

Bees explain floral variation in a recent radiation of *Linaria*

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Abstract

The role of pollinators in floral divergence has long attracted the attention of evolutionary biologists. Although abundant studies have reported the effect of pollinators on flower-shape variation and plant speciation, the influence of pollinators on plant species differentiation during rapid radiations and the specific consequences of shifts among similar pollinators are not well understood. Here, we evaluate the association between pollinators and floral morphology in a closely related and recently diversifying clade of *Linaria* species (sect. *Supinae* subsect. *Supinae*). Our approach combined pollinator observations, functional floral morphometric measures and phylogenetic comparative analyses. The fauna visiting *Linaria* species was determined by extensive surveys and categorized by a modularity algorithm, and the size and shape of flowers were analysed by means of standard and geometric morphometric measures. Standard measures failed to find relationships between the sizes of representative pollinators and flowers. However, discriminant function analyses of geometric morphometric data revealed that pollination niches are finer predictors of flower morphologies in *Linaria* if compared with phylogenetic relationships. Species with the most restrictive flowers displayed the most slender spurs and were pollinated by bees with larger proboscides. These restrictive flower shapes likely appeared more than once during the evolutionary history of the study group. We show that floral variation can be driven by shifts between pollinators that have been traditionally included in a single functional group, and discuss the consequences of such transitions for plant species differentiation during rapid radiations.

Introduction

The evolution of flower shape has been studied by several disciplines that seek to understand the basis of plant speciation, including developmental genetics, phylogeny and evolutionary ecology (e.g. [Cubas et al., 1999](#); [Ree & Donoghue, 1999](#); [Gómez et al., 2008](#)). The remarkable variety of angiosperm flower shapes is

thought to have evolved as a consequence of strong pollinator-driven selection towards greater attractiveness and efficient pollen transfer ([Darwin, 1862](#); [Robertson, 1888](#); [Stebbins, 1970](#); [Faegri & Van der Pijl, 1979](#); [Lehrer et al., 1995](#); [Møller, 1995](#); [Wilson & Thomson, 1996](#); [Endress, 1999](#); [Anderson & Johnson, 2008](#)). The relative importance of pollinator-mediated selection as a source of floral variation and plant speciation has been challenged on the basis of apparent widespread generalization in pollination systems ([Herrera, 1996](#); [Waser et al., 1996](#); [Johnson & Steiner, 2000](#); [Strauss et al., 2005](#); [Gómez et al., 2015](#)), and the evidence of nonpollinator selection agents on flowers ([Harder & Barrett, 2006](#)). Regardless of the

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active debate on their general role in plant speciation, pollinators are still considered to be divergent selection agents in specialist plant groups (Grant, 1949; Kay & Sargent, 2009; Yoder *et al.*, 2013). Moreover, flower specialization may even occur in generalist plant species due to differences in the frequency of flower visits and differences in the pollinator effectiveness for particular plant species (e.g. Stebbins, 1970; Fulton & Hodges, 1999; Fenster *et al.*, 2004; Gómez *et al.*, 2008, 2014).

A great deal of studies can be found that describes the variation in flower forms associated with pollinator fauna (e.g. Armbruster, 1985; Robertson & Wyatt, 1990; Steiner & Whitehead, 1991; Fulton & Hodges, 1999; Schemske & Bradshaw, 1999; Castellanos *et al.*, 2003; Kephart & Theiss, 2004; Pérez-Barrales *et al.*, 2007; Smith *et al.*, 2008; Kay & Sargent, 2009). Most of the studies of flower specialization have focused on major flower-shape changes or morphological combinations (i.e. pollination syndromes), and the recognition of pollinators as functional groups defined by their ecological and morphological similarities (Faegri & Van der Pijl, 1979; Fenster *et al.*, 2004). Pollination syndromes have been studied extensively in the literature. But recently, there has been a call for direct studies on the correlation between fine-scale flower and pollinator features by analysis of specific traits, rather than studies based on evolutionary associations at larger scales (Galen & Cuba, 2001; Gómez *et al.*, 2006; Ollerton *et al.*, 2009; Smith, 2010; van der Niet *et al.*, 2013). In that sense, there is a lack of studies in species with subtle floral divergence that are visited by morphologically similar pollinators. Associations between flower form and pollinators in these systems may provide insights of isolation mechanisms in morphologically similar plant species.

Ecological patterns can only give insights into current (or very recent) differentiation processes. Therefore, it is difficult to infer the adaptive role of specific floral phenotypes from the existing interactions with pollinators (Herrera, 1996; Ollerton, 1996). Studies of recent radiations are particularly appropriate because the pollinators we observe today have higher likelihood to be similar to the ones that occurred during speciation. Currently, there is a lack of species-level phylogenetic analyses combined with pollinator observations in recent plant radiations that consist of partially co-existing relatives (but see Johnson & Steiner, 1997; Valente *et al.*, 2012; van der Niet *et al.*, 2013). These kind of studies may also provide important information on plant groups of recent differentiation that retain inter-fertility, for which floral isolation may be especially relevant (Kay & Sargent, 2009).

The genus *Linaria* is the richest genus (~150 spp.) of the snapdragon tribe (Antirrhineae, Plantaginaceae) (Sutton, 1988), and many species presumably originated from recent radiations (Blanco-Pastor & Vargas, 2013; Fernández-Mazuecos *et al.*, 2013). *Linaria* flowers display a complex spurred corolla that is potentially

linked to pollinator specialization. The flowers of *Linaria* have recently become a research focus in ecological interactions (Sánchez-Lafuente, 2007; Sánchez-Lafuente *et al.*, 2011) and developmental genetics of flower shape (Cubas *et al.*, 1999; Box *et al.*, 2011). Contrasting flower morphologies have been detected previously in *Linaria* and were considered as an adaptation to divergent strategies of pollen placement on nectar-feeding insects (Robertson, 1888; Fernández-Mazuecos *et al.*, 2013). Morphological variation has been primarily associated with narrowing of the corolla tube (Viano, 1969; Sutton, 1988; Fernández-Mazuecos *et al.*, 2013) and is likely driven by selective forces of pollinator types such as bees vs. dipterans/lepidopterans. In this genus, differences in per-visit effectiveness of pollinators of the same functional group (long-tongued bees) have also been documented (Sánchez-Lafuente *et al.*, 2011), but analysis of subtle floral divergence driven by such pollinators has not been addressed. Furthermore, there are few reports in the literature of floral divergence in species pollinated by morphologically similar pollinators (but see Johnson & Steiner, 1997).

Studies focused on fine-scale flower shape have been historically limited by the difficulties involved in the quantification of form (Rohlf, 1990). Flower shape has usually been studied as a qualitative trait or as a feature composed of several linear measurements (Neal *et al.*, 1998; Endress, 1999; Galen & Cuba, 2001). In the last decades, geometric morphometrics has emerged as a useful tool for the study of corolla-shape variation (Herrera, 1993; Gómez *et al.*, 2006; Gómez & Perfectti, 2010; Fernández-Mazuecos *et al.*, 2013). Here, we used geometric morphometrics to study fine-scale shape variation in flowers of closely related *Linaria* species that presumably originated in a recent radiation (Blanco-Pastor *et al.*, 2012, 2013; Blanco-Pastor & Vargas, 2013). Besides, we analysed standard measurements of flowers and their predominant pollinators to evaluate pollinator and flower size fitting or correlation. Our working hypothesis was that shifts between different pollination niches associated with different groups of bees have driven floral divergence. The alternative hypothesis is that flower morphologies have been conserved during the evolution of the group, and therefore, phylogenetic relationships are better predictors of flower morphologies in this recently diverged group of *Linaria* species.

Materials and methods

Study species

We used the following 10 *Linaria* species belonging to subsect. *Supinae* of sect. *Supinae sensu* Blanco-Pastor *et al.* (2012) (hereafter subsect. *Supinae*), a monophyletic group of species inhabiting narrow ranges in southern Iberia (see distribution of each species in

Blanco-Pastor & Vargas (2013)): *L. aeruginea*, *L. almijarensis*, *L. amoi*, *L. anticaria*, *L. depauperata*, *L. lilacina*, *L. platycalyx*, *L. polygalifolia*, *L. tristis* and *L. verticillata* (Fig. 1). Because of their recent diversification in the Quaternary (Blanco-Pastor *et al.*, 2012, 2013; Blanco-Pastor & Vargas, 2013), these species are a suitable study group to test whether changes in mutualistic interactions have fostered differentiation of species. These species present the largest flowers within sect. *Supinae* (Sutton, 1988; Sáez & Bernal, 2009; Blanco-Pastor *et al.*, 2012). They display zygomorphic corollas with a cylindrical tube calcarate (spurred) abaxially at the base and a palate (lower lip) occluding the mouth of the tube. The occluded personate form and consistency of the corolla requires the action of a strong or heavy insect to get access to pollen and nectar reward, which has long been considered as an adaptation to bee pollination (melittophil) (Müller, 1873; Hill, 1909; Sutton, 1988; Vargas *et al.*, 2010). These species are homostylous but with dimorphic stamen heights, protogynous (Valdés, 1970) and self-incompatible (Blanco-Pastor & Vargas, 2013).

Diversification rates

We estimated net diversification rates for subsect. *Supinae* to test for evolutionary radiation in this group. Diversification rates were compared with cases of exceptional plant radiations reported by previous authors (Valente *et al.*, 2010; Bell *et al.*, 2012). We used the method of Magallón & Sanderson (2001)

implemented in the R package *geiger* (Harmon *et al.*, 2008) as similarly carried out by the authors mentioned above. We considered two extremes of the relative extinction rate ($\epsilon = 0$, no extinction and $\epsilon = 0.9$ high extinction rate, where $\epsilon = \text{extinction rate/speciation rate}$). Species richness was estimated from the number of species that fell into subsect. *Supinae* clade (16) as obtained in a recent inclusive species tree analysis of sect. *Supinae* (Blanco-Pastor & Vargas, 2013). Time estimates of Blanco-Pastor & Vargas (2013) were not used because they may be biased towards more recent divergence times as a consequence of the inclusion of plausible hybrid/introgressed species (Leaché *et al.*, 2014) (see Blanco-Pastor & Vargas, 2013). Diversification rates were calculated for crown and stem ages from the *BEAST analysis of Blanco-Pastor *et al.* (2013) that excluded hybrid species.

Flower visitor surveys

We counted flower visits and identified floral visitors in 1–3 populations (or subpopulations) per species. A total of 9609 min of observation evenly distributed among species were carried out during 5 years (2009–2013). Visits were considered legitimate when the insect opened the corolla tube and touched the anthers and stigma. We also made notes of the availability of pollinator groups in the population by indicating those groups that were present but not visited flowers. As an estimate of the sampling effort for each *Linaria* species, we performed accumulation curves using the package

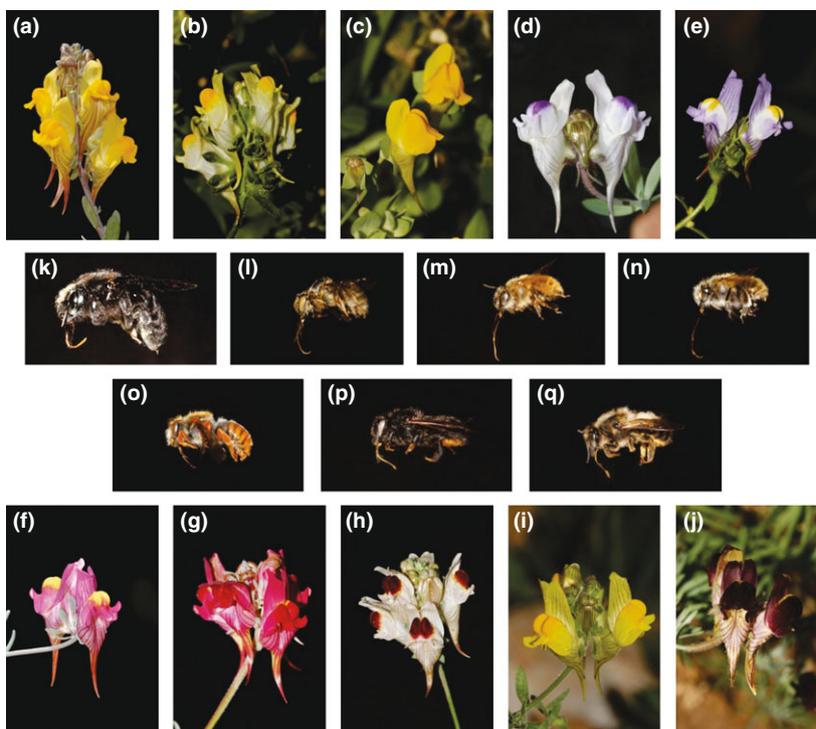


Fig. 1 Photographs of *Linaria* sect. *Supinae* subsect. *Supinae* species: (a) *L. polygalifolia*, (b) *L. verticillata*, (c) *L. platycalyx*, (d) *L. anticaria*, (e) *L. lilacina*, (f) *L. depauperata*, (g) *L. amoi*, (h) *L. almijarensis*, (i) *L. aeruginea* and (j) *L. tristis*, and their most frequent visitors: (k) *Xylocopa violacea*, (l) *Anthophora crassipes*, (m) *Anthophora plagiata*, (n) *Anthophora plumipes*, (o) *Rhodanthidium sticticum*, (p) *Chalicodoma parietina* and (q) *Chalicodoma pyrenaica*. Links among *Linaria* species and their predominant visitors: (a – k), (b – l), (c – m), (d, e – n), (f, g, h – o), (i – p), (j – q).

vegan (Oksanen *et al.*, 2007) of R software (<http://www.R-project.org>), input data available from the Dryad Digital Repository Pollinators were grouped by taxonomy (morphology). The rationale for this is that pollinator taxonomic groups differed in behaviour, fine-scale morphology (proboscis, head, thorax and abdomen structure) and size (see below), and these characteristics were presumed to have an effect on pollination accuracy and effectiveness. That is, taxonomic groups in the current system equal functional groups. This led us to hypothesize the association between pollinator taxonomic groups and fine-scale flower variation.

Determination of pollination niches

We have followed a recently proposed approach to identify pollination niches in our studied species using bipartite modularity, a complex-network metric (Gómez *et al.*, 2014,2015). We constructed a weighted bipartite network including the *Linaria* species with pollinator data. In this network, we pooled the data from the different populations per species and did not consider the time difference involved in sampling across different species. We subsequently determined the modularity level in this weighted bipartite network using the QuanBiMo algorithm (formerly QuaBiMo, Dormann & Strauss, 2014). This method uses a Simulated Annealing Monte Carlo approach to find the best division of populations into modules (niches). A maximum of 10^{10} Markov Chain Monte Carlo steps with a tolerance level = 10^{-10} were used in 100 iterations, retaining the iterations with the highest likelihood value as the optimal modular configuration. We tested 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 (Guimera & Amaral, 2005). Modularity significance was tested for each individual iteration by comparing the empirical vs. the random modularity indices by means of a z-score test (Dormann & Strauss, 2014). After testing the modularity of our network, we determined the number of modules using the approach proposed by Newman (2004). We subsequently identified the pollinator functional groups defining each module and the plant species that were ascribed to each module. Modularity analysis was performed using R package bipartite 2.0 (Dormann & Gruber, 2012).

Pollination niche evolution

To test whether pollination niches depend on the phylogenetic relatedness of the species and analyse the pattern of transition among niches in this group, we performed ancestral state reconstructions (ASRs) of pollination niches in subsect. *Supinae*. For that we used ITS and AGT1 sequences previously obtained in Blanco-

Pastor *et al.* (2013). Gene tree incongruence was found among the two loci (see Blanco-Pastor *et al.*, 2013); therefore, we performed independent ASRs in the two gene trees constructed with MrBayes 3.2 (Ronquist *et al.*, 2012). The unphased sequences of these two data sets were used. Additionally, we used the *BEAST species tree of Blanco-Pastor *et al.* (2013) that was constructed with phased sequences under the multispecies-coalescent model (Heled & Drummond, 2010), which accommodates phylogenetic incongruence assuming that it is exclusively caused by incomplete lineage sorting. Outgroup and *L. glacialis* terminals were pruned from the *BEAST tree because we failed to obtain pollinator data. To account for the topological uncertainty obtained in the Bayesian analyses of MrBayes and *BEAST, the reconstructions of pollination niches were conducted in 1000 trees from the stable posterior distribution of each analysis and summarized in the MrBayes 50% majority rule consensus trees and in the *BEAST maximum clade credibility tree.

We evaluated the phylogenetic signal of three pollination niches (see below), which were linked to three flower morphotypes as moderately to strongly supported by the DFA analysis (89.5%/84.6% of correct classification, see Results). Ancestral states were reconstructed under parsimony in MESQUITE 2.75 (Maddison & Maddison, 2011) using the 'trace character over trees' tool.

Flower and pollinator sizes

The complex corolla of *Linaria* can be separated into two major components: corolla tube and spur. We combined measurements of these two structures to characterize the inter- and intraspecific variability of the corolla size in subsect. *Supinae*. One flower was randomly chosen in 25–36 individuals per species across 1–3 flowering seasons in 1–2 populations or subpopulations per *Linaria* species (a total of 286 flowers). Corolla length was scored in scaled digital photographs. Specifically, spur length was measured from the corolla–calyx insertion to the spur tip (Fig. 2a) and tube length was measured from the corolla–calyx insertion to the stigma placed in the mouth of the tube (Fig. 2a).

The *scutum* (mid-point of mesothorax) of bees is the typical contact zone with the stamens and the stigma during nototribic pollination in *Linaria* (Kampny, 1995) (an effective mode of pollination in this group because it allows the access to nectar while not getting stuck inside the flower). Floral-visitor size was analysed as the distance between the extreme of the buccal apparatus and the *scutum*. For that, we combined two measures: (i) *scutum*-head and (ii) proboscis (Fig. 2b). The sum of both measures is hereafter called 'pollinator contact length' (PCL). Measures were taken from 11–21 individuals of the predominant flower visitors (see Table S1 and Table S3; data available from the Dryad

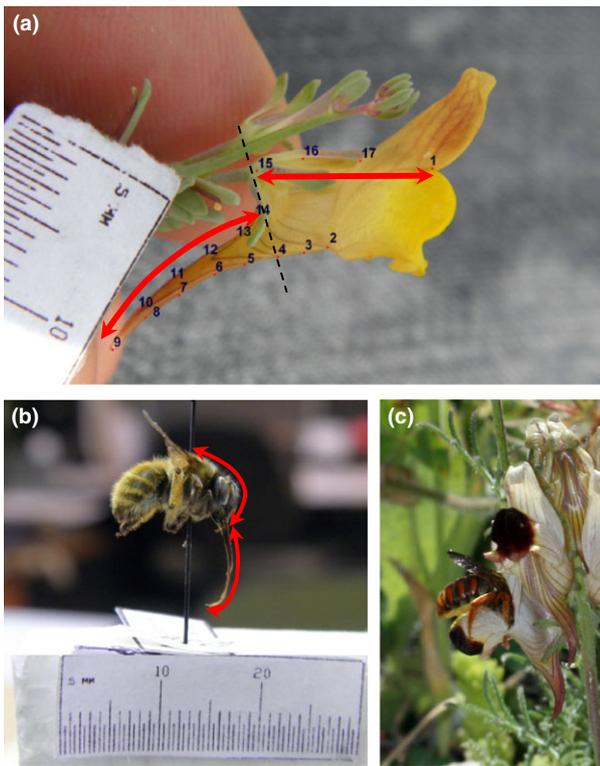


Fig. 2 Measures used in morphometric analyses: (a) metric measures (spur and corolla tube length), landmarks (1, 2, 9, 15) and semilandmarks (3–8, 10–14, 16–17) of flowers; (b) scutum-head and proboscis, (pollinator contact length (*PCL*)) of pollinators; and (c) example of a legitimate visit in a *Linaria* flower (*Rhodanthidium sticticum* visiting *L. almijarensis*).

Digital Repository) (a total of 161 individuals). Individuals were killed with ethyl acetate and dried at room temperature. Bees were rehydrated, and their proboscides manually extended to their maximum length prior to measures. Measures were taken in scaled digital photographs with the software IMAGEJ 1.44p (Abràmoff *et al.*, 2004).

Flower shape

Flower shape was studied by means of geometric morphometric tools, using a landmark-based methodology that eliminates the effect of variation in the location, orientation and scale of the specimens (Bookstein, 1997; Zelditch *et al.*, 2012). We took a digital photograph of one flower per individual in lateral view and planar position (286 flowers). We defined 4 coplanar landmarks and 13 semilandmarks located along the outline of the flowers which were considered satisfactory to define flower shape (Fig. 2a). We captured landmarks and semilandmarks using the software tpsDig 2.16 (Rohlf, 2010a). Landmarks were captured at points of evident homology across species (Zelditch *et al.*,

2012): placement of upper stamens (landmark 1), the hinge of the lower lip (landmark 2), the spur tip (landmark 9) and at the corolla–calyx insertion (landmark 15) (Fig. 2a). The semilandmarks were important for quantifying shape in the corolla that lack clear homologous points. These were placed by increments along the length of the curves typically equal but sometimes varying to reflect the complexity of the curve (Zelditch *et al.*, 2012). We used tpsRelw 1.49 (Rohlf, 2010b) to rotate, translate and scale landmark coordinates through generalized least-squares superimposition (Bookstein *et al.*, 1985; Bookstein, 1997). Semilandmarks were slid along the corolla contours to minimize bending energy. This was carried out because the spacing of the semilandmarks was defined extrinsically. Thus, sliding to minimize the bending energy of the deformation adjusts the spacing of the semilandmarks to minimize the implication of semilandmarks placement on the detection of shape changes (Zelditch *et al.*, 2012). The software tpsRelw calculated, for each individual, a series of shape variables: uniform components and *partial warps* scores (nonuniform components). To reduce dimensionality of the data, the principal components of the covariance matrix of the *partial warps* scores (*relative warps*, RW) were also obtained. We obtained $2p - 4$ ($=30$) orthogonal RWs (where p is the total number of landmarks and semilandmarks) that summarized shape differences among specimens (Zelditch *et al.*, 2012).

Statistical analyses

Size variation of flowers and bees defining each pollination niche was first compared by one-way ANOVA. To investigate the size matching between flowers and representative bees, we summarized their linear measures in box plot graphs and examined the degree of overlap. Additionally, the correlation between the mean *Linaria* corolla length and the mean *PCL* among species was explored using a Pearson's product-moment correlation test.

Corolla-shape differences were assessed by means of a discriminant function analysis (DFA) that used RW scores as shape variables. A first DFA was performed to analyse the ability to differentiate flower morphologies using *phylogenetic lineage* as the grouping variable (lineages with PP > 0.99 obtained in the ITS and AGT1 gene trees and the *BEAST species tree: (i) *L. polygalifolia*; (ii) *L. depauperata*; and (iii) the remaining species). A second DFA was performed to analyse the ability to differentiate flower morphologies using *pollination niches* as the grouping variable (obtained in the QuanBiMo modularity analysis). To compare both results, we first observed the values of Wilks' lambda in both analyses. Additionally, we compared the assignment of specimens to predefined groups (% of correct classification) using two calculations: one computed with the original data set and a cross-validation-based approach in

which the case that is being predicted is left out of the categorization process. The two discriminant functions of the DFAs (the number of functions is $g - 1$, where g is the number of categories in the grouping variable) were represented in a scatterplot, and the relative contribution of each variable (RW) in the discriminant function was explored. Statistical analyses were carried out in the SPSS 21.0 package (IBM Corp., Armonk, NY, USA).

Results

Diversification rates

We estimated the diversification rates of subsect. *Supinae* stem group to be 0.36–1.82 species per million years (sp/Ma) and 1.10–5.52 sp/Ma assuming high extinction and no extinction, respectively. Diversification rates for the crown group were estimated to be 0.48–2.52 sp/Ma and 1.16–6.09 sp/Ma assuming high extinction and no extinction, respectively. Overall, net diversification rate of subsect. *Supinae* ranged from 0.36 to 6.09 sp/Ma (Table 1). These estimates resembled those of the most rapid plant radiations reported to date (reviewed in Valente *et al.*, 2010; and Bell *et al.*, 2012; see also Breitkopf *et al.*, 2015).

Flower visitor surveys

A low number of insect species (2–7 species) visited flowers of *Linaria* species, and all were bees (except the hawk-moth *Macroglossum stellatarum* that visited *L. anticaria* flowers). Five *Linaria* species (*L. tristis*, *L. depauperata*, *L. amoi*, *L. almijarensis* and *L. aeruginea*) were highly visited by a Megachilidae bee, one species (*L. polygalifolia*) was almost exclusively visited by *Xylocopa violacea*, whereas three species (*L. verticillata*, *L. platycalyx* and *L. anticaria*) were mostly visited by *Anthophora* and small bees (see Fig. 3, Table 2 and Table S1). Sampling effort was considered adequate as all species' accumulation curves became nearly asymptotic at the last stages of the sampling (Fig. S1). The distribution of *Linaria* species and their main pollinators did not match geographically because most of the main pollinators were widespread (see Table S6) and covered ranges that included all *Linaria* species.

Survey of pollinator groups' availability revealed 25 cases of zero visitation frequency of a pollinator type

that was available in the *Linaria* population, whereas 17 cases of zero visitation frequency represented cases without availability of the pollinator type in the *Linaria* population (Table 2).

Pollination niches

The network between *Linaria* species and the pollinator functional groups was significantly modular (modularity = 0.484 ± 0.0004 , $N = 100$ iterations), as the empirical modularity values differed from random values in all replicates (z -scores = 47.14 ± 0.005 , all P -values < 0.0001, $N = 100$ iterations). Modularity analysis detected four modules in all the 100 iterations (Fig. 3, Table S4), indicating that this was the most stable configuration. The module identity of the species was consistent across iterations, as they were the same in all iterations. *L. polygalifolia* was grouped in a single module identified by *Xylocopa* visitation (*Xylocopa* niche), whereas *L. lilacina* conformed another single module identified by *Apis* and *Bombus* visitations (*Bombus-Apis* niche). There was a third module associated with high visitation rate by Megachilid bees that was formed by five *Linaria* species (Megachilidae niche), and a fourth module with three *Linaria* species associated with high visitation rate by *Anthophora* and small bees (*Anthophora*–small bees niche).

Pollination niche evolution

The ancestral state reconstruction (ASR) under parsimony performed in ITS and AGT1 gene trees recovered a Megachilidae pollination as ancestral to subsect. *Supinae* (Fig. S2A–B). The ASR performed in the *BEAST trees also recovered Megachilidae pollination niche as ancestral to subsect. *Supinae* (Fig. S2C). In all trees, *Xylocopa* and *Anthophora* niches (associated with more restrictive flowers) appeared independently from an ancestral Megachilidae niche (the *Anthophora* niche probably appeared independently more than once, see Fig. S2C).

Flower and pollinator sizes

The one-way ANOVA indicated significant size differences among groups of pollinators ($F = 339.426$, $P < 0.001$) and flowers ($F = 123.853$, $P < 0.001$). Flower and pollinator sizes were varied among species. Flowers

Table 1 Diversification rates of *Linaria* sect. *Supinae* subsect. *Supinae* based on median crown and stem ages obtained in the *BEAST analysis of Blanco-Pastor *et al.* (2013). Estimates based on 95% highest posterior density are indicated in brackets.

	Diversification rate (stem age, sp/Ma)		Diversification rate (crown age, sp/Ma)	
	$\varepsilon = 0.9$	$\varepsilon = 0$	$\varepsilon = 0.9$	$\varepsilon = 0$
<i>Linaria</i> sect. <i>Supinae</i> subsect. <i>Supinae</i>	0.67 (0.36–1.82)	2.04 (1.10–5.52)	0.97 (0.48–2.52)	2.34 (1.16–6.09)

ε , extinction rate as a fraction of speciation rate.

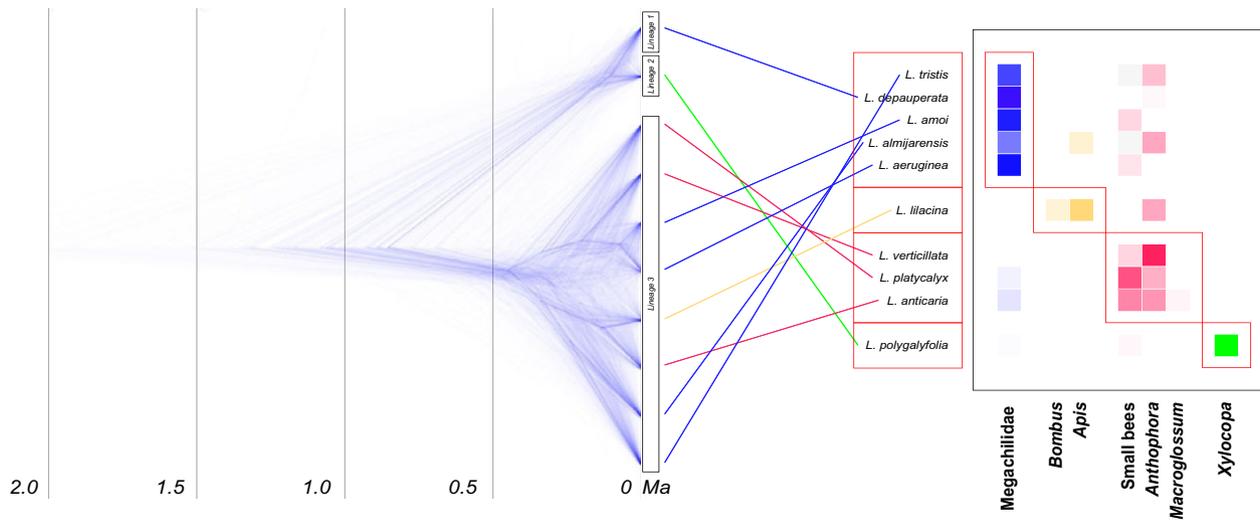


Fig. 3 Left: Posterior distribution of species trees superimposed in a consensus DensiTree plot. Right: Plot showing the average classification (100 replicates) of the study species into different pollinator modules (niches), according to the QuanBiMo analysis of bipartite modularity. The intensity of the colour indicates the relative visitation rate of each functional group per species.

Table 2 Visitation frequency data of pollinator taxonomic groups and their availability for the *Linaria* species.

Plant species	Small bees	<i>Apis</i>	<i>Bombus</i>	Macroglossum	Megachilidae	<i>Anthophora</i>	<i>Xylocopa</i>
<i>L. aeruginea</i>	0.102	0.000	0.000	0.000	0.898	0.000	0.000
<i>L. almijarensis</i>	0.005	0.170	0.000	0.000	0.489	0.332	0.000
<i>L. amoi</i>	0.154	0.000	–	0.000	0.846	–	–
<i>L. anticaria</i>	0.463	0.000	–	0.034	0.103	0.400	0.000
<i>L. depauperata</i>	–	0.000	–	–	0.914	0.086	0.000
<i>L. lilacina</i>	–	0.508	0.157	0.000	0.000	0.335	–
<i>L. platycalyx</i>	0.649	0.000	–	–	0.053	0.298	0.000
<i>L. polygalyfolia</i>	0.027	0.000	–	–	0.012	–	0.961
<i>L. tristis</i>	0.049	0.000	–	–	0.707	0.244	–
<i>L. verticillata</i>	0.159	0.000	0.000	0.000	0.000	0.841	0.000

–, Not available in the study populations.

(spur + tube) ranged from ca. 15 to ca. 35 mm, and pollinators (*PCLs*) ranged from ca. 10 to ca. 25 mm (Fig. S3, Table S2 and S3; data available from the Dryad Digital Repository *Linaria* flowers were larger than the *PCL* of the representative pollinators in all species. In all ten cases, there was no overlap between the interquartile ranges of the flower sizes and the *PCLs* of their representative pollinators (Fig. S3A). Mean values for flower length of *Linaria* species and *PCLs* of the representative pollinators were not significantly correlated ($r = 0.496$; $P = 0.144$). Interquartile ranges of *Xylocopa*, *Anthophora* and Megachilidae showed no overlap in the box plot graphs (Fig. S3B).

Flower shape differences

The DFA that used *phylogenetic lineage* as grouping variable (DFA-phy) revealed statistical significance for the separation of the three predefined *Linaria* groups

(Wilks' lambda of function 1 = 0.306, $P < 0.001$; Wilks' lambda of function 2 = 0.685, $P < 0.001$). The analysis showed that 88.1% of original grouped cases were correctly classified and 83.2% of cross-validated grouped cases were correctly classified (Fig. 4a, Table S5A). DFA-phy showed that individuals from *Lineage 1* and *Lineage 3* were the morphologically closest despite the sister-group relationship between *Lineage 1* and *Lineage 2*.

We performed a DFA analysis using as grouping values the four pollination niches obtained with the QuanBiMo algorithm (DFA-pol). In this analysis, 83.6% of original grouped cases were correctly classified and 75.5% of cross-validated grouped cases were correctly classified (76% and 56% for the *Bombus-Apis* group) (Wilks' lambda of function 1 = 0.136, $P < 0.001$; Wilks' lambda of function 2 = 0.354, $P < 0.001$; Wilks' lambda of function 3 = 0.769, $P < 0.001$). To compare the DFA-phy with the DFA using pollinator niches (DFA-

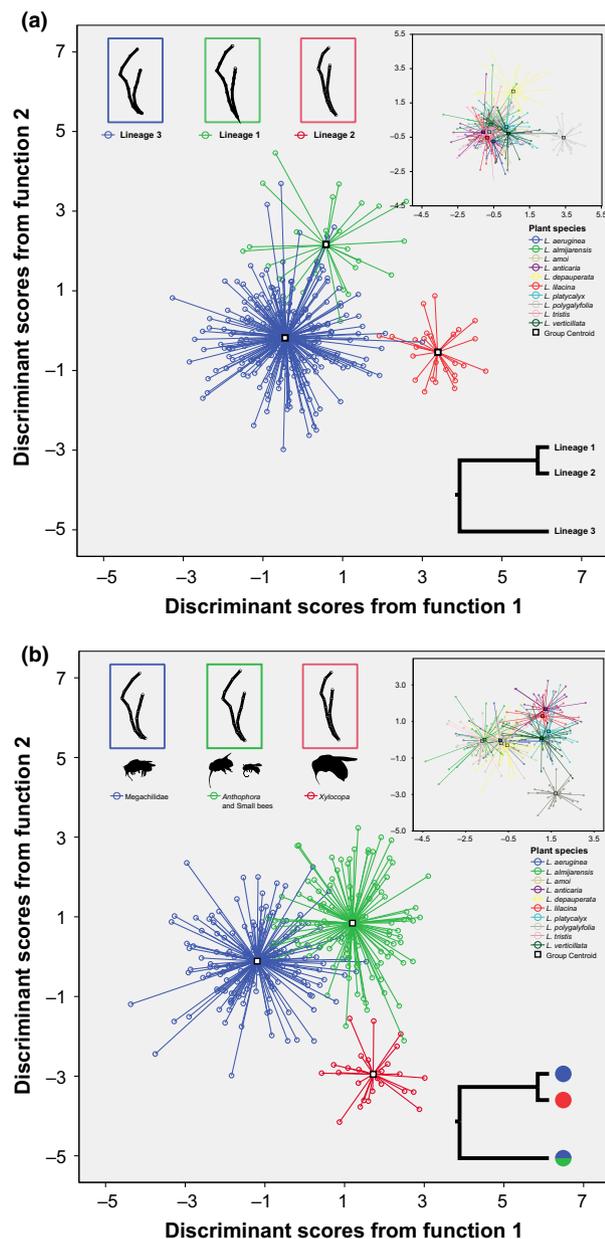


Fig. 4 Discriminant function analyses (DFA) of geometric morphometric variables (relative warps, RWs). (a) DFA using *phylogenetic lineage* as the grouping variable. (b) DFA using *pollination niche* as the grouping variable. Upper left inset: consensus flower shape of each group. Upper right inset: colours representing *Linaria* species (graphic representation of the species, DFA was not run with each species individually as grouping variables). Lower right inset: (a) simplified lineage tree, (b) simplified lineage tree and pollination niches ascribed to each lineage.

pol), it was required to have the same number of groupings in the two analyses. Therefore, we decided to include *L. lilacina* individuals in the *Anthophora*–small bees niche in a second DFA-pol analysis. This was car-

ried out for two reasons: (i) in the first DFA-pol analysis, we observed that *L. lilacina* individuals included in the *Bombus*–*Apis* niche showed strong morphological overlap with the species included in the *Anthophora*–small bees niche (Fig. S4), and (ii) in a study of fitness in *L. lilacina*, *Anthophora* bees showed higher effectiveness as pollinators when compared with *Bombus* and *Apis* bees, despite the higher visitation rate of the latter (Sánchez-Lafuente *et al.*, 2011). When the second DFA-pol was performed, we also observed statistical significance for the separation of the three predefined *Linaria* groups (Wilks' lambda of function 1 = 0.185, $P < 0.001$; Wilks' lambda of function 2 = 0.480, $P < 0.001$). In this case, the 89.5% of original grouped cases were correctly classified and 84.6% of cross-validated grouped cases were correctly classified (Fig. 4b, Table S5B). The second DFA-pol showed that *Linaria* individuals placed in the Megachilidae and *Anthophora*–small bees niches (*Lineage 1* and *Lineage 3*) were the morphologically closest.

Discussion

Restrictive flowers for specialized visitors

The role of nectar spurs in plant specialization and flower restriction has been addressed in many studies during the last two decades (Robertson & Wyatt, 1990; Hodges & Arnold, 1994, 1995; Hodges, 1997; Johnson & Steiner, 1997; Fulton & Hodges, 1999; Whittall & Hodges, 2007). The current study complements the existing literature on the association of spurred flower morphological changes with their representative pollinators. The approach taken here was novel, because flowers were considered as multidimensional traits and were defined by several shape variables (measured as RWs) instead of single variables such as spur length, which was typically measured in previous studies. The flower sizes of closely related *Linaria* species analysed here showed neither overlap nor correlation with the sizes of their representative pollinators (see Results and Fig. S3A). By contrast, analysis of the flower shape in a DFA gave consistent groupings associated with pollinator niches and high percentage of correct classification (84.6–89.5%) (Fig. 4b, Table S5B). This revealed a lack of significant correlation between pollinator sizes and flower sizes but association between pollinator groups and flower shape.

Despite the incongruence displayed in the gene trees and the uncertainty obtained in the species tree, it seems that phylogenetic constraints have not played a crucial role in shaping pollinator preferences in subsect. *Supinae*, as shown by the transition to similar pollination niches in independent lineages (Fig. S2A–C). This result is consistent with a recent study in sect. *Versicolores* (Fernández-Mazuecos *et al.*, 2013), in which flower types associated with divergent strategies of pol-

len placement in different types of insects (broad tubed vs. narrow tubed) exhibited low phylogenetic signal. All species analysed here displayed the typical broad-tubed morphology associated with nototribic pollination (pollen placement in the *scutum*) (Kampny, 1995), which is found in most *Linaria* species of other sections (Type I of sect. *Versicolores*, Fernández-Mazuecos *et al.*, 2013). Even though subsect. *Supinae* species displayed a similar morphology and were primarily visited by similar insects (long-tongued bees) performing the same pollination strategy, we found fine-scale differences in flower shape.

Particularly interesting was the subtle morphological change observed in subsect. *Supinae*. The main difference of flowers visited by *Xylocopa violacea* was their much larger size compared with that of flowers visited by other pollinators (Fig. S3). Because *Xylocopa* bees visited only one species (*L. polygalifolia*), we further discuss on the shape differences between Megachilidae-type and *Anthophora*-small bee-type flowers. The cone-shaped spur of the *Linaria* species studied here is wide open and easily penetrable at the upper part. Interestingly, the spur length is not a functional trait because it is not clearly associated with the restriction to nectar reward. Some spurs are more opened than others, and therefore, spurs with a similar length but different opening show evidence for differences in restriction of the access to nectar. Indeed, differences in spur shape associated with the spur narrowness displayed a restriction effect. The most restrictive flowers, which were characterized by narrow spurs, were found in populations pollinated primarily by *Anthophora* and other small bees (Fig. 4b). The DFA that used three pollination niches as grouping values showed the highest discriminant function coefficients in those morphological variables (RWs) associated with the spur narrowness (see Fig. S5B). Spur shape variation found here may be linked to restriction of the access to nectar reward to certain pollinators, and provide insights for particular cases of subtle floral specialization among spurred flowers that, as far as we know, has never been described. Reward accessibility can affect the attraction of pollinators (Hodges & Arnold, 1995; Armbruster & Muchhala, 2009). Therefore, a morphological change towards more restrictive spurs may have reduced visitation by other bees with shorter proboscides. Visits by extremely long-tongued *Anthophora* bees are more likely to result in effective pollen transfer (Sánchez-Lafuente *et al.*, 2011), which appear to have been a determinant force for spur shape change in this group of self-incompatible species (Blanco-Pastor & Vargas, 2013). We observed that there are plant species not being visited by the bees with the longest proboscis, even when they were available in the population (e.g. *Xylocopa* see Table 2). There are many aspects that can explain the absence of visits. General size of flowers or other plant characteristics such as

robustness of petioles may be key factors. Additionally, the community composition (that is, the accompanying pollinators and flowering plant species) or the availability of resources may additionally influence the behaviour and preferences of pollinators.

Have pollinators driven speciation in the *Linaria* radiation?

Although flower specialization may have played an important role in *Linaria* differentiation, it remains unclear whether flower shape shifts have promoted rapid speciation or whether other factors were also involved. Radiations in many plant groups have been historically thought to be a consequence of adaptive shifts between specialized pollination systems (Stebbins, 1970; Nilsson, 1988; Breitung *et al.*, 2015). Morphological differences in flower traits, such as nectar spurs, could theoretically promote floral isolation and speciation because their variation may reduce or enhance the effectiveness of pollen transfer by different types of pollinators (Nilsson, 1988; Robertson & Wyatt, 1990; Hodges & Arnold, 1995; Hodges, 1997; Johnson & Steiner, 1997; Fulton & Hodges, 1999). Specifically in *Linaria*, it has been recently observed that flower morphologies in sect. *Versicolores* conform two evolutionary optima associated with divergent strategies of pollen placement on pollinators, which may represent a mechanism of reproductive isolation and speciation (Fernández-Mazuecos *et al.*, 2013). Nevertheless, floral divergence is rarely sufficient to cause speciation on its own (Grant, 1949, 1981; Hodges & Arnold, 1994; Kay & Sargent, 2009; Valente *et al.*, 2012; Armbruster *et al.*, 2013). The narrow distributions of subsect. *Supinae* species are mountain and coastal ranges of southern Iberia, with seldom overlap; therefore, allopatric differentiation may have been also involved in the rapid differentiation of this *Linaria* group. In that sense, sympatric speciation (initial-RI model *sensu* Armbruster & Muchhala, 2009) does not appear to be sufficient to explain the species diversity found in subsect. *Supinae*. This is because phenotypic differences associated with pollination niches (Fig. 4b) were not consistent enough to exclude co-occurring bee groups (see Table 2, Fig. 3, Table S1), therefore allowing gene flow among incipient species. Despite the pollinators observed in our study system have widespread distributions (Table S6), the local abundance of each pollinator may vary geographically. According to this, episodes of habitat fragmentation during the Quaternary may have facilitated divergent selection in allopatry driven by geographical differences in pollinator fauna (Grant, 1949; Herrera *et al.*, 2006; Gómez *et al.*, 2008; Breitung *et al.*, 2015). This may have been the major factor that explains flower variation in sect. *Supinae*. Additionally, upon secondary contact, flower specialization may have favoured divergence among incipient species by means

of reinforcement of pre-existing partial reproductive isolation among these interfertile species (Reinforcement model) (Dobzhansky, 1937; Grant, 1949; van der Niet *et al.*, 2006; Armbruster & Muchhala, 2009). This particular model can give rise to increased speciation rates that could explain the elevated diversification rates observed in this group and other species radiations (Armbruster & Muchhala, 2009). A future test of the reinforcement hypothesis should consider several aspects: first, distribution models projected to past climates and/or bio(phylo)geographic analyses would be necessary to confirm allopatric speciation; second, crossing and germination experiments should be performed to confirm intermating ability; and third, additional pollinator surveys in contact zones would be required to confirm segregation of pollinators triggered by flower specialization.

Evolutionary reconstructions of recent radiations enable the linking of current pollinator fauna with morphological transitions as occurs in population-based approaches. Additionally, studies of recent radiations provide species relationships in a treelike manner, facilitating ancestral reconstructions using phylogenetic methods (Nunn, 2011). Nevertheless, we should be cautious with the interpretation of plant radiation phylogenetic reconstructions such as this of subsect. *Supinae*. First, a common limitation may be a low phylogenetic resolution (Fig 3; Fig S2C), which may be a consequence of low levels of genetic differentiation (see Valente *et al.*, 2010; Bell *et al.*, 2012; Breitkopf *et al.*, 2015). Second, reticulate processes affecting phylogenetic reconstructions such as hybridization or introgression (as previously evidenced in genus *Linaria*, Blanco-Pastor *et al.*, 2012) may also be recurrent in plant radiations (Seehausen, 2004), and could hinder more complex scenarios of morphological evolution. High-throughput genotyping (e.g. Baird *et al.*, 2008; Elshire *et al.*, 2011) and future development of analytical methods that account for the shared common ancestry and exchange of migrants between populations (Stone *et al.*, 2011) would greatly contribute to a better understanding of the evolutionary processes occurring in recent radiations such as *Linaria* subsect. *Supinae*.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Plot of cumulative species richness against sampling effort. Asymptotic curves indicate appropriate sampling effort in pollination censuses. It is also shown the complete pollinator censuses dataset used for constructing the accumulation curves.

Figure S2 Phylogenetic analyses and ancestral state reconstructions of pollination niches.

Figure S3 (A) Box plot graphs indicating linear mea-

tures of *Linaria* sect. *Supinae* subsect. *Supinae* species and their most abundant pollinators. (B) Box plot graphs representing linear measures of most frequent visitors grouped in three taxonomic groups.

Figure S4 Discriminant function analyses (DFA) of geometric morphometric variables (relative warps, RWs). DFA using pollination niche (pollination modules as obtained in the QuanBiMo analysis) as the grouping variable. Analysis including the *Bombus-Apis* module.

Figure S5 Standardized canonical discriminant function coefficients of the discriminant function analyses (DFAs).

Table S1 Summary table of pollinator censuses data.

Table S2 Morphometric data of *Linaria* specimens.

Table S3 Morphometric data of pollinator specimens.

Table S4 Outcome of the modularity analysis using QuanBiMo.

Table S5 (A) Predicted group membership obtained in the DFA analysis using phylogenetic lineage as grouping variable. (B) Predicted group membership obtained in the DFA analysis using pollinator module as grouping variable (*Bombus-Apis* module not included).

Table S6 Distribution range of pollinator species.

Data deposited at Dryad: doi:10.5061/dryad.p25m0

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