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Evolutionary transitions of style polymorphisms in *Lithodora* (Boraginaceae)

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Abstract

Floral polymorphisms provide suitable model systems to test hypotheses concerning the evolution of outbreeding in plants. Although heterostyly has evolved in more than 28 angiosperm families, the evolutionary pathways involving related floral conditions have not yet been fully resolved. In this study, the reconstruction of ancestral states of style polymorphism, with both parsimony and maximum likelihood methods, was carried out for Boraginaceae species in the tribe Lithospermeae, particularly in the genus *Lithodora* sensu lato, where species present a wide variety of stylar conditions. Detailed floral morphometric analysis confirm different types of style polymorphism within *Lithodora*. They also reveal a novel style polymorphism (relaxed style dimorphism) in which anther height is variable within a flower (each anther being at a different height), which contrasts to regular distyly (constant anther height within flowers). Style monomorphism is likely to be the ancestral condition in Lithospermeae where the evolution of distyly has occurred several times. Style dimorphism is probably ancestral to distyly, as predicted by certain evolutionary models proposed for heterostyly. However, a reversion from distyly to style dimorphism also appears to occur in this tribe. This is the first documented occurrence of such a transition. This secondary style dimorphism is of the relaxed type and demonstrates the labile nature of floral polymorphisms, which are not necessarily a transition towards heterostyly. We discuss the selective forces involved in the evolution, maintainance and loss of style polymorphisms.

Keywords: Distyly; Heterostyly; ITS; Style dimorphism; *trnK-matK*; $trnL_{UAA}$ intron; *trnL-F*

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Introduction

Heterostyly is a floral polymorphism in which plant populations are composed of two (distyly) or three (tristyly) floral morphs that differ reciprocally in the heights of stigmas and anthers (Ganders, 1979a). Understanding the mechanisms underlying the evolution of this floral polymorphism has challenged evolutionary biologists ever since Darwin's (1877) seminal work on this topic (Barrett, 2002). We now know that heterostyly has evolved in at least 28 angiosperm families (Barrett et al., 2000) and several hypotheses have been proposed to explain its origin and maintenance (Baker, 1966; Charlesworth and Charlesworth, 1979; Ganders, 1979a; Lloyd and Webb, 1992a, b; Richards, 1998; Sakai and Toquenaga, 2004).

Charlesworth and Charlesworth (1979) and Lloyd and Webb (1992a, b) made precise predictions concerning ancestral states, conditions promoting the spread of advantageous style and stamen length mutants and pathways to heterostyly. The model of Lloyd and Webb (1992a, b) has gained increasing support from diverse studies of flower ontogeny (Faivre, 2000), pollination ecology (Stone and Thomson, 1994; Nishihiro et al., 2000; Lau and Bosque, 2003; Pérez-Barrales et al., 2006), mating parameters (Baker et al., 2000a, b; Thompson et al., 2003; Cesaro and Thompson, 2004), and phylogenetics (Kohn et al., 1996; Graham and Barrett, 2004; Mast et al., 2006; Pérez et al., 2004; Pérez-Barrales et al., 2006).

Currently, the most comprehensive phylogenetic studies of heterostylous taxa are those in Pontederiaceae (Kohn et al., 1996), *Amsinckia* (Schoen et al., 1997), *Primula* (Mast et al., 2004, 2006), and *Narcissus* (Graham and Barrett, 2004; Pérez et al., 2004; Barrett and Harder, 2005; Pérez-Barrales et al., 2006). Only *Narcissus* has provided the opportunity to test the pathway proposed by Lloyd and Webb (1992a) because of the persistent presence of style dimorphism as ancestral to distyly, and the lack of heteromorphic incompatibility. Style polymorphisms are also known to exist in Boragineae (*Anchusa*: Dulberger, 1970; Philipp and Schou, 1981; Schou and Philipp, 1983, 1984; Selvi and Bigazzi, 2003; and *Pulmonaria*: Olesen, 1979; Richards and Mitchell, 1990; Brys et al., 2008a,b), Lithospermeae (*Lithospermum* sensu stricto: Johnston, 1952; Ganders, 1979b; Ralston, 1993, *Lithodora* sensu lato: Johnston 1953b, Valdés 1981, and *Arnebia:* Johnston, 1952, 1954b) and Eritricheae (*Amsinckia* and *Cryptantha*: Casper et al., 1988; Schoen et al., 1997; Li and Johnston, 2001).

In this study, we test the evolutionary model proposed by Lloyd and Webb (1992a) using species in Lithospermeae, which shows wide variation of stylar conditions, particularly in the genus *Lithodora.* We predict that distyly would evolve from approach herkogamy via an intermediate state of style dimorphism. First, we test the phylogenetic hypothesis (based in ITS and $trnL_{UAA}$ intron) on the polyphyly of *Lithodora* (Thomas et al., 2008) by independently sampling all the species and subspecies of this genus (except for *Lithodora hispidula* subsp. *cyrenaica*) and sequencing new DNA regions (*trnK-matK*, *trnL-trnF*). Second, based on literature reports of stylar conditions and new flower measurements, we describe the different kinds of style polymorphisms. Finally, we apply methods to reconstruct evolutionary pathways to address the following specific questions: What stylar condition is ancestral to distyly? Is style dimorphism an unstable state and distyly a stable final state, as predicted by Lloyd and Webb (1992a)?

Materials and methods

Characterization of style polymorphism

To characterize taxa according to their style polymorphism we adopted a two-fold approach. First, we

characterized taxa according to taxonomic description provided in monographs by Johnston (1924, 1952, 1953a, b, 1954a, b) who thoroughly studied the tribe Lithospermeae. Characterization of polymorphism was contrasted in voucher specimens at the herbarium of the Royal Botanic Garden in Madrid (MA). As *Lithodora* sensu lato presented the most diverse array of stylar conditions and there were some doubts about the exact reciprocal nature of polymorphisms, we made a morphometric study of taxa of this genus. Detailed morphometric analyses including extensive population sampling will be published elsewhere. Here we present the data of one population for each species (see Table 1), except for *L. hispidula* subsp. *cyrenaica* (endemic to Libya) which we were unable to sample. We collected one fully opened flower per plant on 100 plants in each population, or less if flowering population size was smaller (the minimum number of collected flowers in a population was 57). The population presented here was representative in terms of style polymorphism in a given species (V. Ferrero, unpublished data from a survey of 109 populations). Flowers were preserved in 70% ethanol prior to measurement. We also collected a voucher specimen (deposited in SANT herbarium) and 5–10 leaves (one per plant) for the molecular study.

Flowers were slit longitudinally and measurements were made from digital photos with the image analyzer software *analySIS 5.0*. Floral traits measured were length of corolla and style, and height of all five stamens. Measurements were taken from the bottom of the corolla tube up to the stigmatic surface, to the midpoint of each anther and to the top of one randomly chosen corolla lobe (see Fig. 1a). We calculated the separation between reciprocal whorls for each level by subtracting, for each level, stigma height of one morph from anther height of the other morph. We also applied the method of Sánchez et al. (2008) to calculate the index of reciprocity between complementary sex organs of morphs in each population, in order to have a quantitative estimate of departure from exact reciprocity (i.e. that of an ideal distylous population). For graphical representation, stigma and stamen heights were divided by the length of the corolla of each flower to control for possible allometric effects of flower size.

Based on information in the taxonomic literature, visual inspection of specimens, and values of the reciprocity index, we defined the following classes of stylar conditions: (1) non-herkogamous monomorphism, i.e. anthers and styles at the same height; (2) monomorphic approach herkogamy, i.e. style length exceeds anther height in all individuals in a population; (3) style dimorphism, i.e. two morphs that differ in stigma placement relative to anther height (stigma above or below anthers), with anther height similar in both morphs; (4) distyly, i.e. two morphs for style length with reciprocal anther heights (Fig. 2).

Phylogenetic reconstruction

Plant material

In order to test the polyphyly of *Lithodora* with additional molecular regions to those used by Thomas et al. (2008), we extracted DNA from two different sources: (1) samples from our field collections were used for all species (7) and subspecies (4) of the genus (except *L. hispidula* subsp. *cyrenaica*) and one outgroup (*Echium vulgare*); (2) samples from herbarium collections were used for some other outgroups (*Cerinthe gymnandra*, *Buglossoides purpurocaerulea*, *Lithospermum officinale*, *L. multiflorum, Lobostemon trigonum* and *Cynoglossum magellense*). Sample vouchers are deposited in MA and SANT (Table 1).

DNA extraction, amplification and sequencing

Total DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN Inc.) and amplified using the polymerase chain reaction (PCR) on a Mastercycler® ep gradient S or an MJ Research (Massachusetts) thermal cycler. Approximately 15–20 mg of leaf tissue was used for each extraction. The PCR cycle profile comprised, after 5 min at 94°, 32 cycles at 94° for 1 min, 48–52° for 1–2 min, and 72° for 2 min. Amplifications were performed using the standard primers 17SE and 26SE (Sun et al., 1994) for the ITS; primers (c, d) by Taberlet et al. (1991) for the $trnL_{UAA}$ intron; (e, f) by Taberlet et al. (1991) for the *trnL-F* and *trnK*-3914F; and *matK*-1470R (Johnson and Soltis, 1994) for the *trnK-matK* region. DNA dilutions (1:20–1:50) were necessary for the amplifications of ITS sequences. A volume of 1 µl of dimethyl sulfoxide (DMSO) and 1 µl of bovine serum albumine (BSA) were included in each 25 µl reaction. Products were electrophoresed in 1.5% agarose gel in TAE (Tris–Acetate buffer) and stained with SYBR Green. Amplified products were cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California) following the manufacturer's protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, Little Chalfont, UK) following the manufacturer's protocols and run into polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism Model 3700 automated sequencer. PCR primers were used for cycle sequencing of the $trnL_{UAA}$ intron, *trnL-F* and the *trnK-matK* sequences while ITS5 and ITS4 (Sun et al., 1994) were used for the ITS region.

Molecular analysis

We constructed an initial matrix of four DNA regions for our samples (ITS; *trnK-matK*; $trnL_{UAA}$ intron; *trnL-F*). This matrix was extended by downloading ITS and $trnL_{UAA}$ intron sequences from the GenBank

Table 1. List of species used in the study, localization, analysis carried, voucher information and GenBank accession numbers.

Taxon sequenced in this study	Locality	Coordinates	Elevation (m)	Analysis	Voucher	GenBank Accesion no. ITS	GenBank Accesion no. *trnK-matK*	GenBank Accesion no. *trnL* intron	GenBank Accesion no. *trnL-F*
Lithodora diffusa	Spain: Pl. de Tòro	43°24′57.9″ 04°44′42.3″	15	d, m	SANT58512	FJ789863	FJ789899	FJ789845	FJ789881
Lithodora fruticosa	Spain: Montefrío	37°20′28.4″ 03°51′31.0″	836	d, m	SANT58519	FJ789864	FJ789900	FJ789846	FJ789882
Lithodora hispidula subsp. *hispidula*	Rhodes: Prassonissi	35°55′00.4″ 27°46′47.1″	55	d, m	SANT58510	FJ789865	FJ789901	FJ789848	FJ789883
Lithodora hispidula subsp. *versicolor*	Cyprus: Akanthou	35°20′19.9″ 33°43′03.6″	268	d, m	SANT58516	FJ789866	FJ789902	FJ789847	FJ789884
Lithodora moroccana	Morocco: Akchour	35°14′12.7″ 05°10′20.0″	518	d, m	SANT58495	FJ789867	FJ789903	FJ789849	FJ789885
Lithodora nitida	Spain: Sierra Magina	37°42′06.6″ 03°27′53.0″	1591	d, m	SANT58513	FJ789868	FJ789904	FJ789850	FJ789886
Lithodora oleifolia	Spain: Sant Aniol	42°16′48.9″ 02°35′19.1″	565	d, m	SANT58509	FJ789869	FJ789905	FJ789851	FJ789887
Lithodora prostrata subsp. *lusitanica*	Portugal: Tavira	37°08′14.8″ 07°41′20.7″	91	d, m	SANT58506	FJ789870	FJ789906	FJ789852	FJ789888
Lithodora prostrata subsp. *prostrata*	Spain: Jerte	40°16′18.0″ 05°39′36.0″	604	d, m	SANT58507	FJ789871	FJ789907	FJ789853	FJ789889
Lithodora rosmarinifolia	Sicily: Mt. Cofano	38°06′69.7″ 12.39′88.0″	80	d	–	FJ789872	FJ789908	FJ789854	FJ789890
Lithodora rosmarinifolia	Italy: Capri	40°32′37.3″ 14°12′59.7″	10	m	–	–	–	–	–
Lithodora zahnii	Greece: Ag. Konstantinos	36°55′19.8″ 22°14′58.1″	414	d, m	SANT58494	FJ789873	FJ789909	FJ789855	FJ789891
Buglossoides purpurocaerulea	Turkey: Bolu	–	–	d	111PV06	FJ789859	FJ789895	FJ789841	FJ789877
Cerinthe gymnandra	Spain: Alcaraz	–	–	d	694747 MA	FJ789860	FJ789896	FJ789842	FJ789878
Cynoglossum magellense	Italy: Abruzzo	–	–	d	698375 MA	FJ789861	FJ789897	FJ789843	FJ789879
Echium vulgare	Spain: Ungilde	42°01′36″ 06°37′06″	966	d	SANT58493	FJ789862	FJ789898	FJ789844	FJ789880
Lithospermum multiflorum	Arizona. Apache-Sitgreaves	–	–	d	739222 MA	FJ789874	FJ789910	FJ789856	FJ789892
Lithospermum officinale	Andorra	–	–	d	720052 MA	FJ789875	FJ789911	FJ789857	FJ789893
Lobostemon trigonus	South. Africa: E. Cape	–	–	d	708381 MA	FJ789876	FJ789912	FJ789858	FJ789894

Taxon downloaded from the GenBank[a]

Tribe Boragineae								
Anchusa officinalis	Germany	–	–	–	AY045710	–	AY045703	–
Borago officinalis	Germany	–	–	–	AY383283	–	AY383245	–
Tribe Echiochileae								
Echiochilon fruticosum	Israel	–	–	–	EU044843	–	EU044881	–
Ogastemma pusillum	Tunisia	–	–	–	EU044842	–	EU044880	–
Tribe Lithospermeae								
Alkanna sieberi	Greece	–	–	–	EU044844	–	EU044882	–
Alkanna tuberculata	–	–	–	–	EU044845	–	EU044883	–
Arnebia coerulea	Afghanistan	–	–	–	EU044856	–	EU044894	–
Arnebia decumbens	Tunisia	–	–	–	EU044857	–	EU044895	–
Buglossoides arvensis	Germany	–	–	–	EU044865	–	EU044903	–
Buglossoides incrassata	Greece, Crete	–	–	–	EU044866	–	EU044904	–
Buglossoides tenuiflora	Israel	–	–	–	EU044867	–	EU044905	–
Echium wildpretii	Spain, Tenerife	–	–	–	L43314	–	L43316	–
Halacsya sendtneri	Yugoslavia	–	–	–	EU044847	–	EU044885	–
Lithospermum afromontanum	Tanzania	–	–	–	EU044873	–	EU044911	–
Lithospermum caroliniense	USA	–	–	–	EU044876	–	EU044914	–
Lithospermum cinereum	South Africa	–	–	–	EU044874	–	EU044912	–
Lithospermum distichum	Mexico	–	–	–	EU044879	–	EU044917	–
Lithospermum gayanum	Peru	–	–	–	EU044878	–	EU044916	–
Lithospermum latifolium	USA	–	–	–	EU044887	–	EU044915	–
Lithospermum mirabile	USA	–	–	–	EU044875	–	EU044913	–
Macromeria longiflora	Mexico	–	–	–	EU044871	–	EU044909	–
Macromeria viridiflora	USA	–	–	–	EU044870	–	EU044908	–

Table 1. (*continued*)

Taxon sequenced in this study	Locality	Coordinates	Elevation (m)	Analysis	Voucher	GenBank Accesion no. ITS	GenBank Accesion no. *trnK-matK*	GenBank Accesion no. *trnL* intron	GenBank Accesion no. *trnL-F*
Mairetis microsperma	Morocco	–	–		–	EU044849	–	EU044887	–
Moltkia petraea	–	–	–		–	EU044854	–	EU044892	–
Moltkia suffruticosa	Italy	–	–		–	EU044855	–	EU044893	–
Neatostema apulum	Greece	–	–		–	EU044850	–	EU044888	–
Onosmodium bejariense	USA	–	–		–	EU044868	–	EU044906	–
Onosmodium molle	USA	–	–		–	EU044869	–	EU044907	–
Paramoltkia doerfleri	Albania	–	–		–	EU044848	–	EU044886	–
Podonosma orientalis	Israel	–	–		–	EU044846	–	EU044884	–

Abbreviations: MA, Herbarium of the Royal Botanic Garden of Madrid code; SANT, Herbarium of the University of Santiago de Compostela (Spain); PV, Pablo Vargas personal collection; d, plant material for phylogenetic studies; m, flowers collected for the morphological analysis.

[a]Reported in Böhle et al. (1996), Hilger et al. (2004), and Thomas et al. (2008).

with MEGA 4 software (Tamura et al., 2007) for most of the members of Lithospermeae available from Thomas et al. (2008) and other samples of Boragineae and Echiochileae (Table 1). A matrix of the four DNA regions was constructed with all the sequences and aligned with ClustalW (Thompson et al., 1994) as implemented in MEGA 4 (Tamura et al., 2007). To determine the simplest model of sequence evolution that best fits the sequence data, the Hierarchical Likelihood Ratio Test (hLRT) and Akaike Information Criterion (AIC) were implemented independently for each partition using MrModeltest 3.7 (Posada and Crandall, 1998; Nylander, 2002). A Bayesian inference of phylogeny with Markov chain Monte Carlo sampling was conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). A general time-reversible model of DNA substitution and shape parameter of the gamma distribution (GTR + G) model was used with parameters partitioned across the genes. One cold chain and three heated chains were run simultaneously for 10 million generations, and one tree per 100 generations was sampled (four MCMC, chain temperature = 0.2; sample frequency = 100; and burn-in = 30000). We estimated the 50% majority rule consensus of the remaining trees and used posterior probability (PP) as alternative estimate of robustness.

Moreover, maximum parsimony analyses was run using Fitch parsimony with PAUP* (Swofford, 1999) with equal weighing of all characters and of transitions/transversions. Heuristic searches were replicated 100 times with random taxon addition sequences. Support values were assessed by "full" bootstrapping (1000 replicates) using the heuristic search strategy mentioned above.

Reconstruction of ancestral stylar condition

We conducted ancestral reconstruction of style polymorphism using the phylogeny based on our own sequences and the available ones in GenBank (hereafter referred to as Lithospermeae phylogeny). The evolution of heterostyly under maximum parsimony and maximum likelihood (ML) was reconstructed using the 50 major-rule consensus tree recovered from the Bayesian analysis of the combined nuclear and plastid datasets.

Four different states of sexual polymorphism were considered: 0: non-herkogamous monomorphism; 1: approach herkogamy; 2: style dimorphism; 3: distyly. An ancestral state of style polymorphism was reconstructed using maximum parsimony and maximum likelihood in Mesquite 2.01 (Maddison and Maddison, 2007). Parsimony reconstruction methods find the ancestral states that minimize the number of steps of character change given the tree and observed character distribution. Parsimony reconstruction was carried out under the "Unordered" model, where the cost of change of state is 1, considering all changes among

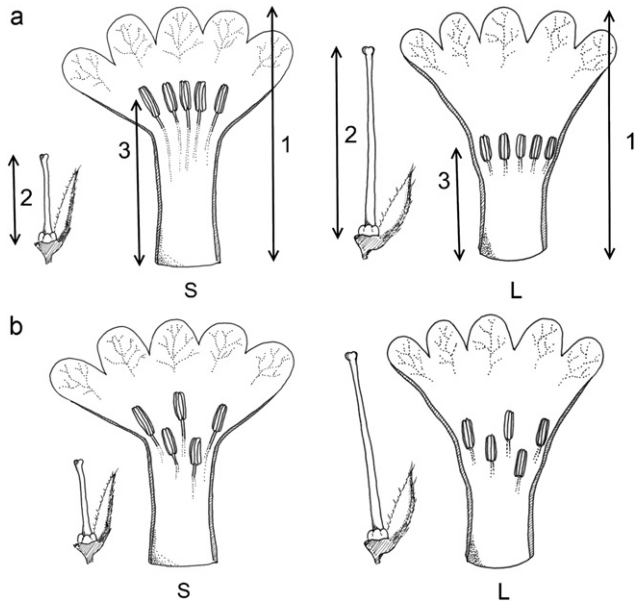

Fig. 1. (a) Long-styled (L) and short-styled (S) flowers of (a) distylous *Lithodora moroccana* and (b) *Lithodora prostrata* subspecies. Numbers correspond to flower measurements: (1) corolla length; (2) style length; (3) anther height.

polymorphism states as equally probable. Likelihood reconstruction methods find the ancestral states that maximize the probability the observed states would evolve under a stochastic model of evolution (Schluter et al., 1997; Pagel, 1999). Polytomies detected were resolved by assigning a branch length of 0.000001. The likelihood reconstruction finds, for each node, the state assignment that maximizes the probability of arriving at the observed states in the terminal taxa, given the model of evolution, and allowing the states at all other nodes to vary. Maximum likelihood reconstruction was carried out based on a Mk1 model (''Markov k-state 1 parameter model''), which is a k-state generalization of the Jukes-Cantor model, and corresponds to Lewis's (2001) Mk model in Mesquite 2.01 (Maddison and Maddison, 2007). In this model the single parameter is the rate of change. Any particular change is equally probable.

Results

Characterization of style polymorphism: morphometric analysis

Among the species analyzed in the tribe Lithospermeae, monomorphism is prevalent, since 28 of 43 species are non-herkogamous or approach-herkogamous. Style polymorphism is found only in species of three genera: *Arnebia, Lithospermum* and *Lithodora* sensu lato.

Within *Lithodora* sensu lato, average values of sex organ position are shown in Table 2. Style monomorphism was not found in the genus. Results from the morphometric measurements in individual plants are plotted in Fig. 2. Polymorphism affects only style length (style dimorphism) or both stigma and stamen height in a reciprocal manner (distyly). *Lithodora fruticosa, L. zahnii* and *L. prostrata* are stylar dimorphic, and *L. hispidula, L. diffusa*, *L. oleifolia*, *L. nitida*, *L. moroccana* and *L. rosmarinifolia* are distylous (Fig. 2). Values of stigma-anther separation between means of reciprocal organs are much higher in stylar dimorphic than in distylous species for both levels (upper and lower). There are significant differences between distylous and stylar-dimorphic species in reciprocity values ($Z = -2.646$; $P < 0.00$), due to the low reciprocity of stylar-dimorphic species (values between 0.040 and 0.077) compared to distylous species (from 0.017 to 0.035) (Table 2).

Phylogenetic reconstruction

Molecular analysis

The alignment of the plastid and nuclear regions for the 48 species of Lithospermeae and the outgroup included a total of 2840 bp positions. In the maximum parsimony analysis, 2220 characters were constant, 316 parsimony-uninformative and 304 parsimony-informative. The consensus tree of the 16,189 MP best trees (results not shown) was fully congruent with the majority rule consensus tree recovered from the Bayesian analysis and both yielded medium to high support values (Fig. 3). Results for the maximum parsimony analysis are not shown. They were congruent with the Bayesian analysis but support values (Bootstraps) were slightly lower, maybe because of missing data.

In our phylogenetic reconstruction of Lithospermeae and related groups, monophyly of the tribe is retrieved, although with low support (0.89 PP) (Fig. 3). Two well-supported groups were recovered. One consists of *Alkanna* and *Podonosma* species (1.00 PP) and the other includes five unresolved subgroups consisting of accessions of: (1) *Arnebia* (1.00 PP), (2) *Moltkia* (1.00 PP), (3) *Echium* and *Lobostemon* (1.00 PP), (4) *Lithodora* I, *Paramoltkia*, *Mairetis*, *Halacksya* and *Neatostema* species (1.00 PP), and (5) *Buglossoides*, *Lithospermum*, *Macromeria*, *Lithodora* II and *Onosmodium* species (1.00 PP). *Lithodora* sensu lato is found to be polyphyletic, which corroborates the results from Thomas et al. (2008), with its species forming part of two independent clades: *Lithodora* I (hereafter genus *Lithodora* because it includes the type species) with *L. fruticosa, L. hispidula* and *L. zahnii*; and *Lithodora* II (hereafter genus *Glandora*) with *G. diffusa*,

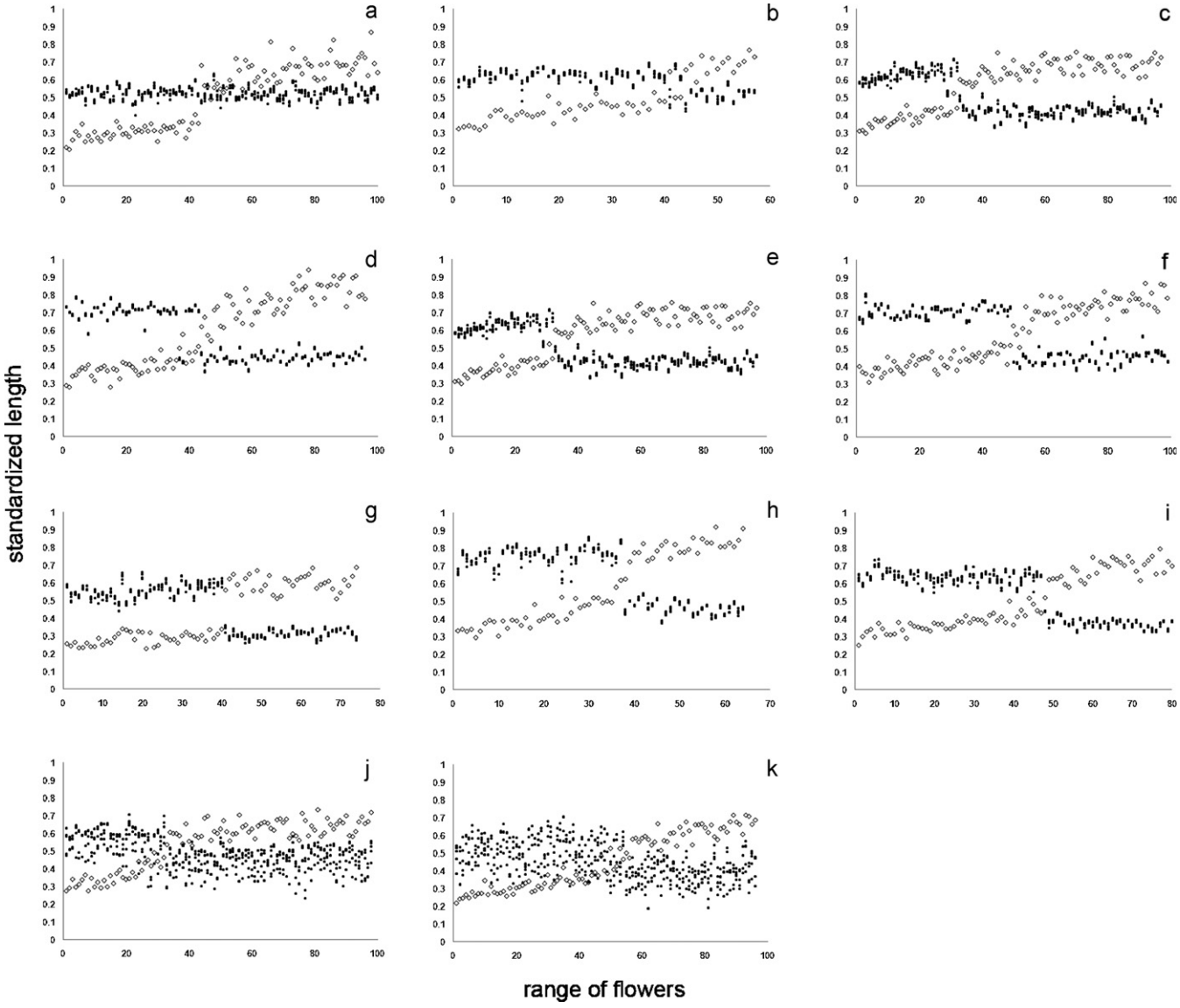

Fig. 2. Stigma (◇) and anther (●) heights adjusted for flower size variation in flowers of (a) *Lithodora fruticosa*, (b) *L. zahnii,* (c) *L. hispidula* subsp. *versicolor*, (d) *L. hispidula* subsp. *hispidula*, (e) *L. diffusa,* (f) *L. moroccana*, (g) *L. nitida*, (h) *L. oleifolia*, (i) *L. rosmarinifolia* (j) *L. prostrata* subsp. *prostrata*, (k) *L. prostrata* subsp. *lusitanica*. Flowers are ranked by stigma height to illustrate the reciprocal correspondence of stigma and anther positions in the L- and S-morphs.

G. nitida, *G. oleifolia*, *G. moroccana*, *G. prostrata* and *G. rosmarinifolia*.

Reconstruction of ancestral polymorphism

Ancestral reconstruction of style polymorphism using ML methods in Lithospermeae and related taxa indicates that monomorphism is the most likely ancestral condition in this tribe; approach-herkogamous and non-herkogamous monomorphism have a similar probability (proportional probability of 0.56 and 0.34, respectively). These analyses revealed that style polymorphism (distyly or style dimorphism) is derived from such states (Fig. 4). Reconstruction with parsimony is, however, equivocal at this node.

Within Lithospermeae, limited sample size prevented reliable reconstruction of the ancestral state (non-herkogamous monomorphism) of the *Podonosma* clade. The other clade contains all the stylar-polymorphic species. Distyly appears at least four independent times in the tribe: in genus *Arnebia*, in *Lithodora hispidula* subspecies, in most of *Glandora* species and in some species of *Lithospermum*, although the latter is not well resolved and a more detailed sampling might reveal more events. Irrespective of missing species of Lithospermeae and problems of phylogenetic resolution in our analysis, the polyphyly of *Lithodora* sensu lato indicates that the evolution of distyly has involved at least two independent processes. In *Lithodora*, distyly appears to

Table 2. Means and coefficients of variation (CV) for the floral traits in the two morphs measured in *Lithodora s*pecies; sample size; Corolla length; style length; stamen length; stigma-anthers separation (difference between mean of stigma length and anther length for each level); ratio of morphs; reciprocity index and kind of stylar polymorphism.

Species	Sample size	Corolla M (CV) (mm)		Style height M (CV) (mm)		Stamen height M (CV) (mm)		Stigma-Anther separation		Morph ratio	Reciprocity index	Stylar polymorphism
Morphs (L, long styled; S, short styled)	L, S	L	S	L	S	L	S	L	S	L:S		
Lithodora diffusa	65, 32	16.32 (9.3)	17.31 (7.7)	10.90 (0.11)	6.69 (0.13)	6.85 (0.11)	10.91 (0.10)	0.01	0.16	67:33	0.017	Distyly
Lithodora fruticosa	53, 47	14.45 (12.2)	14.62 (11.2)	9.53 (0.13)	4.71 (0.21)	7.25 (0.13)	7.74 (0.11)	1.79	2.54	53:47	0.077	Stylar dimorphism
Lithodora hispidula subsp. *hispidula*	56, 41	11.64 (11.1)	11.77 (10.7)	8.88 (0.18)	4.64 (0.14)	5.20 (0.14)	8.49 (0.13)	0.39	0.56	58:42	0.035	Distyly
Lithodora hispidula subsp. *versicolor*	41, 59	12.16 (10.7)	12.47 (8.3)	9.34 (0.13)	6.48 (0.14)	6.45 (0.12)	8.87 (0.12)	0.47	0.03	41:59	0.024	Distyly
Lithodora moroccana	50, 49	18.01 (10.1)	18.01 (9.9)	13.19 (0.14)	7.69 (0.14)	8.12 (0.15)	12.80 (0.11)	0.39	0.43	50:50	0.025	Distyly
Lithodora nitida	34, 40	20.37 (12.4)	20.62 (12.8)	12.14 (0.10)	5.82 (0.14)	6.41 (0.16)	11.53 (0.16)	0.61	0.59	46:54	0.030	Distyly
Lithodora oleifolia	27, 37	17.34 (6.7)	17.73 (9.0)	13.85 (0.10)	7.43 (0.20)	8.02 (0.09)	13.48 (0.12)	0.37	0.59	42:58	0.022	Distyly
Lithodora prostrata subsp. *prostrata*	66, 32	19.67 (8.9)	20.06 (8.1)	12.35 (0.09)	7.24 (0.15)	8.76 (0.18)	11.17 (0.17)	1.18	1.52	67:33	0.040	Stylar dimorphism
Lithodora prostrata subsp. *lusitanica*	44, 52	18.07 (8.0)	18.03 (8.1)	11.03 (0.12)	5.86 (0.19)	7.00 (0.20)	9.25 (0.18)	1.78	1.14	46:54	0.061	Stylar dimorphism
Lithodora rosmarinifolia	34, 47	18.61 (8.6)	18.88 (8.4)	12.65 (0.11)	7.10 (0.15)	6.97 (0.10)	12.04 (0.10)	0.61	0.13	42:58	0.018	Distyly
Lithodora zahnii	15, 42	18.07 (13)	17.81 (10.7)	12.40 (0.15)	7.54 (0.16)	9.30 (0.15)	11.10 (0.13)	1.30	1.76	26:74	0.047	Stylar dimorphism

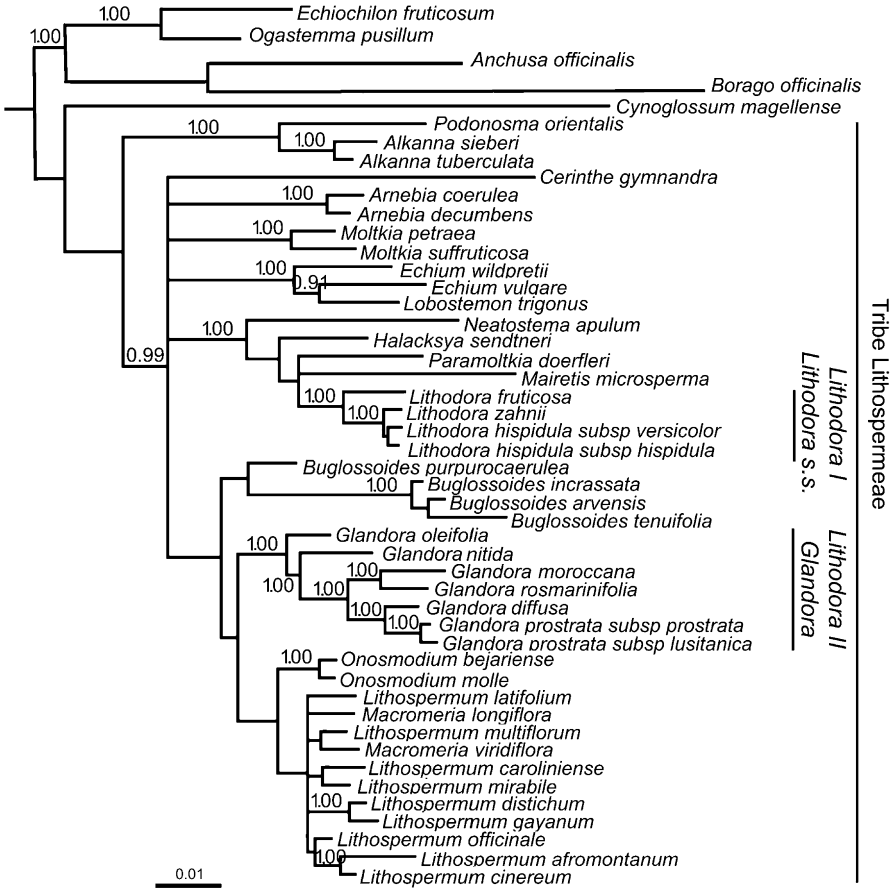

Fig. 3. Majority rule consensus tree recovered from the Bayesian analysis of the combined ITS, *trnK-matK, trnL*$_{UAA}$ intron, *trnL-F* data set. For our sequenced samples we included data for the four regions. For the downloaded samples, only ITS and *trnL*$_{UAA}$ intron sequences were included in the analysis. Numbers above branches show posterior probabilities from the Bayesian analysis.

be a derived state in the parsimony reconstruction (Fig. 4). In the ML reconstruction, the most likely ancestral state for *L. hispidula, L. zahnii* and *L. fruticosa* is style dimorphism (0.52), followed by distyly (0.26). Admittedly, our limited sample did not allow us to infer deep-node ancestral states. In any case, given the predominance of monomorphism in many genera and species of Lithospermeae and the reconstruction of approach herkogamy in deeper nodes of *Lithodora* (*Mairetis–Paramoltkia–Halacksya–Neatostema*) in our analysis, we interpret that distyly has evolved from approach herkogamy. The evolutionary sequence of polymorphisms appears to be different in the *Glandora–Lithospermum* clade, as the ancestor for this clade may have displayed non-herkogamous monomorphism (0.87) (but see the parsimony reconstruction in Fig. 4). Most of the monomorphic species (some *Lithospermum* species) in this clade have this condition, as does the sister clade (*Buglossoides*). Distyly was gained and maintained through the *Glandora* clade, except for two terminal taxa, the two subspecies of *G. prostrata*, which show a reversion to style dimorphism (proportional probability of 0.99).

Discussion

Tribe Lithospermeae shows a range of distinct floral polymorphisms that provide insights in the evolutionary transitions associated with heterostyly. Our confirmation of a phylogenetic hypothesis for the polyphyly of genus *Lithodora* (Thomas et al., 2008) when combined with detailed information on style polymorphic conditions in the group allows us to examine whether approach herkogamy and style dimorphism are ancestral and intermediated stages in the evolution of distyly, as proposed by Lloyd and Webb (1992a).

Phylogenetic relationships

Our Bayesian and maximum parsimony analyses based on ITS, *trnK-matK*, *trnL*$_{UAA}$ intron and *trnL-F*, in Lithospermeae are congruent with the recent analysis carried out by Thomas et al. (2008) based on ITS, and *trnL*$_{UAA}$ intron sequences. The addition of two more sequences for subspecies of *G. prostrata* and *L. hispidula* and other taxa in Boraginales (*Cerinthe gymnandra*,

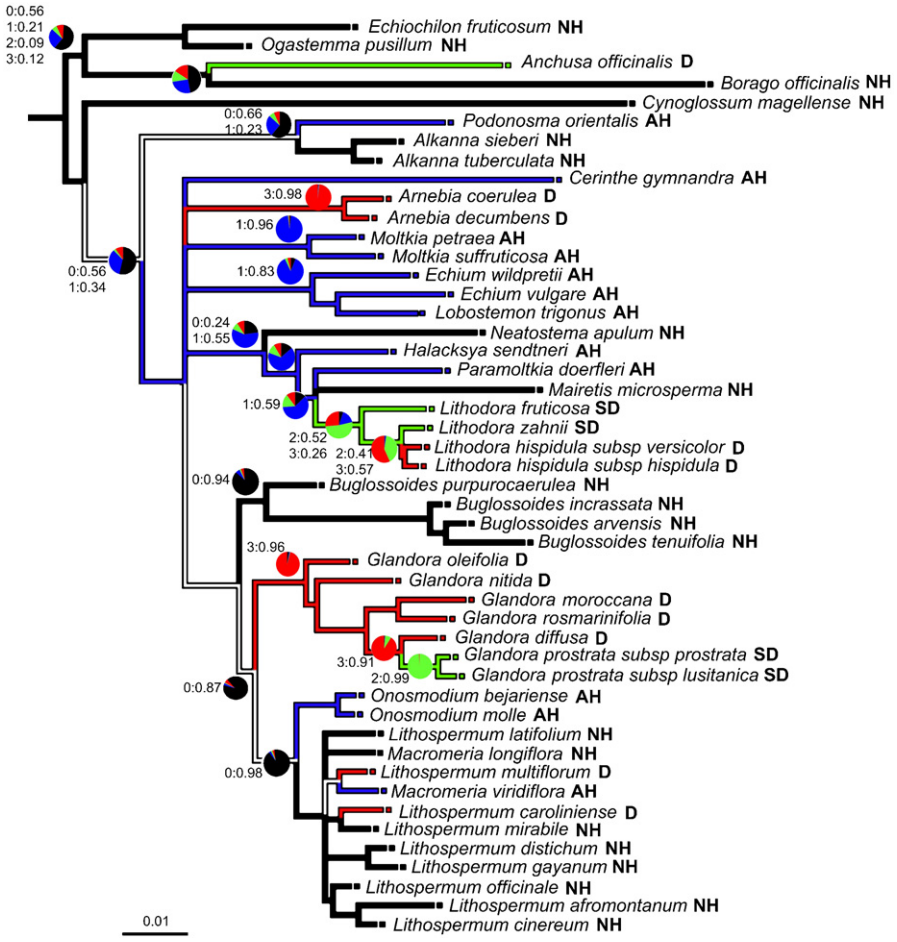

Fig. 4. Evolution of sexual polymorphisms within tribe Lithospermeae under parsimony and maximum-likelihood criteria. The most parsimonious states are shown under the unordered model (coloured lines); probabilities at each node are reported as proportional likelihoods for each character (sectors of the pie charts) using the one-parameter model. Red (3): distyly; green (2): style dimorphism; blue (1): approach-herkogamous monomorphism; black (0): non-herkogamous monomorphism; grey: equivocal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cynoglossum magellense, *Lithospermum multiflorum, Lobostemon trigonus*) did not alter the results with high PP and bootstrap values. In addition, conspecific samples formed monophyletic groups, indicating that species of *Lithodora* and *Glandora* are well defined. Our results confirm the monophyly of Lithospermeae and provide a sound basis for using the inferred phylogenetic relationships for analyzing the evolution of style polymorphism in this group, which was our ultimate goal. The polyphyly of *Lithodora* sensu lato (Thomas et al., 2008) is also supported. Despite recent phylogenetic analysis of Boraginales and particularly Boraginaceae (Långström and Chase, 2002; Långström and Oxelman, 2003; Hilger et al., 2004; Selvi et al., 2004, 2006), future molecular analyses incorporating more taxa could determine the exact number of times a given event has occurred, e.g. the incompatibility system not tightly linked to style morph variation in *Anchusa* (Dulberger, 1970; Philipp and Schou, 1981; Schou and Philipp, 1984) and *Pulmonaria* (Richards and Mitchell, 1990; Brys et al., 2008a), which is also a support for the Lloyd and Webb (1992a) model.

Characterization of style polymorphism in Lithospermeae

Style monomorphism within the tribe Lithospermeae is common, being either non-herkogamous (15 species) or approach-herkogamous (13 species). However, these conditions were ascertained from data in taxonomic monographs and examination of herbarium specimens and detailed population sampling and measurements should be done to confirm the absence of variation. Although monomorphism is probably correct, the distinction between approach herkogamy and non-herkogamy is quantitative and requires further study.

All taxa (species and subspecies) of *Lithodora* and *Glandora* are stylar-polymorphic, four taxa showed style dimorphism and seven taxa distyly. *Lithodora fruticosa*

is a style dimorphic species, displaying invariant stamen height and two morphs for style length. *L. hispidula* subsp. *hispidula* and subsp. *versicolor* are both distylous. In the case of *Glandora* all species are distylous, except for the two subspecies of *G. prostrata* and *G. zahnii*. *Glandora zahnii* had been previously described as distylous (Johnston, 1953b), however our results show that the two morphs have an almost identical stamen height (Fig. 2b), which qualifies this species as stylar dimorphic. Style polymorphism in *G. prostrata* is different from that found in *Lithodora*. In both subspecies anther height is different on each of the five stamens of a flower but constant among flower morphs (Fig. 1b). *Glandora prostrata* was described as distylous by Valdés (1981), however, the lower style-stamen reciprocity in these two subspecies (index values: 0.040–0.061) is higher than that of typical distylous taxa (0.017–0.035) indicative of quantitative variation and stigma height dimorphism. This reciprocity index is correlated with discrete classes; all distylous *Lithodora* and *Glandora* species show higher reciprocity (i.e. lower index values) than stylar-dimorphic species. However, variation within each type is non-negligible.

Evolutionary pathways of style polymorphism

We provide evidence of multiple origins of distyly in the tribe Lithospermeae (Fig. 4). The primitive condition in the tribe is inferred to be monomorphism, although it remains equivocal whether the ancestral state was approach herkogamy or non-herkogamous monomorphism. Hence, we cannot conclude in support of an ancestor with approach herkogamy-a specific tenet of Lloyd and Webb's (1992a) model. Detailed measurements of stigma-anther separation in species of ancestral lineages could throw light on this issue. The complete sequence of events predicted by Lloyd and Webb (1992a) (approach herkogamy-style dimorphism-distyly) is, however, found in the clade *Lithodora–Mairetis–Paramoltkia–Halacksya–Neatostema*. Some of the species (*Neatostema apulum, Mairetis microsperma*) show a reversion to non-herkogamous monomorphism that may allow reproductive assurance in a reversion to selfing (Schoen et al., 1997). The ancestral stylar condition to *Glandora* and *Lithospermum* is equivocal, with somewhat higher support for non-herkogamous monomorphism, which is the condition most frequent among monomorphic sister species in the clade.

Reversion to style dimorphism in *Glandora prostrata*

An important novel result of this study is the unpredicted reversion of distyly to style dimorphism in two subspecies of *G. prostrata* clearly derived from a distylous ancestor, a condition of all other *Glandora* in this clade. When heterostyly is lost, it usually leads to secondary homostyly and selfing for reproductive assurance, e.g. in *Amsinckia* (Schoen et al., 1997), *Primula* (Wedderburn and Richards, 1992; Mast et al., 2004, 2006), *Eichhornia* (Barrett, 1979; Barrett et al., 1989) and *Psychotria* (Sakai and Wright, 2008). In other cases, loss of heterostyly is due to the loss of one of the morphs, usually the short-styled morph, and involves morph compatibility (*Narcissus*: Pérez-Barrales et al., 2006) or clonal propagation (e.g., *Oxalis*: Castro et al., 2007). Some species of *Lithodora* and *Glandora* tested, including *G. prostrata,* are self-incompatible but morph-compatible (V. Ferrero, unpublished data), thus it should be inferred that a high level of disassortative pollen flow and mating probably occurs in populations in order to maintain both distyly and style dimorphism, as indicated by morph-ratios close to isoplethy (Table 2). In fact, in a survey of 42 populations of *G. prostrata,* monomorphism was not found and many of them are indeed isoplethic (V. Ferrero unpublished data). Further work in populations determining the level of assortative and disassortative pollen transfer will reveal the mechanism responsible for style dimorphism maintenance. Style dimorphism in *G. prostrata* is different from that of the ancestral type (*L. fruticosa* and *G. zahnii*) in that anther height is not uniform within the flower, stamens spread along the flower tube to unequal positions (Fig. 1b). One might be tempted to invoke relaxation from stabilizing selection exerted by pollinators, which Lloyd and Webb (1992b) suggest maintains reciprocal positioning of sex organs. However, the high constancy across populations of this morphological pattern in the androecia (V. Ferrero, unpublished data) makes it plausible that such variation could be related to a selective process driven by less efficient pollen transfer. Another Mediterranean Boraginaceae, *Anchusa crispa*, also shows fine-scaled variation in stigma height, including approach herkogamy, reverse herkogamy and non-herkogamy in different populations, which may be related to different outcrossing rates (Quilichini et al., 2004). We suggest that the wider amplitude of the whole anther level in *G. prostrata* may increase opportunities for pollen delivery: the highest anthers may deliver pollen to long-styled flowers and the shorter anthers to short-styled flowers (as suggested for *Narcissus assoanus* by Cesaro and Thompson, 2004). Pollination ecology, pollen flow and mating system studies are being carried out to determine if this is the case.

In this study some of the conclusions could partially be affected by taxon sampling, which can influence conclusions drawn from internal state inferences (Salisbury and Kim, 2001). Having said this, the patterns shown in *Lithodora* and *Glandora* are beyond doubt, since sampling was exhaustive. Determining the ancestral condition in the tribe will, however, require a

greater number of Lithospermeae taxa, especially in the *Alkanna* and *Podonosma* clade. Despite these limitations, this study depicts an evolutionary scenario which fully supports some of the steps proposed by the Lloyd and Webb (1992a) model of evolution of heterostyly, and brings new opportunities for the study of functional significance of intermediate stages.

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