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Corolla morphology influences diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*)

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• *Background and Aims* The role of flower specialization in plant speciation and evolution remains controversial. In this study the evolution of flower traits restricting access to pollinators was analysed in the bifid toadflaxes (*Linaria* sect. *Versicolores*), a monophyletic group of ~30 species and subspecies with highly specialized corollas.
• *Methods* A time-calibrated phylogeny based on both nuclear and plastid DNA sequences was obtained using a coalescent-based method, and flower morphology was characterized by means of morphometric analyses. Directional trends in flower shape evolution and trait-dependent diversification rates were jointly analysed using recently developed methods, and morphological shifts were reconstructed along the phylogeny. Pollinator surveys were conducted for a representative sample of species.
• *Key Results* A restrictive character state (narrow corolla tube) was reconstructed in the most recent common ancestor of *Linaria* sect. *Versicolores*. After its early loss in the most species-rich clade, this character state has been convergently reacquired in multiple lineages of this clade in recent times, yet it seems to have exerted a negative influence on diversification rates. Comparative analyses and pollinator surveys suggest that the narrow- and broad-tubed flowers are evolutionary optima representing divergent strategies of pollen placement on nectar-feeding insects.
• *Conclusions* The results confirm that different forms of floral specialization can lead to dissimilar evolutionary success in terms of diversification. It is additionally suggested that opposing individual-level and species-level selection pressures may have driven the evolution of pollinator-restrictive traits in bifid toadflaxes.

Key words: Convergence, flower specialization, trait-dependent diversification, species selection, pollination, speciation, reversal, nectar spur, flower tube, toadflax, *Linaria* sect. *Versicolores*.

INTRODUCTION

Variation of flower morphological traits has long been considered to drive evolution and diversification of angiosperms (Darwin, 1862, 1877; Grant, 1949; Stebbins, 1970; Kay and Sargent, 2009; van der Niet and Johnson, 2012). Adaptation to different pollinator vectors (particularly animal pollinators) has been hypothesized to be a major force shaping flower morphology. This notion gave rise to the concept of pollination syndromes, i.e. sets of flower traits (shape, colour, nectar, scent) that have convergently evolved in distant plant lineages as an adaptation to particular pollinators (bees, birds, moths, etc.) (Faegri and van der Pijl, 1979; Fenster *et al.*, 2004). However, the concept of pollination syndromes, which relies on flower specialization, has been challenged in recent times (Waser *et al.*, 1996; Ollerton *et al.*, 2009). Specialization may still play a relevant role in plant speciation (Kay and Sargent, 2009), but syndrome shifts may not account for the majority of speciation events (e.g. Valente *et al.*, 2012). Therefore, rather than focusing on the evolution of syndromes, the investigation of particular traits, including their evolutionary trends, shifts and correlations, is probably more fruitful for understanding flower evolution in most plant lineages (Smith, 2010).

Traits that restrict the access of pollinators to flower rewards (nectar, pollen) are of exceptional interest because these physical barriers may have evolved as a specialization to particular pollinators. Variations in length and width of flower tubes and nectar spurs have been the subject of several studies (Herrera, 1990; Johnson and Steiner, 1997; Alexandersson and Johnson, 2002; Pérez *et al.*, 2004; Whittall and Hodges, 2007; Tripp and Manos, 2008). An extreme case of restriction of pollinator access is the personate corolla of snapdragons (*Antirrhinum*) and some relatives of the tribe Antirrhineae and the order Lamiales (Sutton, 1988; Endress, 1994; Kampny, 1995). These species display zygomorphic, gamopetalous, bilabiate corollas in which the lower lip is conspicuously arched upwards, constituting a palate. This structure closes access to pollen and nectar rewards, therefore making the mechanical opening of the corolla necessary for insect pollination. The personate corolla has long been considered as an adaptation to bee pollination (mellitophily), as insects other than bees would not be strong or heavy enough to open it (Hill, 1909; Müller, 1929; Sutton, 1988; Endress, 1992; Vargas *et al.*, 2010).

The relationships between changes in restrictive flower traits and diversification (speciation minus extinction) rates remain poorly understood. Nectar spurs have been hypothesized to

represent a key innovation that promotes species diversification by providing a mechanism of pre-zygotic reproductive isolation through differential pollinator visitation (Hodges and Arnold, 1995; Hodges, 1997; but see Hagen and Kadereit, 2003; Cacho *et al.*, 2010). On the other hand, it has been historically argued that ecological specialists usually evolve from generalists, and that specialization constitutes an evolutionary dead end, i.e. a derived state from which both reversal to a generalist state and shift to a different specialized state would be unlikely (Futuyma and Moreno, 1988). There are, however, many examples that contradict this idea (Gómez and Zamora, 2006). In particular, such a view has been challenged by phylogeny-based analyses of flower evolution (Armbruster and Baldwin, 1998; Tripp and Manos, 2008; Fleming *et al.*, 2009). Simultaneous estimations of rates of character change and state-dependent speciation/extinction rates across phylogenetic trees are crucial for a correct understanding of character evolution (Maddison, 2006; Goldberg and Igić, 2008). Recently developed methods (Maddison *et al.*, 2007; FitzJohn *et al.*, 2009; FitzJohn, 2010) enable such estimations and hold great promise for understanding flower evolution (Smith, 2010), yet they have rarely been applied in this context (but see Armbruster *et al.*, 2009; Smith *et al.*, 2010; Valente *et al.*, 2012).

Toadflaxes (*Linaria*) constitute the most species-rich (~150 species) genus of the snapdragon lineage (tribe Antirrhineae, Plantaginaceae) (Sutton, 1988). *Linaria* pollination has historically attracted the interest of botanists and evolutionary biologists (Sprengel, 1793; Darwin, 1876). Toadflaxes constitute a natural group (Vargas *et al.*, 2004; Fernández-Mazuecos *et al.*, 2013) and display a remarkably diverse array of flower traits whose evolution has not, however, been analysed in a phylogenetic framework to date. Several traits of *Linaria* flowers are potentially linked to pollinator specialization: they have a zygomorphic, bilabiate, usually personate corolla in which a spur of variable length is formed at the base of the lower lip (Fig. 1). The spur contains nectar dripping down from a nectary located at the base of the ovary (Valdés, 1970). The two pairs of anthers are placed at slightly different heights, with the stigma in the space between. While most species have well-developed palates that close access to the corolla throat, in some species belonging to sections *Versicolores*, *Macrocentrum* and *Lectoplectron* the palate is poorly developed, and access to the corolla throat is wide open (e.g. Fig. 1Q). This seems to be usually related to a narrowing of the corolla tube and a broadening of the lower lip (Viano, 1969; Sutton, 1980). Such morphology has been suggested to be related to pollination by long-tongued lepidopterans and dipterans (Hill, 1909; Sutton, 1980, 1988), while the typical personate corolla would be linked to bee pollination (Hill, 1909; Arnold, 1982; Sánchez-Lafuente, 2007; Carrió *et al.*, 2012).

Here we analysed the evolution of flower morphology in a clade of *Linaria* (sect. *Versicolores*) that displays remarkable flower diversity. In particular, we used phylogenetic and comparative methods to achieve the following objectives: (1) to get a deeper insight into the phylogenetic relationships within this lineage; (2) to evaluate intra- and inter-specific morphological variation of traits limiting pollinator access to nectar reward; (3) to analyse whether restrictive traits have exerted an effect on diversification rates; and (4) to reconstruct the evolutionary history of flower morphology and to investigate its potential links to pollinators.

MATERIALS AND METHODS

Study group and taxonomic treatment

Linaria sect. *Versicolores* (bifid toadflaxes) is an assemblage of ~25 species mainly distributed in the western Mediterranean region (Sutton, 1988) (see examples in Fig. 1). According to phylogenetic analyses based on both nuclear and plastid DNA markers (Fernández-Mazuecos and Vargas, 2011; Fernández-Mazuecos *et al.*, 2013) bifid toadflaxes constitute a monophyletic group within *Linaria*, formed by two well-supported sister groups: subsect. *Elegantes* (two species) and subsect. *Versicolores* (~23 species). All species are diploid ($2n = 12$) except for the tetraploid *L. hellenica* (reviewed by Sutton, 1988). Most species seem to be allogamous (Bruun, 1937; Valdés, 1970; Docherty, 1982; M. Fernández-Mazuecos, unpubl. res.). Section *Versicolores* is an ideal system for the evolutionary analysis of several flower traits that restrict the access of pollinators to nectar reward: spur length, tube width and palate development. Although morphological affinities among species have not been analysed in detail, some divergent traits have been described. At least the two species of subsect. *Elegantes* (*L. elegans* and *L. nigricans*) display a widely open corolla mouth and a narrow tube (Fig. 1P, Q), while species of subsect. *Versicolores* usually exhibit typical personate (closed) corollas with a wider tube (e.g. Fig. 1A, K). Some authors, however, have suggested that flowers of certain species of subsect. *Versicolores* (*L. incarnata*, *L. bipartita*; Fig. 1B, E) resemble those of subsect. *Elegantes* regarding their narrow tubes and broad lower lips (Viano, 1969; Sutton, 1988). In addition, sect. *Versicolores* exhibits a wide variation in spur length, including some of the shortest (*L. clementei*; Fig. 1C) and longest (*L. elegans*; Fig. 1P) spurs in the genus (Sutton, 1988; Sáez and Bernal, 2009). These traits seem to be associated with contrasting species diversities: only a few species display narrow tubes, and spurs as short as those of *L. clementei* seem to be rare. Nevertheless, inter- and intra-specific morphological variability has not been quantitatively assessed to date.

The potential effects of alpha taxonomy on diversification rate analyses have been pointed out by some authors (Marazzi and Sanderson, 2010; Valente *et al.*, 2010*b*). Indeed, correct species delimitation is crucial in obtaining accurate estimates of speciation and extinction rates. Therefore, we first conducted a review of the taxonomic literature (Viano, 1978*a*, *b*; Sutton, 1988; Dobignard, 1997; Fennane and Ibn Tattou, 1998; De Leonardis *et al.*, 1999; Tan and Iatrou, 2001; De Leonardis *et al.*, 2003; Gómiz, 2004; Hamdi *et al.*, 2009; Sáez and Bernal, 2009) and a survey of herbarium specimens mainly from two herbaria with a broad representation of *Linaria* sect. *Versicolores* specimens from Iberia (MA) and northern Africa (RNG) (see Supplementary Data Appendix S1). Although the first modern synthesis of the group is due to Viano (1978*a*, *b*), we generally adopted the more inclusive taxonomic treatment of Sutton (1988), except for some modifications detailed in Supplementary Data Appendix S2. In the end, we accepted 30 taxa (including species and subspecies; Table 1) that are morphologically and geographically cohesive.

Phylogenetic relationships and divergence times

Sampling strategy and DNA sequencing. We sampled a total of 45 specimens of *Linaria* sect. *Versicolores*, including

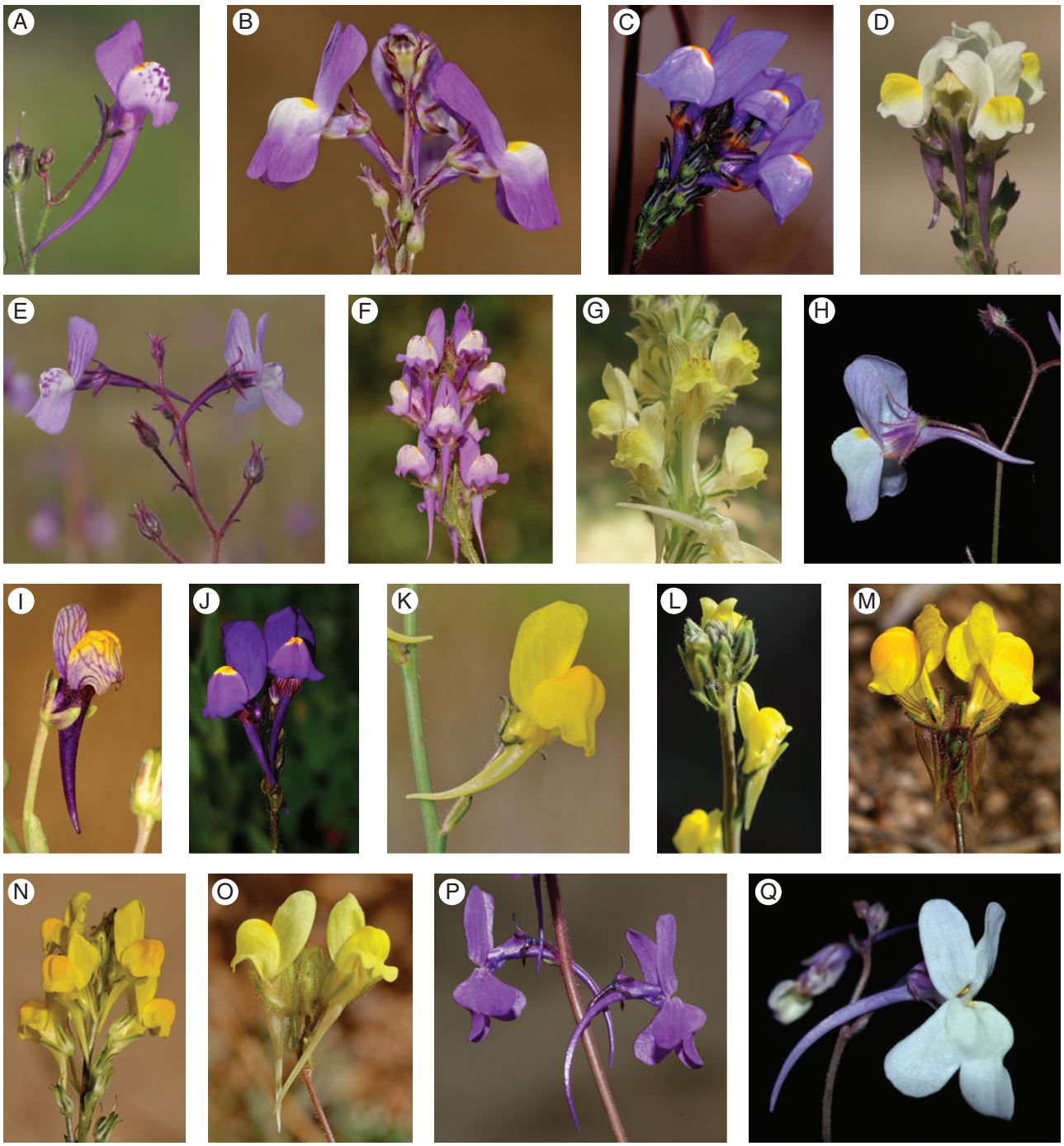


FIG. 1. Representatives of *Linaria* sect. *Versicolores*. Subsect. *Versicolores*: (A) *L. algarviana*; (B) *L. bipartita*; (C) *L. clementei*; (D) *L. gharbensis*; (E) *L. incarnata*; (F) *L. maroccana*; (G) *L. multicaulis* subsp. *heterophylla*; (H) *L. onubensis*; (I) *L. pedunculata*; (J) *L. salzmannii*; (K) *L. spartea*; (L) *L. tenuis*; (M) *L. viscosa* subsp. *spicata*; (N) *L. viscosa* subsp. *viscosa*; (O) *L. weilleri*. Subsect. *Elegantes*: (P) *L. elegans*; (Q) *L. nigricans*. Floral morphological types (see Fig. 4): Type I (broad tube, variable spur: A, D, F, G, I–O); Type II (broad tube, very short spur: C); Type III (narrow tube, variable spur: B, E, H, P, Q). Photographs by A. Fernández-Mazuecos (A, E), J. Quiles (B, F, K, N, O), J. Ramírez (C, I, J, M), J.L. Blanco-Pastor (D), E. Rico (G), P. Vargas (H, P, Q) and O. Fragman-Sapir (L).

representatives of 29 of the 30 recognized species and subspecies (one or two specimens per taxon; Table 1; Supplementary Data Table S1). To minimize the impact of recent hybridization, we selected unambiguously identified individuals, and some with intermediate traits or uncertain identification were discarded. We only failed to sample *L. dissita*, which is a poorly known northern African taxon (Sutton, 1988; Fennane and Ibn Tattou, 1998). We also sampled nine additional species representing six other sections of *Linaria*, one species of *Antirrhinum* and one of *Chaenorhinum* to be used as the outgroup based on previous phylogenetic evidence (Vargas *et al.*, 2004; Fernández-Mazuecos *et al.*, 2013). Plant material was collected in the

TABLE 1. *Taxonomic treatment followed in this paper, taxon distributions, individuals sampled for phylogenetic and morphometric analyses and flower morphological types*

Taxon	Distribution	No. individuals sampled for phylogenetic analyses	No. individuals sampled for spur and tube measures	No. individuals sampled for geometric morphometric analysis	Morphological type
***Linaria* Mill.**					
***Linaria* sect. *Versicolores* (Benth.) Wettst.**					
Subsect. *Versicolores*					
L. algarviana Chav.	SW Portugal (Algarve)	1	7	24	I
L. bipartita (Vent.) Willd.	W Morocco	2	46	—	III
L. bordiana Santa & Simonneau					
subsp. *bordiana*	Algeria	1	8	—	III
subsp. *kralikiana* (Maire) D. A. Sutton	NW Algeria	2	4	—	I
L. clementei Haens.	S Spain (Málaga)	2	22	21	II
L. dissita Pomel	NW Africa	—	6	—	I
L. gattefossei Maire & Weiller	C Morocco	1	6	—	I
L. gharbensis Batt. & Pit.	NW Africa, SW Spain	2	29	23	I
L. hellenica Turrill	S Greece	1	2	—	I
L. imzica Gómiz	S Morocco (Anti Atlas)	1	9	—	I
L. incarnata (Vent.) Spreng.	W Iberian Peninsula	2	36	48	III
L. mamorensis Mazuecos, Vigalondo & L. Sáez	NW Morocco	2	34	—	III
L. maroccana Hook.f.	Morocco (mainly High Atlas)	2	31	—	I
L. multicaulis (L.) Mill.					
subsp. *multicaulis*	Sicily, S Italy (Calabria)	1	4	—	I
subsp. *aurasiaca* (Pomel) D.A.Sutton	Tunisia, NE Algeria	1	3	—	I
subsp. *galioides* (Ball) D. A. Sutton	Morocco (High Atlas)	2	31	—	I
subsp. *heterophylla* (Desf.) D. A. Sutton	NW Africa	2	46	—	I
L. onubensis Pau	SW Spain (Huelva)	2	15	45	III
L. pedunculata (L.) Chaz.	S Iberian Peninsula, NW Africa, Balearic Islands	2	39	27	I
L. pinifolia (Poir.) Thell.	Tunisia, Algeria	1	5	—	I
L. pseudoviscosa Murb.	Tunisia	1	7	—	I
L. salzmannii Boiss.	S Spain (Málaga)	1	5	20	I
L. spartea (L.) Chaz.	Iberian Peninsula, S France	2	87	25	I
L. tenuis (Viv.) Spreng.	N Africa, Middle East	2	3	—	I
L. tingitana Boiss. & Reuter	NW Africa	1	12	—	I
L. viscosa (L.) Chaz.					
subsp. *viscosa*	S Iberian Peninsula	2	60	45	I
subsp. *spicata* (Kunze) D. A. Sutton	SE Iberian Peninsula	1	45	24	I
L. weilleri Emb. & Maire	S Morocco (Anti Atlas)	1	4	—	I
Subsect. *Elegantes* (Viano) D. A. Sutton					
L. elegans Cav.	NW Iberian Peninsula	2	58	24	III
L. nigricans Lange	SE Spain (Almería)	2	32	43	III

field and dried in silica gel or obtained from herbarium collections (RNG, MA, ATH, UPOS; Supplementary Data Table S1).

For phylogenetic analyses, we selected one nuclear (ITS) and three plastid (*rpl*32-*trn*L^{UAG}, *trn*K-*mat*K and *trn*S-*trn*G) DNA regions employed in our previous phylogenetic and phylogeographic analyses of the genus *Linaria* (Fernández-Mazuecos and Vargas, 2011; Blanco-Pastor *et al.*, 2012, 2013; Fernández-Mazuecos *et al.*, 2013; Fernández-Mazuecos and Vargas, 2013). One hundred and eighty-one sequences were taken from our previous studies, while the remaining 43 were newly generated. Procedures used for DNA extraction, amplification and sequencing of DNA regions followed Fernández-Mazuecos and Vargas (2011) and Fernández-Mazuecos *et al.* (2013). Sequences of each DNA region were separately aligned using MAFFT 6 (Katoh *et al.*, 2002) with default parameters, and further adjustments were made by visual inspection. The three ptDNA regions were concatenated in a single matrix after congruence was confirmed in preliminary phylogenetic analyses. All new

sequences have been deposited in the GenBank database (see Supplementary Data Table S1 for accession numbers).

Gene tree estimation and dating. Separate phylogenetic analyses were conducted on the ITS and ptDNA matrices using three methods: Bayesian inference (BI; implemented in MrBayes v3·1·2; Ronquist and Huelsenbeck, 2003); maximum likelihood (ML; implemented in RaxML; Stamatakis, 2006); and maximum parsimony (MP; implemented in TNT 1·1; Goloboff *et al.*, 2003) (see Supplementary Data Appendix S2 for details). Based on previous phylogenetic evidence (Vargas *et al.*, 2004), *Chaenorhinum* was used as the outgroup sequence in all analyses.

In order to obtain time-calibrated gene trees, separate ITS and ptDNA matrices including a single individual per species were analysed through the relaxed molecular clock approach implemented in BEAST 1·6·2 (Drummond *et al.*, 2006; Drummond and Rambaut, 2007). Following previous dating analyses of *Linaria* sect. *Versicolores* (Fernández-Mazuecos and Vargas, 2011, 2013), the root node (divergence between *Chaenorhinum* and *Linaria*) was calibrated using a normal distribution with mean 23 million years ago (Ma) and standard deviation 4 million years (Myr). This was based on a dating analysis of *ndh*F sequences of the tribe Antirrhineae (P. Vargas *et al.*, in prep.), which in turn incorporates a calibration of 74 Ma for the divergence time between Oleaceae and Antirrhineae (Bell *et al.*, 2010), and minimum stem-age constraints for Lamiales families and tribes based on five fossils (see Fernández-Mazuecos and Vargas, 2011 for details). Models of nucleotide substitution were selected for each DNA region under the Akaike information criterion (AIC) in jModelTest 0·1 (Posada, 2008). A birth–death process (Gernhard, 2008) was employed as tree prior. The substitution rate variation was modelled using an uncorrelated log-normal distribution. Based on previous estimates for herbaceous plants, uniform prior distributions were set for the substitution rates, with a range of 5×10^{-4} to 5×10^{-2} substitutions per site per Myr for ITS and 1×10^{-4} to 1×10^{-2} substitutions per site per Myr for ptDNA (see Blanco-Pastor *et al.*, 2012 for details). For each dataset, four Markov chain Monte Carlo (MCMC) analyses with 10 million generations each and a sample frequency of 1000 were run through the CIPRES Science Gateway (Miller *et al.*, 2010). Parameter analysis in Tracer 1·5 (Rambaut and Drummond, 2007) showed adequate chain length, with effective sample sizes above 1000. Chains were combined using LogCombiner 1·6·2 after discarding the first 10 % of sampled generations as burn-in. Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnotator 1·6·2 and visualized in FigTree 1·3·1.

Significant incongruence between loci prevented us from concatenating the ITS and ptDNA sequences for total-evidence phylogenetic and dating analyses (Kubatko and Degnan, 2007; Edwards, 2009). Instead, we implemented a species tree estimation analysis (see below).

Species tree estimation. Phylogenetic incongruence between loci is frequently found in plants, particularly in *Linaria*, due to incomplete lineage sorting and hybridization, among other causes (Blanco-Pastor *et al.*, 2012). While no standard method is currently available for the inference of phylogenetic relationships in the presence of these two processes, a number of coalescent-based methods have been recently proposed for the inference of species trees that account for incongruence between gene trees caused by incomplete lineage sorting (Liu, 2008; Heled and Drummond, 2010). Here we employed the ITS and ptDNA datasets including one or two individuals per taxon to estimate a species phylogeny of *Linaria* sect. *Versicolores* under the multispecies coalescent method *BEAST (Heled and Drummond, 2010), implemented in BEAST 1·6·2.

Haplotypic data are needed for coalescent-based analyses, which posed a challenge in the case of the multi-copy ITS region. Cloning of ITS copies was not considered due to the low quality of DNA extracts and PCR products obtained from herbarium material. Instead, in order to reconstruct haplotypes from the unphased ITS sequences, we employed the Bayesian statistical method PHASE 2·1 (Stephens *et al.*, 2001; Stephens and Donnelly, 2003), as implemented in DnaSP v5 (Librado and Rozas, 2009), with default parameters (recombination model MR0, 100 iterations, 100 burn-in iterations, thinning interval 1). A Bayesian phylogenetic analysis of the inferred haplotypes was conducted in MrBayes. Given that close (or unresolved) relationships between haplotypes of the same individual were recovered in all cases (Supplementary Data Fig. S1), the error introduced by potentially incorrect haplotype inference was considered negligible. Therefore, all ITS haplotypes inferred by PHASE were included in subsequent analyses following Blanco-Pastor *et al.* (2012).

Both datasets (ITS and ptDNA) were included as independent loci in the *BEAST analysis. The tree model and substitution model priors were set as indicated above for dating analyses. Based on results of separate dating analyses of ITS and ptDNA sequences (see above), the crown age of sect. *Versicolores* was calibrated using a normal prior with mean 6·07 Ma and standard deviation 1·85 Myr. Twenty MCMC analyses were run for 100 million generations each, with a sample frequency of 10 000. Analysis with Tracer 1·5 confirmed convergence and adequate sample sizes, with effective sample sizes above 250. Runs were combined using LogCombiner 1·6·2, after discarding the first 10 % of sampled generations as burn-in. Trees were summarized in an MCC tree obtained in TreeAnotator 1·6·2 and visualized in FigTree 1·3·1. In order to visualize the temporal dynamics of *Linaria* sect. *Versicolores* diversification, lineage-through-time (LTT) plots were generated in the R package *ape* (Paradis *et al.*, 2004) for the MCC species tree and a random sample of 1000 trees from the posterior distribution of the *BEAST analysis.

Analysis of corolla shape

Metric measures. In the personate corolla of *Linaria*, the main reward for pollinators (nectar) is located at the end of an abaxial spur of variable length (Fig. 1). Three main traits determine nectar accessibility to pollinators: spur length, tube width and palate development. Of these, spur length and tube width can be readily measured in herbarium specimens. In order to characterize the inter- and intra-specific variability of these traits in bifid toadflaxes, the two variables were scored for 696 herbarium specimens representing the 30 recognized species and subspecies of *Linaria* sect. *Versicolores* (Supplementary Data Appendix S1). Most specimens were provided by the MA, RNG and ATH herbaria. In addition, specimens from the MPU, RAB, FI, K, BM, LD and S herbaria were electronically surveyed through JSTOR Plant Science (Gallagher, 2010). A single, fully developed flower per specimen was measured.

Spur length was measured from the corolla–calyx insertion to the spur tip (Fig. 2A, C). Tube width was measured at the opening level (Fig. 2B, D). In addition, the same variables were measured for 377 living specimens collected from 18 Iberian populations of 12 representative species and subspecies sampled for geometric morphometric analyses (see below). All measurements were obtained from scaled digital photographs using ImageJ 1·44p (Abràmoff *et al.*, 2004).

Geometric morphometrics. Geometric morphometrics provides a powerful tool to assess intra- and inter-specific variation in flower morphology (Shipunov and Bateman, 2005; Gómez *et al.*, 2006; Abdelaziz *et al.*, 2011). We used a landmark-based geometric morphometric analysis to describe corolla shape, including palate development, in 18 populations belonging to 12 species and subspecies of *Linaria* sect. *Versicolores* from the Iberian Peninsula (Supplementary Data Table S2). These species were considered to represent the full range of corolla shapes of sect. *Versicolores* based on our metric measures of herbarium specimens of all 30 species and subspecies (see Results). A total of 369 living specimens (7–25 per population; Supplementary Data Table S2) were sampled, and digital photographs were taken of one completely developed flower per individual in lateral view and planar position. Nine landmarks (Fig. 2A, C) were defined at points of evident homology across species (Zelditch *et al.*, 2004). In addition, one pseudolandmark was defined at the mid-point of the spur. Landmarks were captured using tpsDig 2·16 (Rohlf, 2010). The two-dimensional coordinates of the landmarks were determined for each individual, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was calculated using the generalized Procrustes analysis (GPA) superimposition method (Rohlf and Slice, 1990; Slice, 2001). Corolla shape differences among species were assessed using a canonical variate analysis, a multivariate analysis that optimizes between-group differences relative to within-group variation (Albrecht, 1980; Klingenberg and Monteiro, 2005). It generates several canonical variate axes and computes between-group Procrustes distances in the canonical variate space. The analysis was performed with the software MorphoJ (Klingenberg, 2011). Values of canonical variates 1 and 2 for all individuals were plotted. The statistical significance of the between-groups Procrustes distances was determined by randomization tests using 10 000 permutations.

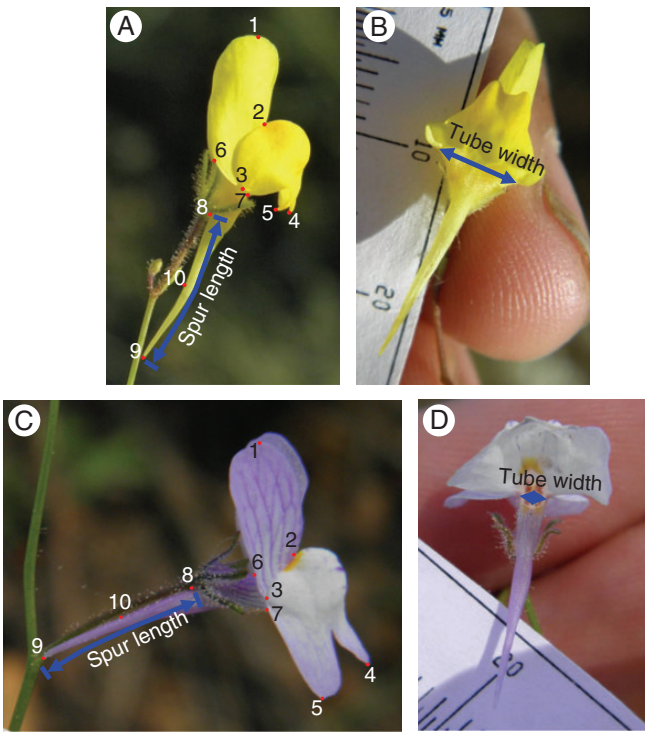


Fig. 2. Metric measures (spur length and tube width) and landmarks (1–10) employed in morphometric analyses, shown in two representative taxa of morphological Types I (*L. spartea*; A, B) and III (*L. onubensis*; C, D). Photos show lateral (A, C) and ventral (B, D) views. Notice the broad tube in B and the narrow tube in D.

Evolution of flower morphology

Effect of flower morphology on diversification rate. Morphometric analyses allowed the identification of three major floral morphological types (see Results). We estimated the effect of flower morphology on *Linaria* sect. *Versicolores* diversification using the binary-state speciation and extinction (BiSSE) model (Maddison *et al.*, 2007; FitzJohn *et al.*, 2009) implemented in the R package *diversitree* v.0·7–2 (FitzJohn, 2012). We defined two character states: (0) wide-tubed flower (Types I and II, see below); and (1) narrow-tubed flower (Type III) (Table 1). The fact that Type II was found in a single species (*L. clementei*) prevented us from using the multiple state speciation and extinction (MuSSE) method, which is an extension of BiSSE for more than two character states (FitzJohn, 2012). Instead, Type II was grouped with Type I based on their morphometric similarity (see below). All recognized taxa (species and subspecies) were included as independent entities in this analysis, based on the fact that subspecies belonging to the same species were usually not closely related in phylogenetic analyses (see below). The MCC species tree from the *BEAST analysis [with nodes with posterior probability (PP) $<0{\cdot}5$ collapsed] and 10 additional species trees randomly chosen from the Bayesian posterior distribution were analysed. For each tree, we compared a model with state-dependent speciation and extinction and asymmetrical transition rates against nested models with speciation, extinction and transition rate parameters constrained to be equal for both states. We calculated ML parameter values of the unconstrained model (full BiSSE model, six parameters) versus the constrained models (five parameters), and the significance of model differences was assessed by performing likelihood ratio tests. Parameter values of the full BiSSE model were additionally explored for the MCC tree and the ten additional trees in a two-step process using ML values as a prior for an MCMC sampling of parameters, a Bayesian approach (MCMC-BiSSE) that provides a measure of parameter uncertainty (FitzJohn *et al.*, 2009). The trees were analysed with 10 000 steps per tree (chain) and a prior for each parameter exponentially distributed (prioritizing small rates of change, in the absence of evidence to the contrary). After discarding the first 2000 steps of each chain as burn-in, parameter values for each tree were summarized and plotted.

Reconstruction of flower morphology shifts. Ancestral state reconstruction (ASR) of the two morphological types analysed in BiSSE was performed in *diversitree* using ML under the BiSSE model (ASR-BiSSE), thus accounting for differential

speciation, extinction and character transition rates in character optimization (Goldberg and Igić, 2008). To account for phylogenetic uncertainty, analyses were conducted separately for the MCC species tree and the same ten additional trees for which parameter values were estimated using MCMC-BiSSE. A higher number of trees was not analysed due to the high computational demands of this method. Given that the sister group to sect. *Versicolores* is currently unknown (Fernández-Mazuecos *et al.*, 2013), outgroup taxa, potentially biasing ASRs, were pruned from the analysed trees. Parameter distributions obtained in MCMC-BiSSE analyses were used to account for parameter uncertainty.

For comparison with the ASR-BiSSE approach described above, and to fully account for phylogenetic uncertainty, ASR was also performed using parsimony in Mesquite 2·75 (Maddison and Maddison, 2011). In this case, the three morphological types were included, thus treating Type II as a separate state. Reconstructions were conducted on the full set of species trees of the *BEAST posterior distribution using the 'trace character over trees' option. Additionally, the 'summarize state changes over trees' option was used to summarize the number of changes between character states across the Bayesian posterior distribution of trees.

Models of spur length and tube width evolution. We tested for the existence of one or more evolutionary optima for spur length and tube width using the ML method implemented in the R package *ouch* (Butler and King, 2004; King and Butler, 2009). Two models based on an Ornstein–Uhlenbeck process (Hansen, 1997) with one and two optima respectively were tested against a null Brownian motion model. Morphological Types I/II and III were defined as hypothetical selective regimes, and ancestral states were included based on the ASR-BiSSE reconstruction. Model comparisons were performed using AIC values. Analyses were conducted for the MCC species tree and the ten randomly sampled trees.

Pollinator observations

We performed flower visitor surveys in populations of the 12 Iberian species and subspecies of *Linaria* sect. *Versicolores*, which represent the full range of corolla shapes of the group. A total of 4618 min of observations (267–941 min per taxon) were performed in 2009, 2010, and 2011 in 14 populations that were also included in geometric morphometric and phylogenetic analyses. Visits were considered legitimate when the visitor touched the anthers and stigma. The placement of pollen on the insect body (thorax or proboscis) was recorded.

RESULTS

Phylogenetic relationships and divergence times

The ITS and ptDNA datasets had total aligned lengths of 610 and 2679 bp respectively (see characteristics of the four DNA regions in Table 2). Overall, the BI, ML and MP analyses yielded congruent topologies, except for some weakly supported clades. The separate phylogenetic analyses of nuclear ITS (Fig. 3A) and ptDNA (Fig. 3B) sequences consistently retrieved sect. *Versicolores* as a monophyletic group with strong statistical support [PP = 1; bootstrap support (BS) > 90 % in ML and MP analyses] and subsections *Elegantes* and *Versicolores* also as monophyletic (PP = 1; BS > 95 %) and sister to each other. However, topological incongruence between both datasets was extensive within subsect. *Versicolores*. In general, higher clade support values were obtained in ptDNA analyses.

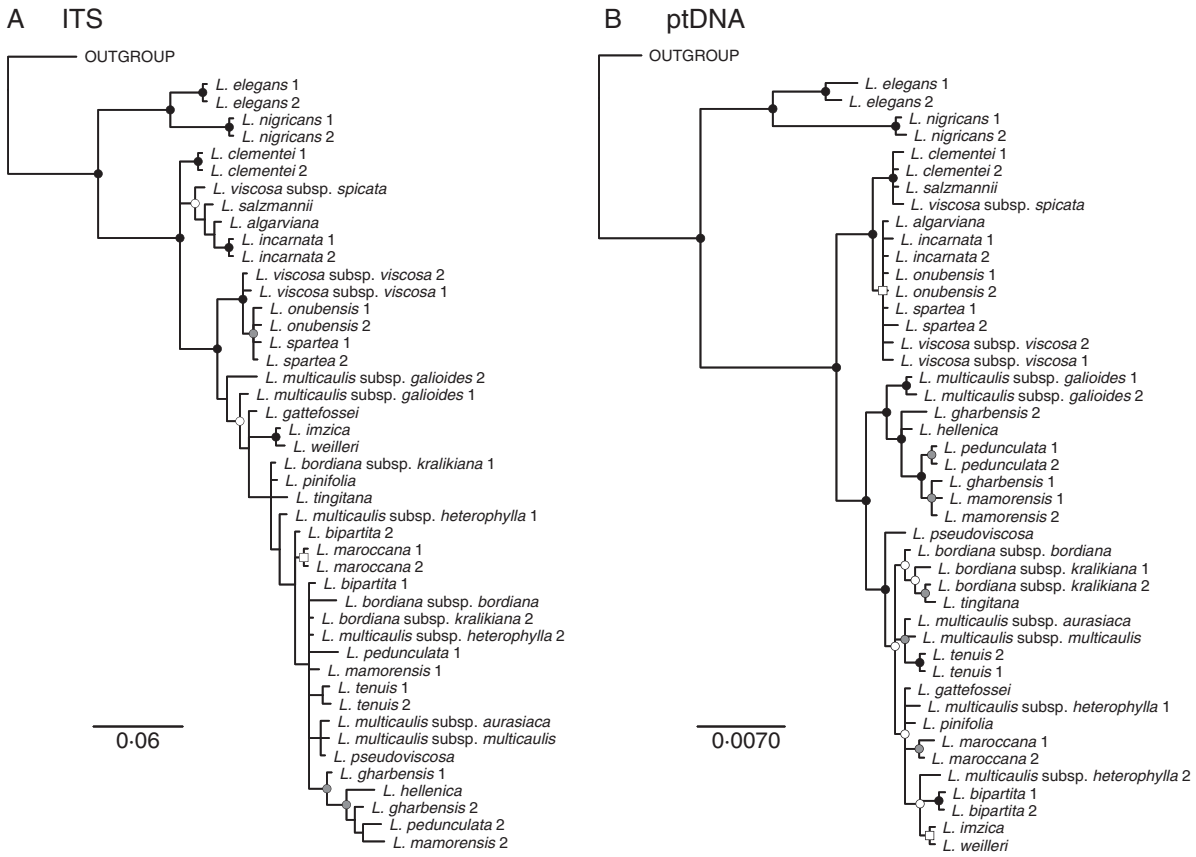
Separate dating analyses of the two loci (Supplementary Data Fig. S2) consistently recovered a crown age for sect. *Versicolores* around 6 Ma. This age was then used to calibrate the species tree analysis. The time-calibrated species tree obtained in *BEAST (Fig. 3C) strongly supported major clades of *Linaria* sect. *Versicolores*, but displayed lower resolution at shallow phylogenetic levels. Divergence between subsections *Elegantes* (PP = 1) and *Versicolores* (PP = 1) was dated back to the late Miocene or Pliocene. Two strongly supported sister clades were recognized within subsect. *Versicolores* (Fig. 3C): the 'Iberian clade' (PP = 1) included all species that are endemic or subendemic to the Iberian Peninsula, while the 'northern African clade' (PP = 1) included all northern African endemics, plus the Ibero-North African *L. pedunculata* and *L. gharbensis*, the southern Italian *L. multicaulis* subsp. *multicaulis* and the Greek *L. hellenica*. A Quaternary diversification of subsect. *Versicolores* was estimated, with the Iberian and northern African clades diverging 0·69–2·24 Ma and both clades diversifying in the last million years. The LTT plots (Fig. 3C) clearly depicted diversification of *Linaria* sect. *Versicolores* since the late Miocene–Pliocene, and primarily during the Quaternary.

Analysis of corolla shape

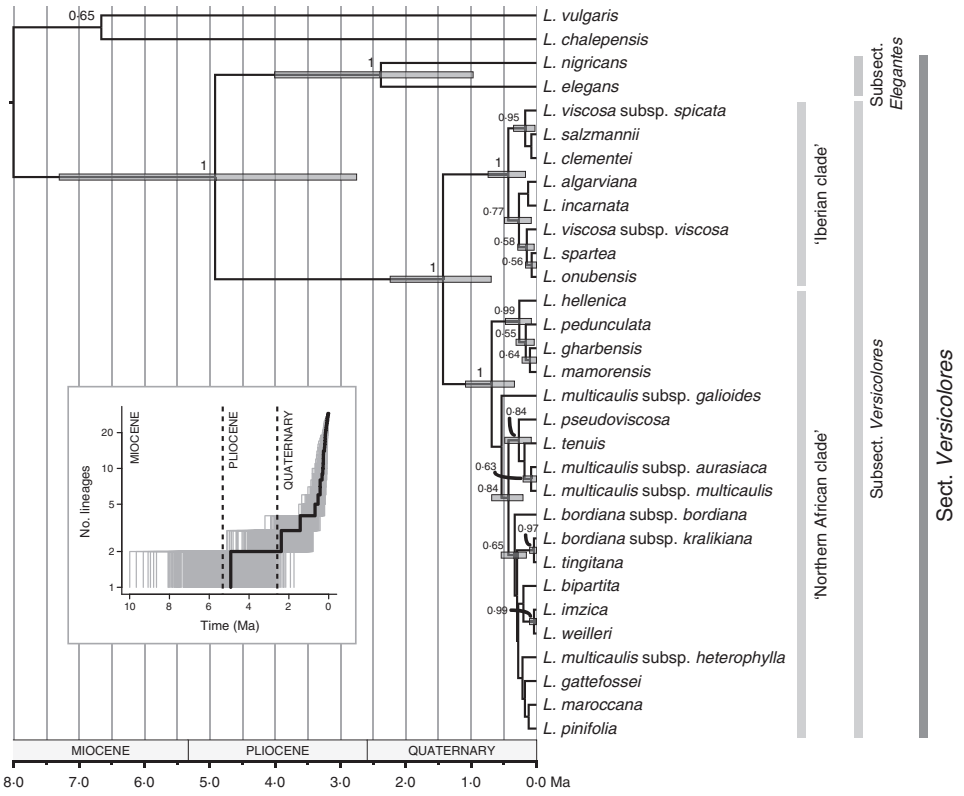
Measures of spur length and tube width from herbarium specimens of the 30 recognized species and subspecies of sect.

TABLE 2. *Characteristics of the four DNA regions sequenced for 45 individuals of* Linaria *sect.* Versicolores *and 11 outgroup taxa, and employed in phylogenetic analyses*

		Plastid DNA		
	Nuclear DNA, ITS (ITS1-5·8S-ITS2)	*rpl*32-*trn*L^{UAG}	*trn*K-*mat*K	*trn*S-*trn*G
Aligned length (bp)	610	830	1230	619
Ungapped length range (bp)	577–597	582–768	1206–1221	466–590
Pairwise identity (%)	90·7	94·0	98·2	92·7
Variable characters	199	211	186	139
Parsimony-informative characters	134	106	82	59
Mean G + C content (%)	56·1	23·6	32·3	27·4
Substitution model	GTR + G	GTR	GTR + G	HKI + I + G



C Species tree (ITS/ptDNA)



Versicolores (Fig. 4A; Supplementary Data Table S3) revealed three major morphological types. Type I was the most frequent (22 species and subspecies) and was characterized by a broad tube (>2 mm) and wide variation in spur length (4–18 mm). Type II was only found in *L. clementei*, and displayed a broad tube (4–7 mm) and a very short spur (1–4·5 mm). Type III, found in seven taxa, had a narrow tube (1–3 mm) and variable spur length (5–19 mm). The same three groups were consistently recovered when measuring living specimens of the 12 taxa present in the Iberian Peninsula (Supplementary Data Fig. S3).

The canonical variate analysis of landmark-based geometric morphometric data of these 12 Iberian taxa (Fig. 4B) recovered the same three morphological types. The first two canonical variates explained 83·6 % of the variance, and all between-taxa Procrustes distances were statistically significant ($P < 0{\cdot}001$) based on randomization tests. Variation along canonical variate 1 was related to palate development, since it affected the relative position and shape of the upper and lower lips (Fig. 4B; Supplementary Data Fig. S4A). Variation along canonical variate 2 was related to the shape of the spur and the relative sizes and positions of the upper and lower lips (Fig. 4B; Supplementary Data Fig. S4B). Taxa of Types I and II displayed well-developed palates, while Type III had broader and expanded lower lips. Type II (*L. clementei*) was clearly related to Type I, from which it was mainly differentiated by its short spur.

Evolution of flower morphology

An effect of flower morphology on diversification rates was supported by the likelihood ratio tests of BiSSE models (Table 3). In particular, we detected a significant effect on speciation rates, as the 'symmetric speciation' model was rejected for the MCC species tree and nine out of ten randomly chosen trees. Bayesian estimation of BiSSE parameters revealed significantly higher speciation rates for morphological Type I/II than for Type III, as shown by the non-overlapping 95 % credibility intervals obtained when analysing the MCC species tree (Fig. 5A) and the ten randomly chosen trees (Supplementary Data Fig. S5). No effect on extinction rates was detected (see the widely overlapping 95 % credibility intervals in Fig. 5A and Supplementary Data Fig. S5). Diversification (speciation minus extinction) rates were higher for Type I/II, although with certain overlap of the 95 % credibility intervals for three out of ten trees. No significant difference between transition rates was found.

Ancestral state reconstruction under state-dependent diversification (ASR-BiSSE) using the MCC species tree (Fig. 5B) recovered morphological Type III as ancestral to sect. *Versicolores* and subsect. *Elegantes*. Type I/II was inferred as ancestral to subsect. *Versicolores*, which implied an old shift from Type III to Type I/II. Four to five shifts from Type I/II to Type III were inferred within subsect. *Versicolores*, in both the Iberian and the northern African clade. Similar results were obtained when conducting ASR-BiSSE analysis on ten randomly chosen trees, although with variable ancestral state probabilities (Supplementary Data Fig. S6). Parsimony-based reconstructions yielded congruent results, although with equivocal reconstruction at the root node (Supplementary Data Fig. S7). Accordingly, zero to two shifts from Type III to Type I were estimated when accounting for topological uncertainty (Fig. 5C). Four to six shifts from Type I to Type III were estimated, and one shift from Type I to Type II was unequivocally reconstructed. No shifts were obtained from Type II to Types I and III, and from Type III to Type II (Fig. 5C).

Different models of trait evolution were supported for spur length and tube width in relation to the two main morphological types (Table 4). When analysing the MCC species tree, the Ornstein–Uhlenbeck model with one optimum at 8·98 mm was supported for spur length, while the Ornstein–Uhlenbeck model with two optima at 4·67 (Type I/II) and 1·86 mm (Type III) was preferred for tube width. Similar results were obtained for the ten additionally analysed trees (Table 4).

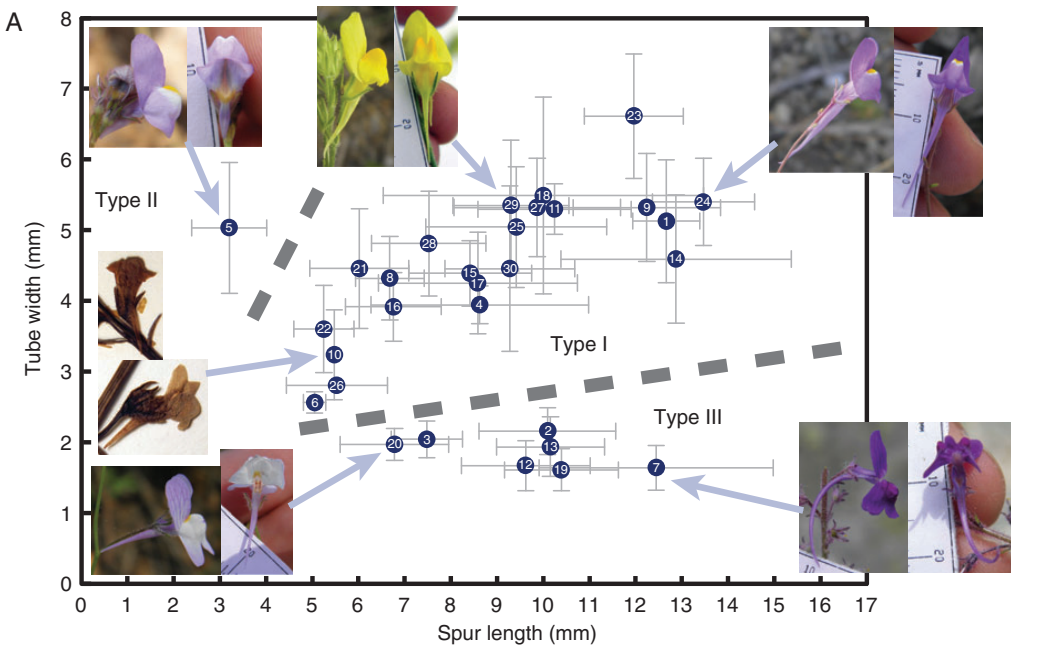
Pollinator observations

Observations of flower visitors in the 12 Iberian species and subspecies of *Linaria* sect. *Versicolores* (Table 5; Fig. 6) suggested that flowers of Types I and II are mainly pollinated by bees (Hymenoptera) carrying pollen on the back of the thorax (Fig. 6A), although sporadic visits by nectar-feeding butterflies (Lepidoptera; Fig. 6B) and bee flies (Bombyliidae, Diptera) carrying pollen on the proboscis were also recorded for three taxa: *L. spartea*, *L. viscosa* subsp. *viscosa* (Type I) and *L. clementei* (Type II). For the four Type III species, a wide variety of flower visitors were observed, most of them displaying a long proboscis: hawk moths (Sphingidae, Lepidoptera), *Anthophora*-like bees (Anthophorini, Apidae, Hymenoptera) and bee flies (Bombyliidae, Diptera). All of them carried pollen on the proboscis (Fig. 6C).

DISCUSSION

This study provides key insights into the evolution and diversification of bifid toadflaxes, with important consequences for understanding the relationship between floral specialization and species diversification. Species with narrow-tubed flowers were found to have evolved recurrently from broad-tubed ancestors, suggesting that similar selective pressures have driven flower evolution in independent lineages. However, the increasing pollinator specialization associated with narrower corolla tubes appears to have prevented narrow-tubed lineages from further diversification. Therefore, our results support a significant effect of floral specialization on the evolutionary success of flowering plant lineages.

FIG. 3. Phylogenetic analyses of *Linaria* sect. *Versicolores*. (A, B) Gene trees of ITS (A) and ptDNA (B) sequences. The 50 % majority rule consensus trees obtained in Bayesian analyses are shown. A black dot indicates clade support in BI (PP > 0·95), ML (ML – BS > 70 %) and MP (MP – BS > 70 %) analyses. A grey dot indicates support only in BI and ML analyses. A white dot indicates support only in the BI analysis. A white square indicates support only in the ML analysis. (C) Time-calibrated maximum clade credibility species tree obtained in the Bayesian *BEAST analysis based on ITS and ptDNA sequences. Node bars represent the 95 % highest posterior density intervals for the divergence time estimates. Numbers along the branches are Bayesian posterior probabilities. Major clades are named, including subsections following Sutton (1988). The inset shows a log-lineage-through-time plot for *Linaria* sect. *Versicolores*, based on 1000 trees randomly sampled from the posterior distribution of the *BEAST analysis. The thick line corresponds to the MCC species tree.



1 *L. algarviana*
2 *L. bipartita*
3 *L. bordiana* subsp. *bordiana*
4 *L. bordiana* subsp. *kralikiana*
5 *L. clementei*
6 *L. dissita*
7 *L. elegans*
8 *L. gattefossei*
9 *L. gharbensis*
10 *L. hellenica*
11 *L. imzica*
12 *L. incarnata*
13 *L. mamorensis*
14 *L. maroccana*
15 *L. multicaulis* subsp. *aurasiaca*
16 *L. multicaulis* subsp. *galioides*
17 *L. multicaulis* subsp. *heterophylla*
18 *L. multicaulis* subsp. *multicaulis*
19 *L. nigricans*
20 *L. onubensis*
21 *L. pedunculata*
22 *L. pinifolia*
23 *L. pseudoviscosa*
24 *L. salzmannii*
25 *L. spartea*
26 *L. tenuis*
27 *L. tingitana*
28 *L. viscosa* subsp. *spicata*
29 *L. viscosa* subsp. *viscosa*
30 *L. weilleri*

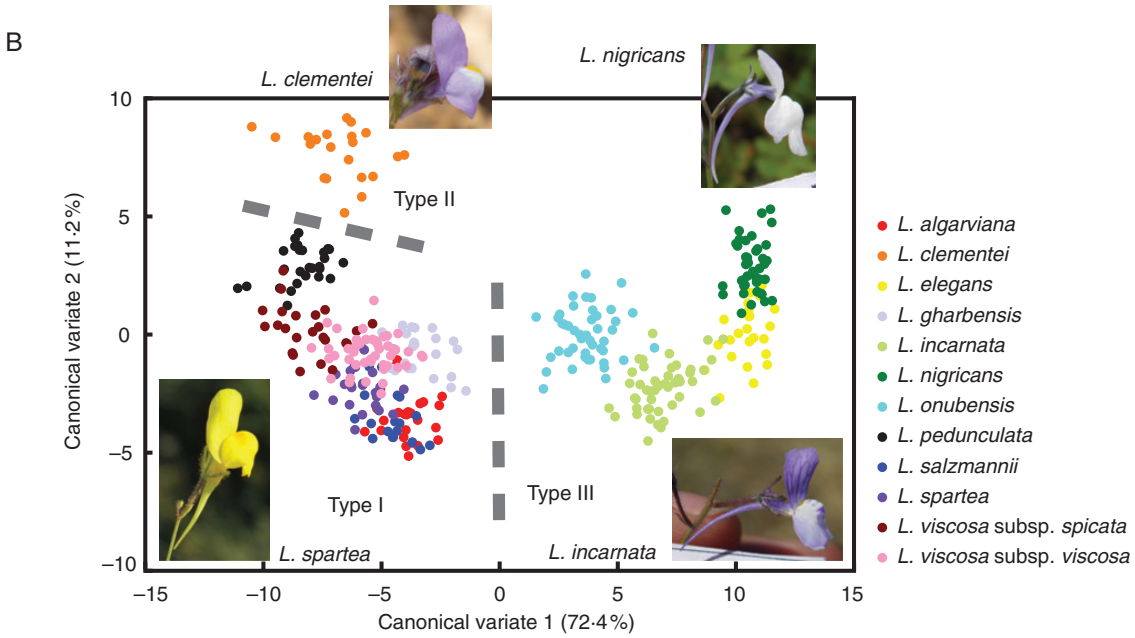


FIG. 4. Morphological traits of *Linaria* sect. *Versicolores* flowers. (A) Scatter plot of tube width versus spur length measured in 696 herbarium specimens representing the 30 species and subspecies of *Linaria* sect. *Versicolores*. Means (numbered dots) and standard deviations (bars) for each taxon are plotted. Notice the broad tubes of Types I and II, and the narrow tubes of Type III. (B) Canonical variate analysis of the landmark-based geometric morphometric dataset. Scatter plot of canonical variate 2 versus canonical variate 1 for 369 living specimens sampled in 18 populations belonging to the 12 Iberian species and subspecies (colours), which represent the full range of corolla shapes of *Linaria* sect. *Versicolores*. The variance explained by each axis is shown in brackets. In both plots, the three major morphological types discussed in the text are indicated, and representative taxa are shown.

TABLE 3. *Maximum likelihood estimates of BiSSE parameters and likelihood ratio tests of alternative models based on the maximum clade credibility tree of the *BEAST analysis*

	df	$\lambda_{I/II}$	λ_{III}	$\mu_{I/II}$	μ_{III}	$q_{I/II \to III}$	$q_{III \to I/II}$	lnL	AIC	χ^2	*P* value	No. trees with $P < 0.05$
Unconstrained model	6	3·75	2.83×10^{-6}	3·02	2·33	1·03	7.51×10^{-6}	−23·05	58·10	—	—	—
Symmetric speciation ($\lambda_{I/II} = \lambda_{III}$)	5	2·97	2·97	1·17	6·45	2·05	5.25×10^{-8}	−25·54	61·08	4·97	0·03*	9/10
Symmetric extinction ($\mu_{I/II} = \mu_{III}$)	5	3·53	1.93×10^{-9}	2·61	2·61	1·19	1.55×10^{-7}	−23·09	56·19	0·09	0·77 ns	0/10
Symmetric transition rate ($q_{I/II \to III} = q_{III \to I/II}$)	5	4·20	4.45×10^{-9}	3·87	1·77	0·88	0·88	−23·88	57·75	1·65	0·20 ns	0/10

The last column shows the number of trees (out of a random sample of ten trees from the posterior distribution of the *BEAST analysis) where each model was significantly worse ($P < 0.05$) than the unconstrained model according to a likelihood ratio test. Abbreviations and symbols: df, degrees of freedom; λ, speciation rates; μ, extinction rates; q, character transition rates; lnL, log likelihood; AIC, Akaike information criterion.

*$0.01 < P < 0.05$; ns, not significant.

Recent diversification of bifid toadflaxes

Our phylogenetic analyses based on nuclear and plastid sequences confirmed the monophyly of *Linaria* sect. *Versicolores* (Fernández-Mazuecos and Vargas, 2011; Fernández-Mazuecos *et al.*, 2013). Dating results indicate that diversification of bifid toadflaxes began in the late Miocene or Pliocene. Most inferred cladogenetic events occurred since the onset of the Mediterranean climate (~3·2 Ma; Suc, 1984) and particularly during the Quaternary (i.e. the last 2·6 Ma), as previously suggested based on plastid markers alone (Fernández-Mazuecos and Vargas, 2011; Fiz-Palacios and Valcárcel, 2013). While our species tree analysis provided strong support for several clades within sect. *Versicolores* (particularly the two subsections; Fig. 3C), fine-scale relationships between species were mostly unresolved or poorly supported. Low phylogenetic resolution is a common feature of recent radiations (e.g. Hughes and Eastwood, 2006; Scherson *et al.*, 2008; Valente *et al.*, 2010*a*). It is well known that rapid diversification brings about processes, such as hybridization and incomplete lineage sorting, that cause incongruence between gene trees and therefore may obscure phylogenetic relationships (Degnan and Rosenberg, 2009). This has recently been demonstrated for a different clade of toadflaxes, *Linaria* sect. *Supinae* (Blanco-Pastor *et al.*, 2012). Methods based on the multi-species coalescent (such as the one implemented in *BEAST), rather than concatenated analyses, currently constitute the best approach for the inference of species phylogenies in the presence of incongruent gene trees, because they account for incomplete lineage sorting (Edwards, 2009; Leaché and Rannala, 2011). We cannot rule out hybridization as a source of incongruence between gene trees (Fig. 3A, B). However, all phylogenetic comparative methods currently available are tree-based (Nunn, 2011). Incorporation of hybridization will strengthen phylogenetic inference (Yu *et al.*, 2011, 2012), and the development of comparative methods able to deal with reticulate phylogenies will probably lead to the reconstruction of more realistic evolutionary scenarios in the future. At present, however, the assumption of a tree-like phylogeny must be made in order to test evolutionary hypotheses using available tools. To account for uncertainty about phylogenetic relationships at shallow levels (part of which might be due to hybridization), we performed all comparative analyses on the consensus species tree and a random sample of ten species trees from the posterior distribution of the *BEAST analysis. Congruence of results across such sample was interpreted as evidence for a strong evolutionary signal in spite of phylogenetic uncertainty (Huelsenbeck *et al.*, 2000).

Convergence in the evolution of flower shape

Different biotic interactions affecting *Linaria* flowers have been studied previously, including floral herbivory, nectar robbery and insect pollination (Arnold, 1982; Stout *et al.*, 2000; Newman and Thomson, 2005*a*, *b*; Sánchez-Lafuente, 2007). The last is likely the most relevant factor affecting the evolution of morphological traits studied here (Sánchez-Lafuente, 2007). Our phylogenetic comparative analyses suggest that the evolution of flower morphology in bifid toadflaxes has been dominated by shifts between two morphological types mainly differentiated by the width of the tube and the development of the palate (Figs 4 and 5). It is suggested (Table 5; Fig. 6) that these two types constitute divergent strategies of pollen placement on nectar-feeding insects (Armbruster *et al.*, 1994; Grant, 1994; Kay, 2006; Yang *et al.*, 2007). These two strategies of *Linaria* pollination (Fig. 6) were first identified by Robertson (1888) and Hill (1909). One strategy corresponds to the typically nototribic pollination of broad-tubed species (Type I/II), in which pollen is deposited on the back of the thorax (scutum) of the nectar-feeding insect (Fig. 6A). This is the strategy found in most *Linaria* species of other sections, and has been demonstrated to result in effective pollination (Macior, 1967; Arnold, 1982; Stout *et al.*, 2000; Newman and Thomson, 2005*a*; Sánchez-Lafuente, 2007; Sánchez-Lafuente *et al.*, 2011). In sect. *Versicolores*, the placement of the first optimum inferred by the two-peak Ornstein–Uhlenbeck model (~4·67 mm; Table 4) suggests an adaptive adjustment to the thorax width of frequent pollinators. Indeed, closely related species pollinated by similar insects should be similarly selected for the floral phenotype that most efficiently uses these pollinators (Kay and Sargent, 2009). In our case, an adjustment of flower tube size to pollinator size probably maximizes pollen transfer in personate, wide-tubed corollas (but see Vargas *et al.* (2010) for *Antirrhinum*). The other strategy is displayed by narrow-tubed species (Type III), in which nectar-feeding insects usually carry pollen on the proboscis (Fig. 6C). In this situation, pollen transfer is maximized by narrowing the tube, so that contact of the proboscis with the anthers and the stigma is guaranteed when the insect reaches for nectar contained in the spur (Robertson, 1888; Kampny, 1995). This would lead to the

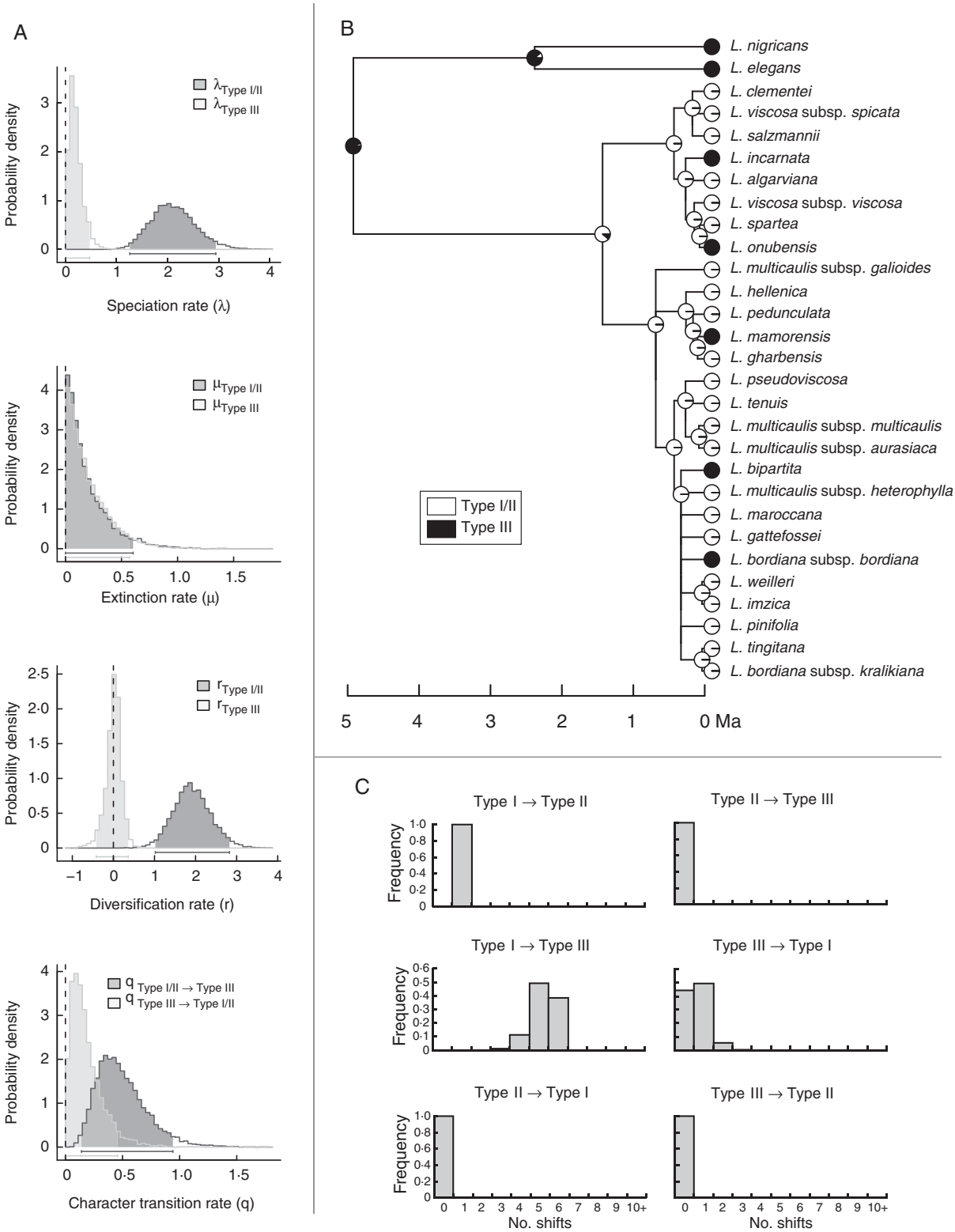


FIG. 5. Analyses of diversification rates and flower type transitions in *Linaria* sect. *Versicolores*. (A) Results of the binary-state speciation and extinction (BiSSE) analysis of the MCC species tree (Fig. 3C), considering two character states corresponding to morphological Types I/II (broad tube) and III (narrow tube). Posterior distributions of parameters (speciation rates, extinction rates, diversification rates and character transition rates) obtained in the MCMC-BiSSE analysis are shown. Horizontal bars indicate the 95 % credibility interval for each parameter. (B) Maximum likelihood ancestral state reconstruction of morphological Types I/II and III under state-dependent diversification (ASR-BiSSE), based on parameter estimates shown in A. The tree is the MCC species tree with nodes with PP $< 0{\cdot}5$ collapsed. (C) Summary distributions of the number of shifts between flower types inferred when implementing parsimony optimization over the full posterior distribution of trees obtained in the *BEAST analysis.

TABLE 4. *Testing of evolution models for spur length and tube width*

	lnL	AICc	σ^2	α	Optima (mm)	No. trees supporting the model
Spur length						
BM	−83·98	172·43	230·45	NA	NA	0/10
OU1*	−68·36	143·68	4891·41	374·84	8·98 (8·94–9·06)	10/10
OU2	−68·10	145·86	2253·02	175·54	8·77 (8·72–8·87) 9·61 (9·57–9·78)	0/10
Tube width						
BM	−62·33	129·12	51·76	NA	NA	0/0
OU1	−51·12	109·20	6301·93	1584·62	3·99 (3·81–4·00)	0/0
OU2*	−32·27	74·20	726·20	669·87	4·67 (4·66–4·68) 1·86 (1·83–1·86)	10/10

Three models were tested: Brownian motion (BM), Ornstein–Uhlenbeck process with one optimum (OU1) and Ornstein–Uhlenbeck process with two optima (OU2). Values for the MCC species tree obtained in the *BEAST analysis are shown. Optimum values for the MCC species tree and minimum and maximum values obtained for ten randomly chosen species trees from the Bayesian posterior distribution (in brackets) are indicated. The last column summarizes the number of trees of the same sample where each model was significantly supported based on corrected AICc values. Abbreviations and symbols: *preferred model; lnL, log likelihood; AICc, corrected Akaike information criterion; σ, α, model parameters.

TABLE 5. *Potential pollinators of Iberian species of* Linaria *sect.* Versicolores*, recorded after 4618 min of observations in 2009, 2010 and 2011*

Taxon	Pollinators
Subsect. *Versicolores*	
L. algarviana (I)	Hymenoptera: *Ceratina saundersi* (S) + Coleoptera: *Attagenus* sp. (S)
L. clementei (II)	Hymenoptera: *Amegilla quadrifasciata* (T), *Rhodanthidium sticticum* (T), *Bombus ruderatus* (T), *Xylocopa* sp. (T), *Heliophila bimaculata* (T) −, *Ceratina mocsaryi* (S) − Diptera: *Dischistus separatus* (P) Lepidoptera (P)
L. gharbensis (I)	Hymenoptera: *Anthophora plumipes* (T) +
L. incarnata (III)	Diptera: *Systoechus gradatus* (P), *Amictus variegatus* (P) Lepidoptera: *Thymelicus* spp. (P) Hymenoptera: *Lasioglossum* sp. (S), *Ceratina* sp. (S) −
L. onubensis (III)	Hymenoptera: *Eucera nigrilabris* (P) +
L. pedunculata (I)	Not seen (autogamous species)
L. salzmannii (I)	Hymenoptera: *Heliophila bimaculata* (T) +, *Rhodanthidium sticticum* (T), *Lasioglossum* sp. (S), *Hoplitis* sp. (S) −
L. spartea (I)	Hymenoptera: *Apis mellifera* (T), *Heliophila bimaculata* (T), *Ceratina cucurbitina* (S) −, *Lasioglossum* sp. (S) − Lepidoptera: *Euchloe crameri* (P) −
L. viscosa subsp. *viscosa* (I)	Hymenoptera: *Apis mellifera* (T) +, *Hoplitis* sp. (T), *Xylocopa uclesiensis* (T) −, *Heliophila bimaculata* (T) −, *Lasioglossum* sp. (S) − Lepidoptera: *Euchloe crameri* (P) −
L. viscosa subsp. *spicata* (I)	Hymenoptera: *Rhodanthidium sticticum* (T) +, *Osmia andrenoides* (T)
Subsect. *Elegantes*	
L. elegans (III)	Lepidoptera: *Macroglossum stellatarum* (P) +, *Lasiommata megera* (P) − Hymenoptera: *Anthophora retusa* (P) − Diptera: *Bombylius major* (P) −
L. nigricans (III)	Hymenoptera: *Eucera nigrilabris* (P) +, *Apis mellifera* (P) Lepidoptera: *Colias croceus* (P) −, *Pontia daplidice* (P) −

Observed strategies of pollen placement on insect body are coded as follows: (T) pollen placed on the back of the thorax; (P) pollen placed on the proboscis; (S) small insects with variable pollen placement. +, >50 % of flower visits; −, <5 % of flower visits. Morphological types are indicated in brackets after taxon names.

second optimum of tube width (∼1·86 mm; Table 4), which is large enough to fit the anthers (∼0·5 mm) but narrow enough to guarantee pollen transfer by long-proboscis visitors. Similar strategies of pollen placement on pollinators are frequently found in angiosperm lineages with spurred flowers, particularly those pollinated by long-proboscis insects (Herrera, 1993; Johnson and Steiner, 1997; Schiestl and Schlüter, 2009). In *Linaria*, the narrow-tubed strategy has been previously related to pollination by butterflies (Robertson, 1888; Hill, 1909) and moths (Sutton, 1988; Kampny, 1995). Our observations partially support these predictions (see *L. elegans* and *L. incarnata* in Table 5). Additionally, we have found the narrow-tube morphology to be also suited to other long-proboscis pollinators, such as long-tongued bees (mainly from tribe Anthophorini) and bee flies (Bombyliidae) (Table 5; see Supplementary Data Appendix S2 for further discussion on flower visitors).

Several independent origins of similarly configured narrow-tubed flowers have been inferred by ancestral state reconstructions (Fig. 5B, C and Supplementary Data Figs S6 and S7). Therefore, this is definitely a case of phenotypic homoplasy, i.e. convergence *sensu* Scotland (2011). In addition, reversal to an ancestral state (which can be regarded as a special case of convergence; Scotland, 2011) is suggested by the ancestral narrow-tubed flower inferred by the ASR-BiSSE analysis, together with its loss and subsequent repeated reappearance in subsect. *Versicolores* (Fig. 5B) (Hall, 2003; Porter and Crandall, 2003). The convergent evolution of narrow-tubed phenotypes in several different lineages of bifid toadflaxes may have involved similar genetic and developmental mechanisms (Hall, 2012), in which case it would be interpreted as an instance of parallel evolution *sensu* Scotland (2011) (note that the definitions of convergence and parallelism are controversial; see also Hall, 2003; Arendt and Reznick, 2008; Wake *et al.*, 2011; Hall, 2012). Homoplasy of morphological traits can result from common adaptive responses to similar selection pressures, coupled with genetic and developmental constraints (Wake, 1991; Brakefield, 2006; Wake *et al.*, 2011). While no information about the latter is yet available for the study group, pollinator observations suggest an adaptive meaning of the two morphological types, as they correspond to different strategies of pollen placement on pollinators. Indeed, the recurrent shifts

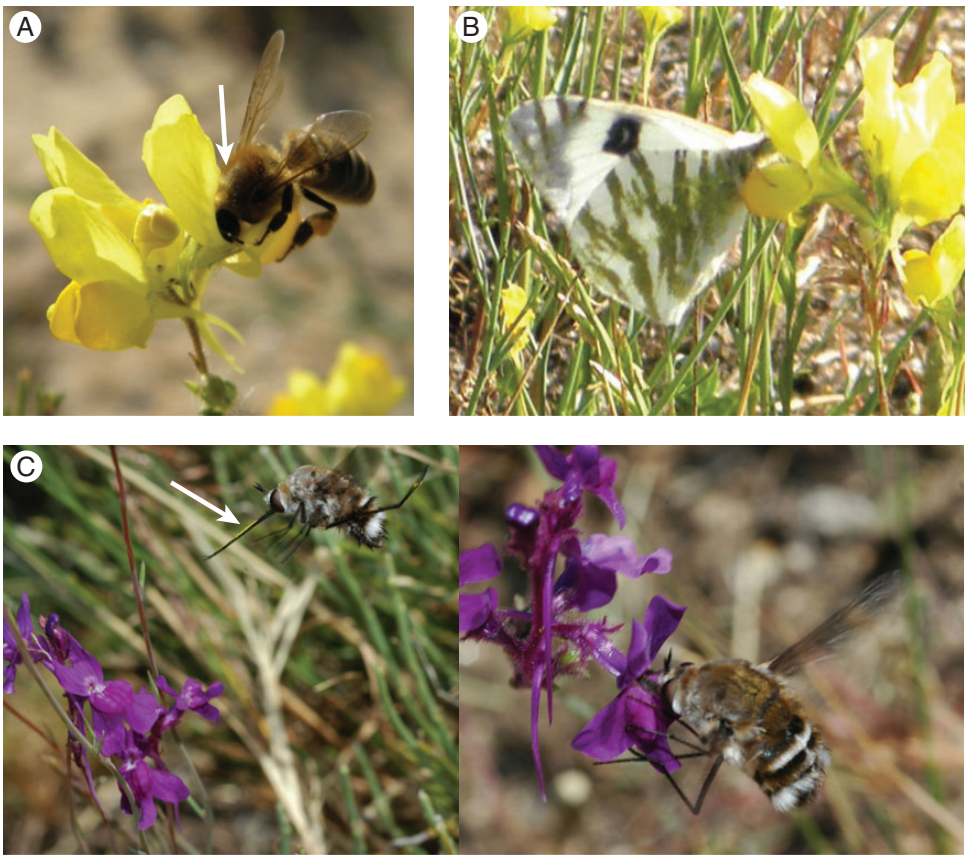


Fig. 6. Different behaviours of potential pollinators of morphological Types I (broad tube) and III (narrow tube). (A) *Apis mellifera* (Hymenoptera) in *L. viscosa* subsp. *viscosa* (Type I). (B) *Euchloe* sp. (Lepidoptera) in *L. viscosa* subsp. *viscosa*. (C) Bombyliidae (Diptera) in *L. elegans* (Type III). White arrows indicate pollen placement on pollinators. Photographs by M. Fernández-Mazuecos (A, B) and P. Vargas (C).

from broad-tubed flowers to narrow-tubed ones in subsect. *Versicolores* are not surprising given the fact that broad-tubed species are visited at a low rate by long-proboscis insects, including butterflies and even bee flies, which carry pollen on their proboscis (Table 5; Fig. 6B). Following the 'pollinator shift' hypothesis (Grant and Grant, 1965; Stebbins, 1970; Campbell, 2008), when a broad-tubed species is faced with an environment in which such long-proboscis insects are dominant, natural selection would favour a narrowing of the tube that would maximize pollen transfer, a mechanism similar to that previously invoked to explain spur elongation in American columbines (Whittall and Hodges, 2007). Additional research on reproductive and pollination biology at the population level will be needed to shed further light on the microevolutionary mechanisms (including selective pressures) involved in these putatively adaptive morphological shifts.

Trait dependent diversification?

Even though we have inferred a higher number of shifts from broad- to narrow-tubed flowers (4–6) than in the opposite direction (0–2) (Fig. 5B, C), a directional trend in the evolution of morphological types is not supported by BiSSE analyses (Fig. 5A and Supplementary Data Fig. S5). Instead, our analyses of trait-dependent diversification revealed a dissimilar evolutionary success of the broad- and narrow-tube strategies, as shown by the consistently higher speciation rate of broad-tubed species found in BiSSE analyses (Fig. 5A and Supplementary Data Fig. S5). Thus, despite having repeatedly evolved, the narrow-tubed strategy seems to display limited success in terms of speciation. In fact, radiation of subsect. *Versicolores* (23–28 species) may have been triggered not only by the onset of the Mediterranean climate (see above), but also by a shift from the ancestral narrow tube (as inferred by the ASR-BiSSE analysis; Fig. 5B) to the broad tube in the common ancestor of this clade. This is illustrated by the fact that the sister subsect. *Elegantes*, which maintained the ancestral state (narrow tube), yielded only two species during the same period of time and under similar climatic conditions (Fig. 3C; see Supplementary Data Appendix S2 for additional analyses and discussion).

BiSSE results suggest that trait-dependent diversification rates, rather than asymmetrical rates of change, are responsible for the contrasting species diversities of the two morphological types. Caution should be exercised in this interpretation, given the small size of our study group (Maddison *et al.*, 2007; Wertheim and Sanderson, 2011; Davis *et al.*, 2013). It is important to note that our analyses were able to reject the null hypothesis of symmetrical speciation rates (Table 3; Fig. 5A) despite the reported low statistical power of BiSSE with small datasets (Davis *et al.*, 2013). However, confounding effects of asymmetrical extinction rates and/or rates of change cannot be ruled out. In any case, the differential diversification success of morphological types is further suggested at the genus level (~150 species), as shown by the fact that additional *Linaria* clades

displaying narrow tubes are remarkably species-poor (sects *Macrocentrum* and *Lectoplectron*, six species) compared with broad-tubed clades (sects *Linaria*, *Speciosae*, *Supinae*, *Diffusae* and *Pelisserianae*, >120 species) (Sutton, 1988; Fernández-Mazuecos *et al.*, 2013).

Several flower traits have been found previously to influence diversification rates, including flower symmetry (Sargent, 2004), biotic/abiotic pollination mode (Dodd *et al.*, 1999) and the presence of nectar spurs (Hodges and Arnold, 1995; Hodges, 1997; but see Hagen and Kadereit, 2003; Cacho *et al.*, 2010). In general, it has been proposed that floral specialization promotes diversification (but see Smith *et al.*, 2008), although it has in turn been suggested that high species diversity may promote floral specialization (Armbruster and Muchhala, 2009). Our results suggest that traits restricting pollinator access to rewards and pollen placement on pollinators have significant effects on diversification rates of *Linaria* sect. *Versicolores*. Mechanisms of species selection (Stanley, 1975; Jablonski, 2008; FitzJohn, 2010; Rabosky and McCune, 2010) causing such differences in trait-dependent 'emergent fitness' (i.e. heritable differences in net diversification rates) are different from those involved in selection at the individual level (Rabosky and McCune, 2010). Indeed, the narrow-tube strategy in bifid toadflaxes has recurrently evolved by means of individual-level selection mechanisms, yet it may have exerted a negative influence on diversification rates, thus leading to the low frequency of this character state (Fig. 5). Specific mechanisms of species selection acting in bifid toadflaxes may include differential opportunities for exploitation of pollinator fauna (e.g. higher diversity of pollinators carrying pollen on the thorax than on the proboscis) and differential extinction risks (e.g. due to the likely higher specialization of narrow-tubed species; Johnson and Steiner, 2000). Even though our analyses have detected significant effects on speciation rather than extinction rates, the latter cannot be ruled out given the reported limitations of phylogeny-based methods to detect variation in extinction rates (FitzJohn, 2010; Rabosky, 2010). Further research will be needed to understand the mechanisms that account for the effects of pollinator-restrictive traits on diversification rates of *Linaria* and other genera with highly specialized corollas.

Our results support a relationship between resource specialization and evolutionary success in terms of diversification. The intriguing finding that opposing individual-level and species-level selection pressures may drive the evolution of specialized traits is worth further investigation in additional model systems.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Appendix S1: Herbarium specimens studied for taxonomic delimitation and measurement of flower traits. Appendix S2: Additional methods, results and discussion. Fig. S1: Bayesian phylogenetic analysis of ITS haplotypes inferred by PHASE. Fig. S2: Maximum clade credibility trees produced by relaxed molecular-clock analyses of ITS and ptDNA sequences in BEAST. Fig. S3: Scatter plot of tube width versus spur length measured from living specimens of the 12 Iberian species and subspecies of *Linaria* sect. *Versicolores*. Fig. S4: Visualization of landmark displacements along canonical variates 1 and 2 of the geometric morphometric analysis. Fig. S5: Results of the BiSSE analysis of ten species trees randomly chosen from the posterior distribution of the *BEAST analysis. Fig. S6: Ancestral state reconstructions of morphological Types I/II and III under state-dependent diversification, performed on ten trees randomly chosen from the posterior distribution of the *BEAST analysis. Fig. S7: Summary of parsimony-based ancestral state reconstruction of the three major flower types of *Linaria* sect. *Versicolores*, conducted over the full posterior distribution of trees obtained in the *BEAST analysis. Table S1: Voucher specimens and GenBank accession numbers of species and subspecies of *Linaria* sect. *Versicolores* and the outgroup sampled for DNA sequencing. Table S2: Voucher specimens of Iberian species and subspecies of *Linaria* sect. *Versicolores* and the outgroup sampled for geometric morphometric analyses. Table S3: Measures of spur length and tube width obtained from herbarium specimens of the 30 species and subspecies of *Linaria* sect. *Versicolores*.

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