plant populations or pollination races (Waser and Campbell 2004; Kay and Sargent 2009). As a result of this theoretical framework, pollinator-mediated divergence in floral traits has seldom 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). However, the study of specialized pollination systems will only provide a partial picture of the role played by pollinators in the evolution and diversification of flowers. It is thereby fundamental to also investigate the adaptive nature of floral traits in generalist plants (Waser et al. 1996).

In this study we explore the occurrence of pollinator-mediated floral evolution and diversification in a pollination generalist plant genus, *Erysimum* L. We focus on a monophyletic clade distributed in Western Europe and North Africa (Moazzeni et al. 2014; Gómez et al. 2015). Although sharing the floral

bauplan typical of most members of Brassicaceae, their flowers vary in shape, color and size (Fig. 1). In addition, despite being pollinated by a diverse assemblage of insects, *Erysimum* species differ in the relative frequency of interactions with different pollinator functional groups (Gómez et al. 2008, 2015). These species can be sorted according to their similarity in pollinator faunas into several pollination niches (Gómez et al. 2015). It seems that the evolution of these pollination niches has occurred mostly by recurrent shifts between slightly different generalized pollinator assemblages varying spatially as a mosaic and without much change in specialization degree (Gómez et al. 2015). As a consequence of this evolutionary pattern, *Erysimum* pollination niches show weak phylogenetic signal and are evolutionary labile (Gómez et al. 2015). Despite being generalist, pollinators act as selective agents of many floral traits in several species of *Erysimum* (Abdelaziz 2013; Alarcón et al. 2008; Gómez et al. 2006, 2008, 2009b; Lay et al. 2011, 2013). The spatial variation in pollinators prompts the occurrence of a geographic mosaic in selection (Gómez et al. 2009a), pollinator-mediated local adaptation (Gómez et al. 2009b; Lay et al. 2013), and between-population divergent selection (Abdelaziz 2013, Gómez et al. 2009a). Consequently, correlated evolution between pollinators and some floral traits has been detected in some *Erysimum* species, causing the rise of intraspecific generalist pollination ecotypes (Gómez et al. 2014b). Taking into account this preliminary information, we postulate that pollinator-mediated floral diversification and evolution may be invoked in *Erysimum* when the following three conditions are fulfilled: (1) The evolution of floral traits is evolutionary labile, (2) there is correlated evolution between floral traits and pollination niches, and (3) this evolutionary association is caused by shifts between some pollination niches. We test these predictions using a phylogenetic comparative approach. Our main goal is to determine whether pollinators may also mediate floral evolution in generalist plant clades.

Materials and Methods

STUDY SPECIES

The 40 *Erysimum* species studied here (Table S1) represent over 90% of the species inhabiting Western Europe and Northwest Africa (Tutin et al. 1964). They inhabit different environments, from pure alpine habitats above treeline in the Alps, Sierra Nevada, Pyrenees or Atlas mountains, to oak and pine mountain forests in Mediterranean areas, and lowland and coastal habitats in Central Europe and North Africa. The phylogenetic relationship between the studied species of *Erysimum* is already known (Fig. S1; Gómez et al. 2014b).

The studied species are grouped into six different pollination niches (niches A to F in Table 1 and Fig. S2; see Gómez et al. 2015 for details about how these niches were obtained using a

modularity algorithm). Each niche is associated with some pollinator functional groups (Table 1), although all *Erysimum* species are visited by many functional groups (the flowers of all *Erysimum* species were visited by more than 10 functional groups; Table 1). Consequently, pollinator diversity is very high in all pollination niches (Gómez et al. 2015; Table 1).

We studied two populations per species except when they were very narrowly distributed or highly endangered (Table S1). We avoided sampling populations from marginal habitats or in the border of the species distribution range. In all cases, we studied at least 30 individual plants per species (2113 plants in total; Table S1).

PLANT PHENOTYPIC TRAITS

We quantified the following phenotypic traits of each studied individual: (1) *Flowering stalk height*, the height in cm of the tallest flowering stalk. (2) *Flower number*, the total number of flowers and floral buds produced by each plant. (3) *Corolla diameter*, the distance in mm between the apical edges of two opposite petals. (4) *Corolla tube length*, the distance in mm between the corolla tube aperture and the base of the sepals. (5) *Corolla tube width*, the inner diameter of the corolla tube at the aperture. (6) *Corolla color*, considered as a discrete trait based on human vision, with three states (yellow, lilac, white). Flowers of *Erysimum*, irrespective of their color, do not reflect UV light (Fig. S3; Abdelaziz et al. 2011). (7) *Corolla shape*, characterized using landmark-based geometric morphometrics (Zelditch et al. 2012). For one flower per individual, we digitized 32 landmarks along the midrib, primary, and secondary veins and petal edges and connections (Gómez and Perfectti 2010). Landmark data were subjected to a full Procrustes fit and projection into tangent space using the MorphoJ software (Klingenberg 2011). After sampling, we obtained the species mean value of each continuous trait. Because corolla color did not vary between individuals of the same species, we categorized each species according to its corolla color. In this study we have not decomposed shape variation in its symmetric and asymmetric components. However, to ensure that our analyses were robust, we repeated all comparative analysis with the symmetric component of the corolla shapes (data not shown but available upon request).

TESTING THE EVOLUTIONARY LABILITY OF FLORAL TRAITS

We tested the evolutionary lability of floral traits by calculating their phylogenetic signal and examining how floral traits changed along the phylogeny. We used the phylogeny (Fig. S1) reported by Gómez et al. (2014b), built with two nuclear (*ITS1* and *ITS2*) and two plastidial (*trnT-L* and *ndhF*) markers. To incorporate phylogenetic uncertainty, all comparative analyses were performed using not just the consensus phylogenetic tree (Fig. S1), but also a

Table 1. Pollination niches identified in the studied species.

Pollination niches	<i>Erysimum</i> species	Main pollinator functional groups ¹	Number of pollinator functional groups ²	Pollinator diversity ³
A	<i>E. merxmuelieri</i> , <i>E. ruscinnonense</i> , <i>E. semperflorens</i> , <i>E. penyalarens</i> , <i>E. nevadense</i> , <i>E. gorbeanum</i> , <i>E. crepidifolium</i> , <i>E. bonannianum</i>	Beetles, grasshoppers, bugs, moths	15 ± 1	0.88 ± 0.03 ^a
B	<i>E. myriophyllum</i> , <i>E. crassistylum</i>	Ants	10 ± 2	0.69 ± 0.09 ^{a,b}
C	<i>E. baeticum</i> , <i>E. riphaeum</i> , <i>E. odoratum</i> , <i>E. nervosum</i> , <i>E. cheiri</i> , <i>E. wilczekianum</i>	Large wasps, butterflies	15 ± 4	0.93 ± 0.01 ^a
D	<i>E. rhaeticum</i> , <i>E. fitzii</i> , <i>E. mediohispanicum</i> , <i>E. gomezcampoi</i> , <i>E. pseudorhaeticum</i> , <i>E. collisparsum</i>	Medium-sized bees, small bees, beeflies, Extra-small bees	13 ± 4	0.87 ± 0.03 ^a
E	<i>E. lagascae</i> , <i>E. linifolium</i> , <i>E. scoparium</i> , <i>E. metlesicsii</i> , <i>E. bastetanum</i> , <i>E. rondae</i> , <i>E. cazorlense</i> , <i>E. bicolor</i> , <i>E. popovii</i>	Long-tongued large bees, short-tongued large bees	12 ± 4	0.73 ± 0.06 ^b
F	<i>E. jugicola</i> , <i>E. cheiranthoides</i> , <i>E. duriaei</i> , <i>E. gesleri</i> , <i>E. etnense</i>	Small flies, thrips, small wasps, large flies, hoverflies, others	13 ± 3	0.90 ± 0.03 ^a

¹Pollinator functional groups associated with each pollination niche according to the modularity analysis used to detect niches (Gómez et al. 2015).

²Mean number (± 1 SE) of pollinator functional groups visiting the flowers of *Erysimum* species belonging to each pollination niche.

³Different superscript letters in the last column indicate significant differences in pollinator diversity (quantified as Hurlbert's PIE) among pollination niches (see Gómez et al. 2015 for details).

set of 6400 phylogenetic trees sampled from the stationary phase of the MCMC Bayesian analysis (Gómez et al. 2014b).

The phylogenetic signal of standard floral traits was tested using Pagel's λ and Blomberg's K (Pagel 1999; Freckleton et al. 2002; Blomberg et al. 2003). Pagel's λ is a tree transformation that assesses the degree of phylogenetic signal within the trait by multiplying the internal branches of the tree by values ranging between 0 and 1. A $\lambda = 1$ indicates a Brownian motion model and the tree is returned with its branch lengths untransformed. A

$\lambda = 0$ indicates no patterning as the tree is collapsed to a single large polytomy. To test phylogenetic signal, we compared a model generating a maximum likelihood estimate of Pagel's λ for floral traits with a model constraining λ to 0. A significant departure from the model with $\lambda = 0$ would indicate phylogenetic correlation (Freckleton et al. 2002). Blomberg's K expresses the strength of phylogenetic signal as the ratio of the mean squared error of the tip data measured from the phylogenetic corrected mean and the mean squared error based on the variance–covariance matrix

derived from the given phylogeny under the assumption of Brownian motion (BM; Münkemüller et al. 2012). The phylogenetic signal of the corolla shape was tested by using K_{mult} , a method proposed by Adams (2014a) to generalize Blomberg's K to multidimensional traits. Statistical significance of K_{mult} was determined using 1000 permutations. These analyses were performed using the “fitDiscrete” command in the R package *geiger* 1.99–3 (Harmon et al., 2008) for corolla color, “physignal” function in the R package *geomorph* 2.1.1 (Adams and Otarola-Castillo 2013) for corolla shape, and “phylosig” function in the R package *phytools* 0.2–14 (Revell 2012) for the remaining unidimensional continuous traits.

We reconstructed the ancestral states of corolla color along the phylogenetic tree by using an updated version of the ‘anc-Threshold’ command implemented in the R package *phytools* 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. We ran four chains of 106 generations each. The posterior probabilities for each character state at each internal node were obtained with the ‘ace’ command in R packages *ape* 3.0–6 (Paradis et al. 2004) and *phytools* 0.2–14 (Revell 2012).

Ancestral state reconstruction of the continuous unidimensional floral traits was done using the ‘anc.Bayes’ function implemented in the R package *phytools* 0.2–14 (Revell 2012). This function uses Bayesian MCMC to sample from the posterior probability for the states at internal nodes in the tree.

Finally, we reconstructed the ancestral multidimensional shapes of the *Erysimum* corollas using squared-change parsimony, a robust method for multidimensional morphometric traits (Webster and Purvis 2002; Astúa 2009; Klingenberg and Gidaszewski 2010). The ancestral shapes are inferred by minimizing the length of the phylogenetic tree as measured in units of squared Procrustes distances, obtaining a landmark configuration for each internal node (Rohlf 2002; Klingenberg and Gidaszewski 2010). Afterward, the reconstructed ancestral shapes and the phylogenetic trees were plotted into the corolla shape space obtained in a PCA to generate a phylomorphospace (Astúa 2009; Klingenberg and Gidaszewski 2010).

CORRELATED EVOLUTION OF POLLINATION NICHES AND FLORAL TRAITS

We explored the correlated evolution between pollination niche and *Erysimum* floral traits using phylogenetic generalized least square (PGLS) models (Grafen 1989; Martins and Hansen 1997). This analysis optimizes the phylogenetic signal

meanwhile performing the analysis (Revell 2010). We performed a PGLS model for each different trait, including the phenotypic trait as a dependent variable and pollination niche as explanatory variable. PGLSs for unidimensional traits were performed using the R package *caper* 0.5.2 (Orme 2013). However, corolla color is a categorical trait and cannot be analyzed using PGLS. For this reason, we explore its correlation with pollination niche by means of a phylogenetic Markov chain Monte Carlo generalized linear mixed model (MCMCglmm), a model that allow including discrete traits both as independent and dependent variables (Hadfield and Nakagawa 2010). We used 10^5 MCMC steps with burn-in = 50,000 and a thinning interval = 10. These analyses were performed using the R package *MCMCglmm* 2.17 (Hadfield 2013). Finally, corolla shape is a multidimensional trait, and consequently, cannot either be analyzed using standard PGLSs. To explore the relationship between corolla shape and pollination niche we used D-PGLS, a robust comparative method recently proposed for high-dimensional traits (Adams 2014c; Adams and Collyer 2015). D-PGLS was performed using the R package *geomorph* 2.1.1, doing 1000 iterations to test significance (Adams and Otarola-Castillo 2013).

We explored the evolutionary correlation between corolla color and shape using D-PGLS. In addition, we checked whether the evolutionary rate in corolla shape varied between corollas of different color using σ^2_{mult} , a multidimensional generalization of the one-dimensional index (Adams 2014b). This estimate of multivariate evolutionary rate is based on distances rather than covariances and has proven suitable for describing evolution occurring in multivariate trait space (Adams 2014b). This analysis was performed using the “compare.evol.rates” command in the R package *geomorph* 2.1.1, doing 1000 iterations to test significance (Adams and Otarola-Castillo 2013).

TESTING ALTERNATIVE SCENARIOS OF MACROEVOLUTIONARY ASSOCIATION BETWEEN POLLINATION NICHE AND COROLLA COLOR

We checked whether any association between pollination niche and floral phenotype was a consequence of the selection exerted by pollinators or was instead a consequence of other nonadaptive processes by evaluating alternative evolutionary scenarios. We used a method proposed by Armbruster (2002) and based on the comparison between transition rates of two phylogenetically correlated binary traits. We adapted this approach to our case in which one selective agent, the pollinators, putatively affects the evolution of plant traits. However, we can apply this method only to corolla color, because the other *Erysimum* floral traits are continuous. To apply Armbruster's (2002) approach, we made both pollination niche and corolla color binary traits. We considered floral color as a discrete character with two states, lilac (1) and yellow (0), removing *E. semperflorens* from the analyses because

it was the only species with white flowers. Pollination niche was also considered as a discrete character with two states, pollination niche E (1) and the other pollination niches combined (0). We lumped together all the non-E pollination niches because the PGLS detected a significant association between pollination niche E and lilac flowers (see Results). According to previous information (Moazzeni et al. 2014; Gómez et al. 2015), the ancestral state (0,0) seems to be yellow flowers belonging to non-E pollination niches.

We consider two alternative evolutionary scenarios: (A) Scenario A postulates that changes in corolla color precede changes in pollination niche, suggesting that pollinators do not cause corolla color evolution. An ancestral population (0,0) evolves lilac corollas (1,0), and as a consequence of this change in corolla color, subsequently moves toward niche E (1,1). Under this hypothesis the transitions from state 0,0 to 0,1 (transition q_{12}) and from 0,1 to 1,1 (transition q_{24}) should be zero. (B) Scenario B postulates that changes in pollinator niche precede changes in corolla color, suggesting that pollinators drive evolution of corolla color. An ancestral population (0,0) moves first toward niche E (0,1) and afterwards evolves lilac corollas (1,1). Under this hypothesis the transitions from state 0,0 to 1,0 (transition q_{13}) and from 1,0 to 1,1 (transition q_{34}) should be zero. To test these two scenarios, we compared the Akaike Information Criterion (AICs) and likelihood of a full model assuming free changes between all possible states with two alternative models constraining some transition rates to zero following the postulates of each hypothesis. These analyses were performed using the R package diversitree 0.9–3 (FitzJohn 2012).

Results

There was ample variation in most phenotypic traits across plant species (Table 2). The color of the corolla was yellow in most species, lilac in six taxa from the Iberian Peninsula and the Canary Islands, and white in the Moroccan *E. semperflorens* (Fig. 1). There was not intraspecific, between-individual variation in floral color. Only the two Macaronesian species, *E. bicolor* and *E. scoparium*, produce flowers that are white at the beginning of the development but turn into lilac during their anthesis (Fig. 1). Consequently, they were categorized as lilac-flowered species, agreeing with their standard taxonomic description.

The studied species also varied in corolla shape (Fig. 1). Some species, such as *E. lagascae*, *E. linifolium*, and *E. semperflorens*, have corollas with parallel petals. Other species, such as *E. cheiranthoides* and *E. myriophyllum*, have open corollas with nonparallel narrow petals. There were also species, such as *E. bonannianum* and *E. seipkae*, with rounded corollas and wide, overlapping petals (Fig. 1).

The species also varied in the other floral traits. Some species had short flowering stalks (e.g., *E. nervosum* and *E. riphaeum*), whereas other species produced long flowering stalks (e.g., *E. bastetianum*, *E. crassistylum* and *E. crepidifolium*). Some species produced few flowers (e.g., *E. wilczekianum* and *E. penyalarensis*), whereas other species produced many flowers (e.g., *E. collisparsum* and *E. odoratum*). The corolla diameter and tube length ranged between 6 mm and 3 mm, respectively, in *E. cheiranthoides*, and around 20 mm and 15 mm, respectively, in *E. raethicum* (Table 2).

EVOLUTIONARY LABILITY OF FLORAL TRAITS

The phylogenetic signal was very weak for corolla color, obtaining a Pagel's lambda nonsignificantly different from zero (Table 3). The phylogenetic signal was also very weak for unidimensional floral traits (Table 3). In fact, only corolla tube width showed a marginally significant phylogenetic signal and only when analyzed through Pagel's lambda (Table 3). Finally, phylogenetic signal was significant for corolla shape when estimated with K_{mult} (Table 3).

The ancestral reconstruction analysis suggests that the ancestral corolla of the studied *Erysimum* lineage was open and with nonparallel petals (Fig. 2). From this ancestral corolla, it seems that the corollas evolved toward parallel petals in certain subclades, although without an apparent pattern across clades (Fig. 2; see Fig. S4 for visualizing changes in landmark positions from the ancestral shape to the shape of each studied *Erysimum* species).

The ancestral corolla color of the pool of species studied here was yellow, whereas white and lilac corollas were derived states (Fig. 2). Lilac flowers seem to have appeared several times in different clades (Fig. 2). More interesting, our reconstruction analysis suggests that yellow corollas have secondarily appeared from ancestral lilac-flowered species in some subclades (Fig. 2). Hence, the most likely color of the corolla of the ancestral species of the subclade containing the species from Canary Island, Morocco and most Southern Spain species was lilac, despite the fact that in this subclade there are species with yellow flowers (*E. rondae*, *E. myriophyllum*, *E. fitzii*, *E. nervosum* and *E. mediohispanicum*; Fig. 2).

The reconstruction of the remaining unidimensional floral traits suggests the absence of important changes along the phylogeny, except for number of flowers (Table S3). It seems that ancestral *Erysimum* had very few flowers (less than 10), and from there many-flowered species evolved (Table S3).

CORRELATED EVOLUTION OF POLLINATION NICHES AND FLORAL TRAITS

Corolla shape significantly correlated with pollination niches according to D-PGLS (Table 4). The phylomorphospace shows that

Table 2. Phenotypic traits of the studied taxa.

Species	Corolla color	Flowering stalk height (cm)	Number of flowers	Corolla diameter (mm)	Corolla tube length (mm)	Corolla tube width (mm)
<i>E. baeticum</i>	Lilac	16.95	52.17	16.78	12.78	0.85
<i>E. bastetanum</i>	Lilac	47.63	56.63	14.22	11.97	0.93
<i>E. bicolor</i>	Lilac	35.55	169.46	14.95	9.27	1.36
<i>E. bonannianum</i>	Yellow	35.39	106.12	14.42	10.93	1.02
<i>E. cazorlense</i>	Lilac	22.20	55.61	15.22	11.50	0.63
<i>E. cheiranthoides</i>	Yellow	50.90	99.93	6.41	3.83	0.96
<i>E. cheiri</i>	Yellow					
<i>E. collisparsum</i>	Yellow	47.40	280.20	16.40	13.20	2.44
<i>E. crassistylum</i>	Yellow	51.42	130.62	15.62	10.54	1.30
<i>E. crepidifolium</i>	Yellow	68.60	84.68	14.04	9.14	0.98
<i>E. duriaei</i>	Yellow	14.03	43.43	16.50	14.12	1.61
<i>E. etnense</i>	Yellow	27.76	62.37	16.61	11.87	1.95
<i>E. fitzii</i>	Yellow	26.57	44.50	10.59	9.67	0.99
<i>E. geisleri</i>	Yellow	22.25	65.78	18.03	13.97	1.33
<i>E. gomezcampoi</i>	Yellow	39.50	86.15	12.28	11.37	0.11
<i>E. gorbeanum</i>	Yellow	17.85	48.67	13.29	11.71	1.67
<i>E. incanum</i>	Yellow	6.52	15.91	5.28	4.25	0.10
<i>E. jugicola</i>	Yellow	21.17	36.63	16.59	12.18	1.10
<i>E. lagascae</i>	Lilac	43.85	144.33	14.89	10.15	0.21
<i>E. linifolium</i>	Lilac	31.91	61.90	17.07	9.69	2.40
<i>E. mediodhispanicum</i>	Yellow	30.46	35.66	11.23	10.84	0.43
<i>E. metlesicsii</i>	Yellow	30.63	136.37	14.60	12.90	1.61
<i>E. merxmülleri</i>	Yellow	47.14	69.35	13.54	11.60	0.77
<i>E. myriophyllum</i>	Yellow	11.95	56.50	9.42	9.88	0.00
<i>E. nervosum</i>	Yellow	9.96	129.28	12.62	10.58	0.73
<i>E. nevadense</i>	Yellow	13.85	54.68	14.32	11.13	0.95
<i>E. odoratum</i>	Yellow	74.92	180.13	16.56	10.62	1.54
<i>E. penyalarens</i>	Yellow	11.79	27.17	13.49	10.33	1.25
<i>E. popovii</i>	Lilac	15.90	26.07	11.44	9.55	0.79
<i>E. pseudorhaeticum</i>	Yellow	25.95	74.07	16.89	13.02	2.59
<i>E. rhaeticum</i>	Yellow	38.24	64.18	19.17	15.05	3.44
<i>E. riphaeum</i>	Yellow	8.67	90.55	13.70	10.75	0.92
<i>E. rondae</i>	Yellow	32.42	88.47	14.37	13.26	1.00
<i>E. ruscinonense</i>	Yellow	24.32	55.45	10.98	8.71	1.02
<i>E. scoparium</i>	Lilac					
<i>E. semperflorens</i>	White	41.24	93.31	16.49	12.20	0.54
<i>E. seipkae</i>	Yellow					
<i>E. sylvestre</i>	Yellow	11.58	12.23	14.79	12.33	0.70
<i>E. virgatum</i>	Yellow					
<i>E. wilczekianum</i>	Yellow	7.04	20.71	12.42	7.42	1.20

plants belonging to niche E had zygomorphic corollas with parallel petals, plants belonging to niche B had slightly zygomorphic corollas, and plants from the other niches had similar symmetric corollas with open petals (Fig. 3). Species from niche E are located in the lower half of the phylomorphospace, separated from species belonging to other niches.

We also found a significant association between corolla color and pollination niches (Table 4). In particular, there was a strong

association between lilac corolla and the pollination niche E, because all lilac-flowered species but one, *E. baeticum*, belonged to this niche (Fig. 4).

We found a significant evolutionary correlation between color and shape ($F = 5.15$, $df = 2,32$, $P = 0.029$, D-PGLS). Species with lilac flowers had more parallel petals than species with yellow flowers (Fig. 5). In addition, the net evolutionary rate in corolla shape differed between yellow ($\sigma^2_{mult} = 0.00057$) and

Table 3. Outcome of the phylogenetic signal analyses. Shown are the mean values of each variable across the 6400 tested trees as well as the range in brackets.

Traits	Blomberg's <i>K</i>	<i>P</i> value	Pagel's λ	<i>P</i> value	% trees <i>p</i> < 0.05	
					<i>K</i>	λ
Corolla color			0.000 [0.000–0.000]	1.000 [1.000–1.000]		0
Corolla shape ¹	0.406 [0.060–0.628]	0.003 [0.002–0.337]			99.5	
Stalk height	0.266 [0.043–0.480]	0.415 [0.053–0.867]	0.432 [0.000–0.779]	0.969 [0.207–1.000]	0	0
Flower number	0.367 [0.181–0.735]	0.185 [0.021–0.859]	0.419 [0.000–1.000]	0.980 [0.021–1.000]	10	1
Corolla diameter	0.314 [0.058–0.497]	0.247 [0.045–0.994]	0.036 [0.000–0.627]	0.938 [0.223–1.000]	0.5	0
Corolla tube length	0.346 [0.074–0.591]	0.211 [0.014–0.977]	0.011 [0.000–0.826]	0.994 [0.186–1.000]	10	0
Corolla tube width	0.371 [0.105–0.754]	0.170 [0.005–0.897]	0.385 [0.282–0.624]	0.050 [0.020–0.195]	10	55

¹Phylogenetic signal of corolla shape was estimated using Adams' *K_{mult}* statistic.

Table 4. Outcome of the models exploring the correlated evolution between pollination niches and *Erysimum* floral traits. The analyses were performed using phylogenetic generalized least square (PGLS) analyses for all traits, except for corolla shape and corolla color. The significance of lambda in the PGLS analyses was contrasted against lower bound = 0.

Traits	PGLS			
	RSE ¹	<i>F</i> _{6,27}	<i>P</i>	λ
Corolla shape ²	0.71	4.26	0.047	0.359
Corolla color ³	-	-	0.001	0.126
Flower number	9.91	1.40	0.251	0.780
Stalk height	7.52	2.53	0.450	0
Corolla diameter	4.53	0.19	0.317	0.949
Corolla tube length	3.19	1.06	0.412	0
Corolla tube width	8.44	0.65	0.769	0.226

¹RSE = Residual standard error.

²The multidimensional trait corolla shape was analyzed using D-PGLS (Adams 2014c).

³The corolla color, due to its categorical nature, was analyzed using MCM-Cglmm. The posterior mean was 192.2, with a 95% CI = [53.0–361.3].

lilac flowers ($\sigma^2_{mult} = 0.00141$), being more than two times faster in the latter group of plants (σ^2_{mult} lilac flowers/ σ^2_{mult} yellow flowers = 2.47, *P* = 0.001).

TESTING ALTERNATIVE SCENARIOS OF MACROEVOLUTIONARY ASSOCIATION BETWEEN POLLINATION NICHE AND COROLLA COLOR

Our analysis suggests that changes in corolla color preceded changes in pollination niche (Table 5). Although the transition rate from ancestral state (yellow corolla and non-E pollination niche) to state 0,1 with yellow corolla and pollination niche E (Fig. 6) was 1.7, the transition rate from ancestral state to state 1,0

with lilac corolla and non-E pollination niche was 4.3. Similarly, the transition rate from state 0,1 to 1,1 (lilac corolla and pollination niche E) was 0, whereas the transition rate from state 1,0 to 1,1 was 100 (Fig. 6). Consequently, when constraining to zero the transition rates *q*₁₂ and *q*₂₄ (scenario A), the model performed better than the full model, indicating that this evolutionary pathway was very unlikely. In contrast, the model obtained by constraining to zero the transition rates *q*₁₃ and *q*₃₄ (scenario B) performed much worse than the full model, having a much higher AIC value and a significantly lower likelihood (Table 5). This outcome suggests that these two transitions rates were significantly above zero.

Discussion

Most of the *Erysimum* floral traits studied here showed very weak phylogenetic signals, both when calculated with Blomberg's *K* or with Pagel's λ . Although the phylogenetic signal of the corolla shape was significant when considered as a single, multidimensional trait, its low value, much lower than one (0.406), suggests that close relatives are less similar phenotypically than expected under Brownian motion (Adams 2014a). Certain measurement errors related to the uncertainty of the phylogenetic relationships of the taxa included in the analyses may sometimes cause low phylogenetic signal (Blomberg et al. 2003; Ives et al. 2007). We think that this methodological drawback is not critical in our study, because the phylogeny was obtained with two nuclear and two plastidial genes (Gómez et al. 2014b), and the analyses were performed considering the phylogenetic uncertainty by using a set of 6400 Bayesian trees. Hence, it is very probable that the observed low phylogenetic signal is evidence of evolutionary lability in *Erysimum* floral traits (Losos, 2008; McEwen and Vamوسي 2010).

Weak phylogenetic signal and evolutionary lability is not rare in plant phenotypic traits related to pollination, such as floral display size (Smith et al. 2008; Roncal et al. 2012), floral color (Beardsley et al. 2003; McEwen and Vamوسي 2010; Muchhala

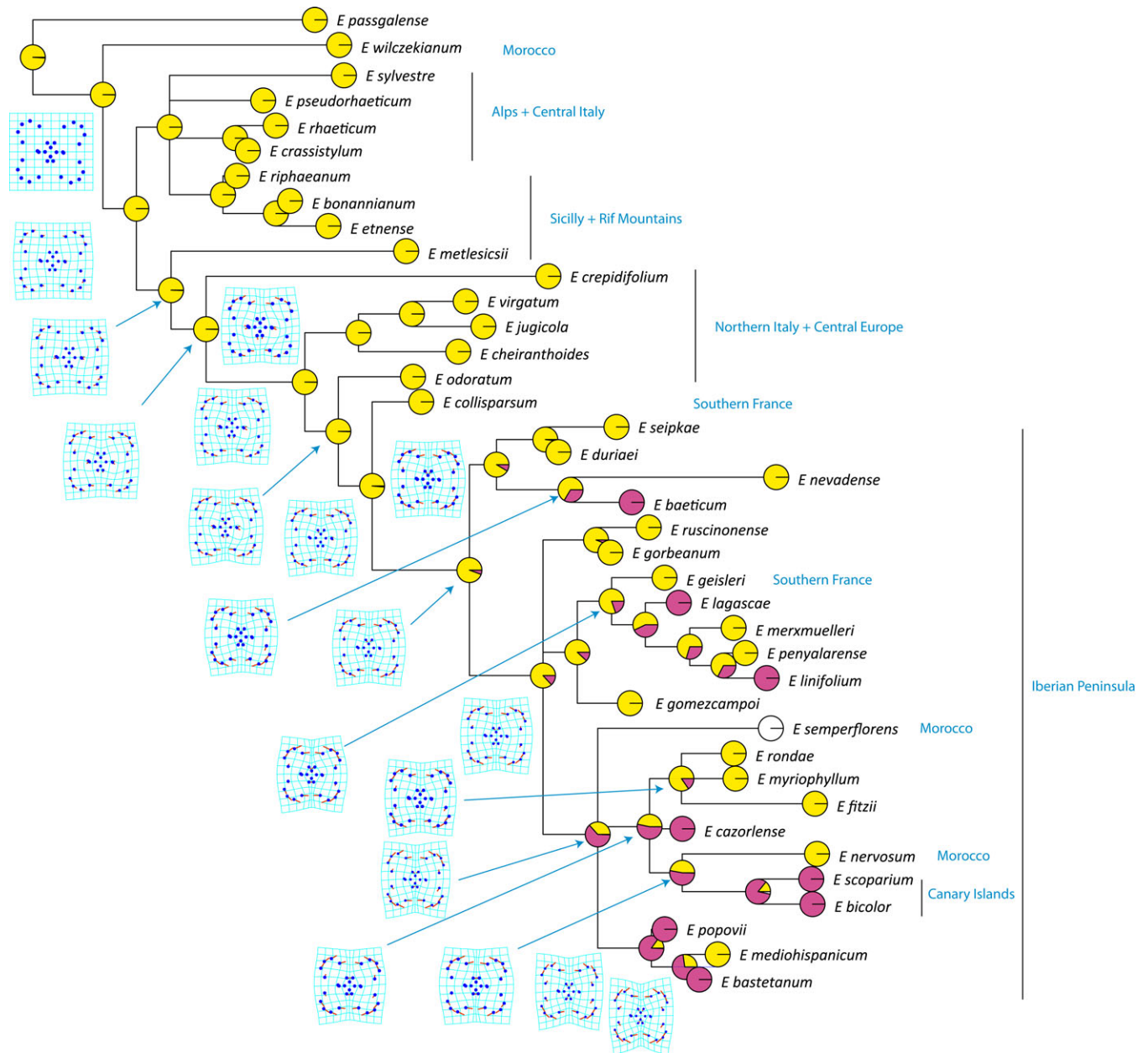


Figure 2. Evolution of corolla shape and color in *Erysimum*. Shown are the corolla color (yellow, purple, white) of present-day species as well as the Bayesian color reconstruction of the internal nodes (see Table S2 for values). It is also shown the MorphoJ shape reconstruction of some internal nodes as changes in landmark positions (red lines) with respect to the most ancestral shape (blue dots).

et al. 2014), and floral shape (Alcantara and Lohmann 2010). Although several genetic and ecological factors may cause high lability in trait evolution (O'Meara et al. 2006; Losos 2008; Bell 2010; Cooper et al. 2010; Crisp and Cook 2012; Hansen 2012), low phylogenetic signal is sometimes associated with punctuated divergent selection (Revell et al. 2008). This process may happen when daughter lineages face different selective scenarios and evolve to two different optima after every bifurcation (Revell et al. 2008; Ackerly 2009). Under this scenario, low phylogenetic signal is expected when there is recurrent short-term variation in

pollinator-mediated selection (Beardsley et al. 2003; Ornelas et al. 2007; Wilson et al. 2007; Harder and Johnson 2009). Pollination niches have evolved in *Erysimum* through recurrent shifts between slightly different generalized pollinator assemblages (Gómez et al. 2015). This evolutionary pattern has probably entailed the occurrence of frequent divergent and convergent pollinator-mediated selective pressures on plant traits (Gómez et al. 2009a,b). The observed weak phylogenetic signal and evolutionary lability in *Erysimum* floral phenotype can thereby be a consequence of a concomitant lability in pollination niche evolution. However, it is

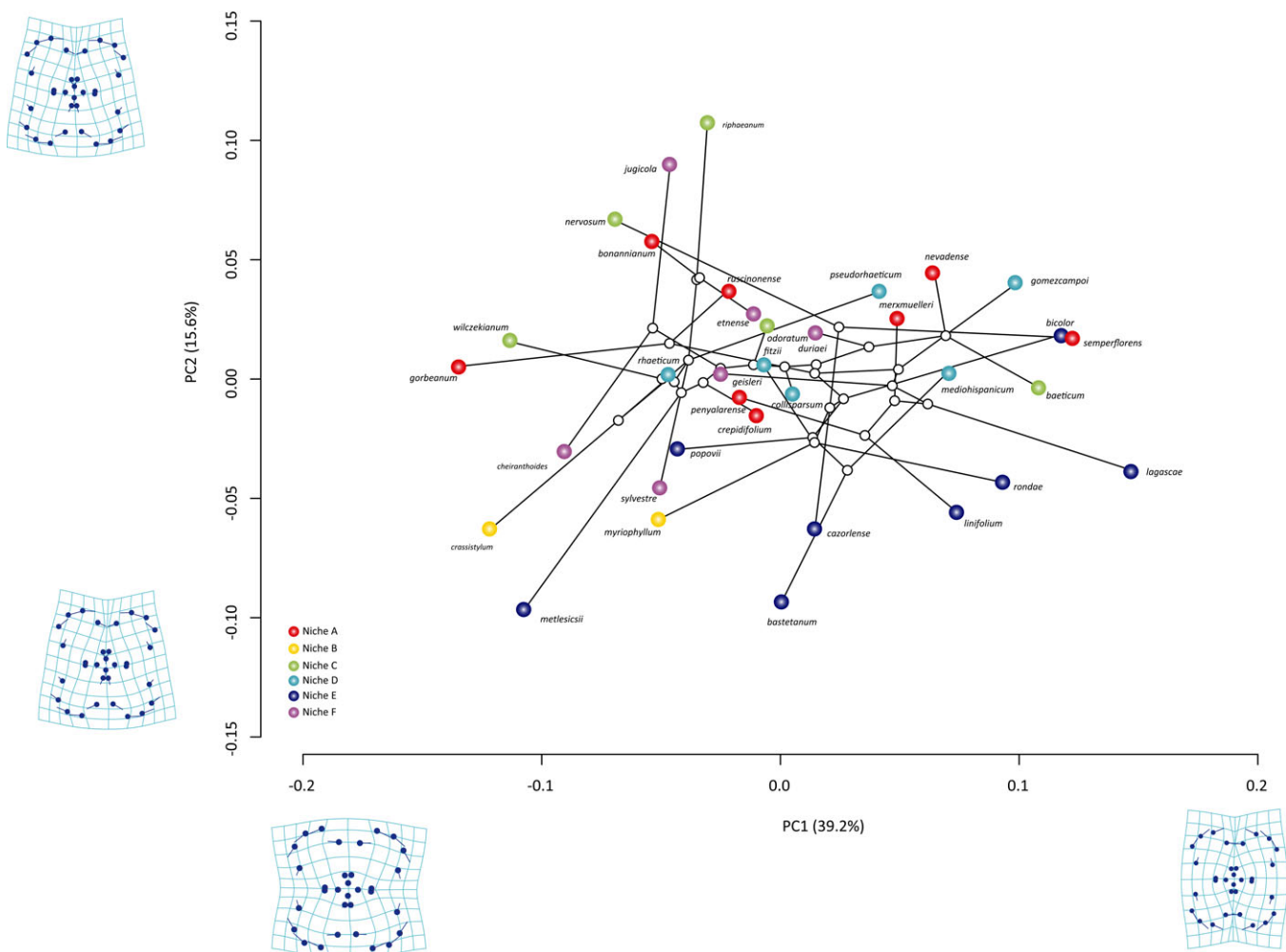


Figure 3. Phylomorphospace of the first two PC axes (PC1 vs. PC2) showing the association between *Erysimum* corolla shapes and pollination niches.

Table 5. Outcome of the likelihood ratio test testing the two evolutionary scenarios explaining the correlated evolution between corolla color and pollination niche.

Models	Transition rates constrained to zero	df ¹	AIC ²	lnLik ³	χ ²	P
Null model		14	63.583	−17.792		
Scenario A [Change in corolla color precedes change in pollination niche]	q ₁₂ + q ₂₄	12	61.572	−18.786	1.99	0.37
Scenario B [Change in pollination niche precedes change in corolla color]	q ₁₃ + q ₃₄	12	96.838	−36.419	37.25	0.0001

¹df = degree of freedom of the model.

²AIC = Akaike Information Criterion of each model.

³lnLik = likelihood of the model.

necessary to know whether this pattern has actually been caused by adaptive responses to the selection exerted by pollinators.

Corolla shape and color were associated with changes in pollination niches. Previous evolutionary studies have found that pollinators may exert significant selection on corolla shape in

some *Erysimum* species, such as *E. mediohispanicum* and *E. nevadense* (Gómez et al. 2006; Abdelaziz 2013), and may affect the morphological integration of corolla shape in several species of *Erysimum* (Gómez et al. 2014b). The geographic variation in pollinators with contrasting preference patterns causes

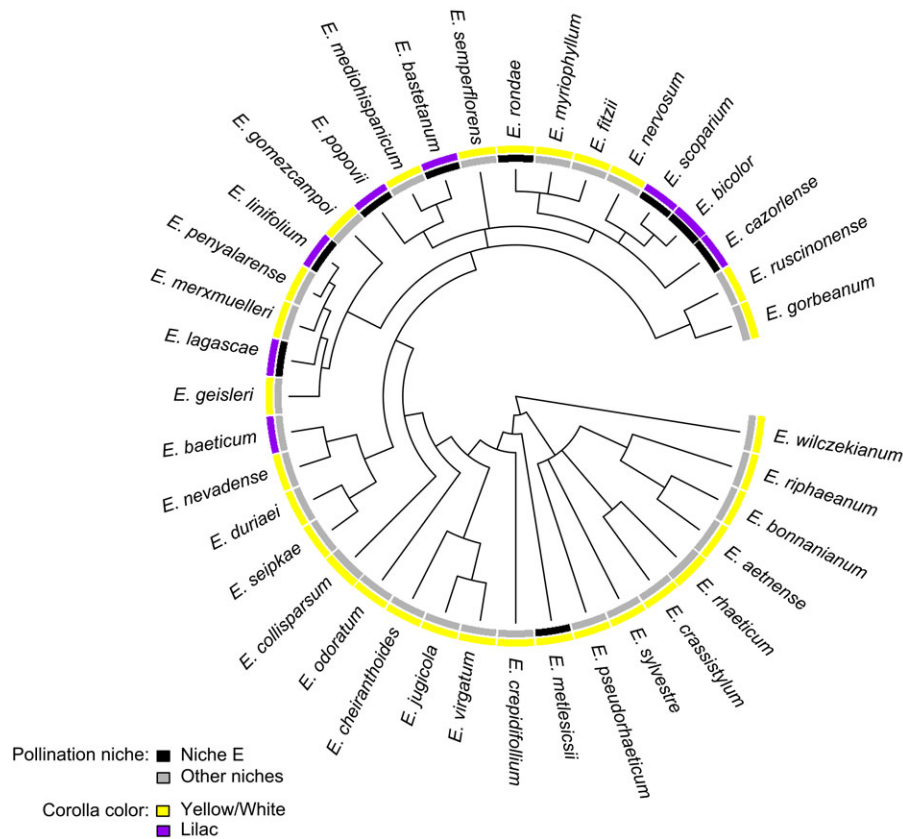


Figure 4. Phylogram showing the among-species distribution of corolla color and the pollination niche.

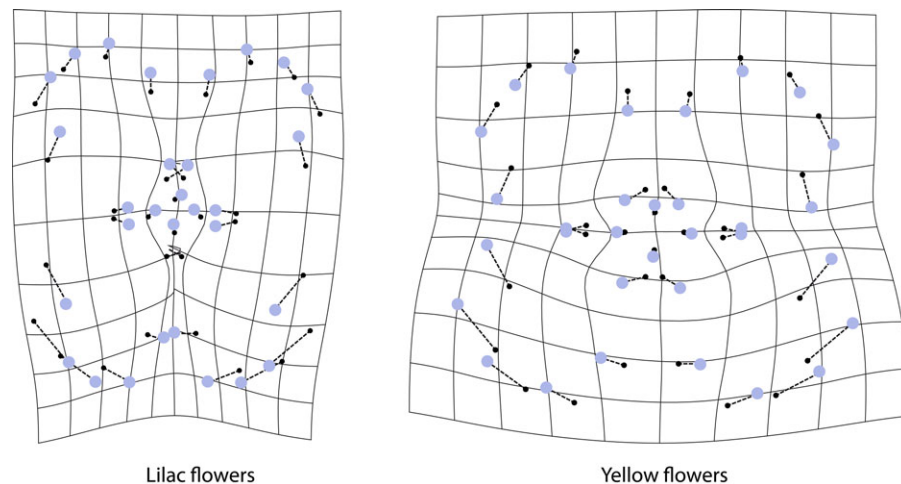


Figure 5. Differences in corolla shape between lilac- and yellow-flowered *Erysimum* species visualized as deformation grids showing changed between the overall mean corolla shape (small black dots) and the corolla shape of each corolla color (large blue dots).

even spatially divergent selection and local adaptation in *Erysimum* corolla shape (Gómez et al. 2009a,b). Consequently, the geographic variation in pollination niches has probably contributed to the observed floral divergence in some species, such as *E. mediohispanicum* (Gómez et al. 2014a). This previous evidence supports the idea that the observed phylogenetic correlation between pollination niche and corolla shape is a

consequence of pollinators shaping the evolution of this floral trait. Although pollinator-mediated floral shape evolution has been demonstrated in specialized plant clades (Neal et al. 1998; Endress 2001; Sargent 2004; Papadopoulos et al. 2013), it has not been documented in generalist plant lineages. Our finding is thus the first evidence of pollinators playing a role in the evolution of corolla shape in generalist plants. Nevertheless, further

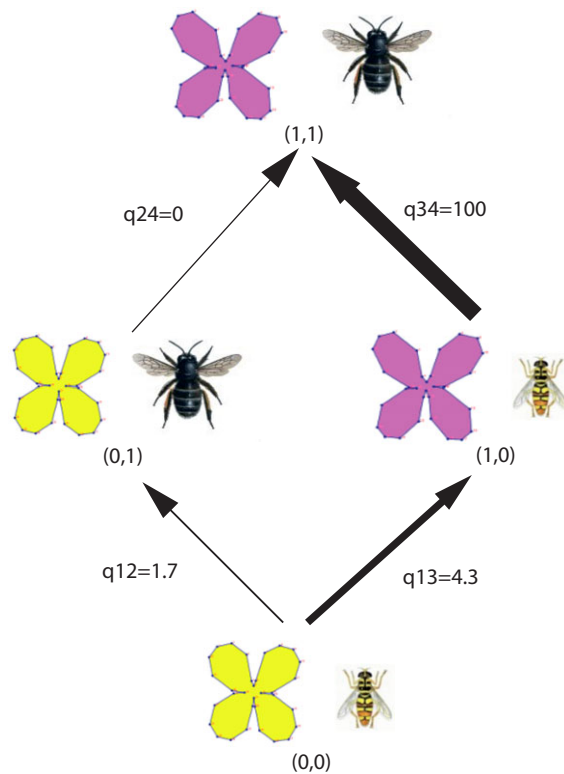


Figure 6. Scheme depicting the possible evolutionary transitions between different corolla color and pollination niche states. Arrow width is proportional to the value of the transition rate.

studies are necessary to disentangle whether this correlation actually means that pollinators drive corolla shape or is just the consequence of a hidden factor affecting both traits in the same direction.

Corolla color was also associated with pollinators. Specifically, most plant species displaying lilac corollas belonged to pollination niche E, a pollination niche formed mostly by long-tongued large bees belonging to the genus *Anthophora*. Lilac and purple corollas are traditionally associated with bee pollination (Faegri and van der Pijl 1979; Willmer 2011). Consequently, the current association between lilac corollas and niche E in *Erysimum* could be interpreted as evidence of bee-mediated corolla color evolution. Nevertheless, according to our analysis of evolutionary scenarios, floral color evolution seems to have preceded shifts in pollination niche in *Erysimum*. Under these circumstances, we believe that the correlated evolution between corolla color and pollination niche has occurred in two steps. First, lilac flowers evolve recurrently in some populations belonging to any pollination niche. Our ancestral reconstruction analysis effectively shows that lilac color has evolved several times in different Iberian lineages, indicating the occurrence of evolutionary convergence in this trait. At this moment, pollinators did not play a major role in the evolution of this trait. Rather, other factors have

been involved in the early evolution of lilac corolla. This color is produced in Brassicaceae by acylated anthocyanins (Tatsuzawa et al. 2012a, b, c, 2013, 2014). Besides their role as a signal for pollinators, floral anthocyanins also function as protection agents against stresses induced by radiation, drought, and temperature (Chalker-Scott 1999; Dick et al. 2011), and as defense against floral herbivores (Irwin et al. 2003; Strauss et al. 2004; Lev-Yadun and Gould 2009). The evolution of lilac corollas may be related to these factors. It is remarkable that only a few of the Old-World *Erysimum* species have lilac flowers (23 out of 200 species; Polatschek 2010, 2011, 2012, 2014), and most of them inhabit arid, steppe, and dry alpine ecosystems in the southernmost areas of the genus' distribution (Iberian Peninsula, the Macaronesian Islands, Near East and Central Asia—Taiyan et al. 2001; Polatschek 2010, 2011). This is circumstantial evidence suggesting that lilac corollas may also function to protect *Erysimum* against drought and harmful radiation frequent in those places.

In a second phase, most lilac-flowered populations may gradually evolve toward pollination niches with high frequency of long-tongued large bees (pollination niche E in our system). This occurs because (1) these bees visit the flowers of most *Erysimum* species, irrespective of the corolla color and the niche they belong to (Figs. 4 and S2)—the boundaries between generalist pollination niches are not sharp and the niches differ among them in the relative frequency of pollinator functional groups (Gómez et al. 2014a, 2015), but (2) these bees are highly attracted to *Erysimum* lilac corollas (Ollerton et al. 2007). Therefore, when lilac corollas evolve in a population or species, the abundance of those bees at flowers increases gradually, causing a steady shift to a new pollination niche. At this moment, because long-tongued large bees are very efficient pollinators of *Erysimum* (Ollerton et al. 2007; Gómez et al. 2010; Lay et al. 2011) and act as selective agents on these plants (Gómez et al. 2009a,b), it is possible that a subsequent adaptive fit between flowers and bees driven by the selective pressures is exerted by these pollinators. In this respect, the evolution toward corollas with parallel petals observed in lilac-flowered *Erysimum* might be caused by the strong selection exerted by long-tongued large bees. Large bees can discriminate among *Erysimum* plants according to their corolla shape, preferring those with narrow and parallel petals (Gómez et al. 2008). Consequently, selection on corolla shape will be stronger in those *Erysimum* species being visited more frequently by this kind of bees. By exerting consistent selection on corolla shape, pollinators may thus influence the evolutionary correlation found between corolla color and shape.

Conclusion

Several features describe the evolution of floral traits in *Erysimum*. First, as expected by the currently accepted theoretical framework,

pollinators did not drive the diversification of many of their floral traits. In contrast, the evolution of these floral traits was not associated with any pollination niche shift. Nevertheless, we did find that some floral traits, such as corolla shape, evolved as a consequence of strong and consistent selection exerted by generalist pollinator assemblages. This is a remarkable finding, because it is widely assumed that pollinators play a fleeting role in the evolution of floral traits in generalist plant clades (Waser 2001; Waser and Campbell 2004; Kay and Sargent 2009). Even so, not all pollination niches are equally able to drive floral evolution. In our study, only the niche in which long-tongued large bees were highly abundant (niche E) was associated with *Erysimum* floral evolution, while the other pollination niches did not affect any floral traits. This outcome is probably common to most generalist systems, suggesting that pollinator-mediated evolution can happen in just a few “hot” niches (Gómez et al. 2014a). To make this evolutionary scenario more complex, we found that it is even possible to find correlated evolution between pollination niches and some traits as a consequence of pollinators tracking the evolution of those traits rather than being drivers of their diversification. In our system, this pattern was found with corolla color evolution. Finally, floral evolution in *Erysimum* has occurred without any trend toward pollination specialization. Lilac species are not more evolutionary specialized than yellow species, whereas plants from niche E being visited by as many functional groups as plants from any other pollination niche. We presume that many of these features describing the evolution of *Erysimum* floral traits are common to other plants displaying generalist pollination systems. Altogether, our findings suggest that floral evolution in generalist plants may be more complex than traditionally thought. More studies are necessary to conclude whether our findings can be extrapolated to other generalist plants.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.rv08g.

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Supporting Information

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

Figure S1. Consensus Bayesian tree showing the phylogenetic relationships among the studied species of *Erysimum* according to one nuclear (*ITS1* and *ITS2* and the 5.8 *rDNA* between both ITSs sequences) and two plastidial (*ndhF* gene and *trnT-trnL* intergenic spacer) DNA regions.

Figure S2. Plot showing the classification of the studied species into different pollinator niches, according to the analysis of bipartite modularity QuanBiMo.

Figure S3. Spectral reflectance of one yellow-flowered *Erysimum* species (*E. nevadense*) and one purple-flowered species (*E. popovii*).

Figure S4. Shape transformation of the corolla of each studied *Erysimum* species from the reconstructed ancestral corolla.

Table S1. Location and sample size of studied *Erysimum* species.

Table S2. Ancestral reconstruction of the corolla color.

Table S3. Ancestral reconstruction of quantitative corolla traits.