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Unusual heterostyly: style dimorphism and self-incompatibility are not tightly associated in *Lithodora* and *Glandora* (Boraginaceae)

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- **Background and Aims** Heterostyly is a floral polymorphism characterized by the reciprocal position of stamens and stigmas in different flower morphs in a population. This reciprocal herkogamy is usually associated with an incompatibility system that prevents selfing and intra-morph fertilization, termed a heteromorphic incompatibility system. In different evolutionary models explaining heterostyly, it has been alternately argued that heteromorphic incompatibility either preceded or followed the evolution of reciprocal herkogamy. In some models, reciprocal herkogamy and incompatibility have been hypothesized to be linked together during the evolution of the heterostylous system.
- **Methods** We examine the incompatibility systems in species with different stylar polymorphisms from the genera *Lithodora* and *Glandora* (Boraginaceae). We then test whether evolution towards reciprocal herkogamy is associated with the acquisition of incompatibility. To this end, a phylogeny of these genera and related species is reconstructed and the morphological and reproductive changes that occurred during the course of evolution are assessed.
- **Key Results** Both self-compatibility and self-incompatibility are found within the studied genera, along with different degrees of intra-morph compatibility. We report for the first time extensive variability among members of the genus *Glandora* and related species in terms of the presence or absence of intraspecific polymorphism and heteromorphic incompatibility. Overall, our results do not support a tight link between floral polymorphism and incompatibility systems.
- **Conclusions** The independent evolution of stylar polymorphism and incompatibility appears to have occurred in this group of plants. This refutes the canonical view that there is strong linkage between these reproductive traits.

Key words: Character transitions, *Lithodora*, *Glandora*, Boragineae, evolution, heterostyly, incompatibility system, ITS, Lithospermeae, phylogenetic reconstruction, *trnL_{UAA}* intron.

INTRODUCTION

Despite a century and a half of studies on the evolutionary significance of heterostyly following the extensive treatment by Darwin (1877), there are still many open questions, and there is a lack of general agreement about the significance of and steps necessary to build this fascinating reproductive system in plants. Heterostyly is a morphological and reproductive polymorphism in which plant populations are composed of two (distyly) or three (tristyly) floral morphs that differ reciprocally in the height of their stigmas and anthers (Ganders, 1979). Additionally, heterostylous species usually possess a sporophytic diallelic incompatibility system, which prevents self- and intra-morph fertilization; this is termed a heteromorphic incompatibility system (Mather and de Winton, 1941). There is a set of ancillary traits that often accompany such a system (e.g. stigmatic papillae and pollen grain polymorphisms), although a number of deviations from the complete set of traits have been observed (for a review, see Ganders, 1979).

Several hypotheses about the evolution of heterostyly have been proposed, but two of them, representing quite different

alternatives, are the most noteworthy: those proposed by Charlesworth and Charlesworth (1979) and by Lloyd and Webb (1992a, b). In these models, the incompatibility system has been interpreted differently. Darwin considered the evolution of incompatibility in heterostylous plants to be an ‘incidental and purposeless’ result of dissimilarities in pollen growth patterns in styles of different length (Darwin, 1877, p. 265). Later, incompatibility was considered of primary importance in the evolution of heterostyly. In particular, Charlesworth and Charlesworth (1979), following Baker (1966), proposed that the acquisition of a very simple, diallelic incompatibility system is the first necessary step in the evolutionary process, serving as a mechanism to avoid inbreeding effects in selfing plants. This system is considered highly inefficient because the plants are separated into two isolated mating groups within a population, as determined by the possession of different incompatibility alleles. The appearance of reciprocal herkogamy would be the consequence of two mutations tightly linked to the incompatibility locus that are selected to guarantee mating between plants of opposite groups. In contrast, and following Darwin (1877), Lloyd and Webb (1992a) proposed that heterostyly is mostly a

morphological device that promotes precise, legitimate pollinations between morphs. Inbreeding is not invoked as the key selective factor, but an increase in the precision of cross-pollination and avoidance of interference between sex organs within the flower are instead considered to be the selected features (Barrett, 2002). Incompatibility reactions would evolve after the emergence of reciprocal herkogamy as a result of pollen specialization for legitimate pollination (Lloyd and Webb, 1992b). Heteromorphic incompatibility operates through the failure of each class of pollen (legitimate or illegitimate) to grow in a particular type of style, allowing growth only if legitimate cross-pollination is achieved. In this context, it is at least possible for the morphological changes to occur without any accompanying heteromorphic incompatibility. Recent studies using character reconstruction in *Narcissus* species have provided evidence for the Darwinian Lloyd and Webb (1992a) hypothesis (Pérez-Barrales et al., 2006). However, aside from the wide occurrence of heterostyly across families of angiosperms, the phenomenon has not been well documented in taxonomic groups outside the Amaryllidaceae.

The Boraginaceae is known to be one of the lineages in which heterostyly evolved (e.g. Ganders, 1979; Schoen et al., 1997; Ferrero et al., 2009; Cohen, 2011), and this family displays a wide variety of reproductive systems involving stylar polymorphism (Ray and Chisaki, 1957; Opler et al., 1975; Philipp and Schou, 1981; Schou and Philipp, 1983, 1984; Casper, 1985; De Jong and Klinkamer, 1989; Weller and Ornduff, 1991; Schoen et al., 1997; Ferrero et al., 2009, 2011a). Within the heterostylous taxa of the Boraginaceae, full self-compatibility appears to be common, as it has been observed in the distylous *Cryptantha flava* (Casper, 1985) and in some species of *Pulmonaria* (although with different strengths, Olesen, 1979; Brys et al., 2008). In most of these cases, however, self-compatibility seems to be a derived condition selected by reproductive assurance (see, for example, Schoen et al., 1997), produced through recombination within the heterostyly incompatibility supergene (Mather and de Winton, 1941).

Recent studies show extensive variation in style polymorphism within the tribe Lithospermeae, specifically in the genera *Glandora* and *Lithodora* (Thompson, 2005; Thomas et al., 2008; Ferrero et al., 2009; Cohen, 2011), suggesting that this group is well suited as a model system to test hypotheses about the evolution of heterostyly. In all species within the genera *Lithodora* and *Glandora*, two morphs with different style lengths are observed, called long (L) and short (S) morphs, although anther position varies widely. Thus, in stylar dimorphic species, the anthers remain at approximately the same height in both morphs, and in distylous species the anther height tends to be reciprocal to that of the stigma of the opposite morph (Ferrero et al., 2009, 2011a). Facing such diversity, we analyse the incompatibility systems found in six species of *Glandora* and *Lithodora* exhibiting different degrees of stylar polymorphism, which allows us to examine whether the incompatibility reaction is linked to style polymorphism, as predicted by the Charlesworth and Charlesworth (1979) model, or whether the two traits are not necessarily associated, as predicted by Lloyd and Webb (1992a). To that end, we have conducted hand pollination experiments using style dimorphic and distylous species, and

we have investigated the reproductive and morphological shifts that occurred during the course of evolution using phylogenetic analysis of sequences from the nuclear internal transcribed spacer (ITS) region and the plastid *trnL*_{UAA} intron.

MATERIALS AND METHODS

Study species

Traditionally, *Lithodora* (Griseb.) has formerly consisted of seven species and five subspecies distributed around the Mediterranean Basin. Recently, based on molecular data, this genus has been split into *Glandora* D.C. Thomas, Weigend and Hilger and *Lithodora* (Griseb.) D.C. Thomas, Weigend and Hilger (Thomas et al., 2008). All species in these two genera are perennial shrubs with style dimorphic flowers showing different degrees of reciprocity (Ferrero et al., 2009, 2011a). The plants occur mainly on rocky limestone habitats and cliffs, except for the widespread *Glandora prostrata*, which grows on acid soils. Their flowering period extends from February to July depending on the species and location. Like many heterostylous plants, these species present actinomorphic, tubular flowers that are pollinated by long-tongued pollinators (mainly solitary bees, bee-flies and butterflies; Ferrero et al., 2011b). Single flower life span is 3–5 d. Pollen is released from the first day onward, and nectar is secreted abundantly at the base of the ovaries at a rate of 1.2–3.6 μ L per flower per day, depending on the species (Ferrero, 2011b). We carried out our study using six populations located in Spain, Portugal and Morocco, where most of the floral diversity occurs. We selected one population of each species from the genus *Glandora* (except *G. rosmarinifolia*, endemic to Italy) and one species from the genus *Lithodora* (*L. fruticosa*), thus accounting for much of the polymorphism present in the genus *Lithodora* as it was formerly defined (Ferrero et al., 2009, 2011a) (see Table 1 for details). Other species from tribe Lithospermeae and the tribe Anchuseae, for which information on morphological and reproductive traits as well as DNA sequence data were available, were also included in the study to serve as outgroups (see Table 1 and the Molecular analysis section for details).

Floral polymorphism and reciprocity

In order to describe the types of stylar polymorphisms observed in the studied species, we calculate the degree of reciprocity between sexual whorls for each population, following Sánchez et al. (2008). This index is based on a comparison of the positions of every anther of each flower with the stigmas of all plants of the opposite morph in a population, thus accounting for both the reciprocal positions of organs and their variability within the flower and within the population. *Lithodora* and *Glandora* show a variety of stylar polymorphisms and degrees of reciprocity, distyly being the most common polymorphism within both genera (Ferrero et al., 2009, 2011a). However, *L. fruticosa* is style dimorphic, i.e. its flowers have two different lengths for the style but only one for the anthers. In addition, *G. prostrata* shows relaxed style dimorphism, a recently described polymorphism in which there are also two lengths for the style and two similar average lengths for the stamens in each morph, but

TABLE 1. Taxa included in the study with details on the studied populations of *Glandora* and *Lithodora* species: type of stylar polymorphism, location and co-ordinates of the studied localities where the breeding systems were carried out and GenBank accession numbers are provided

Species	Stylar polymorphism	Locality (reference)	Co-ordinates	GenBank accession no. ITS*	GenBank accession no. <i>trnL</i> intron*
<i>Anchusa officinalis</i>	Stylar dimorphism	Denmark (Philipp and Schou, 1981)	–	AY045710	AY045703
<i>Borago officinalis</i>	Non-herkogamous monomorphism	Spain (Montaner et al., 2000)	–	AY383283	AY383245
<i>Echium vulgare</i>	Approach herkogamy	UK (Corbet, 1978)	–	FJ789862	FJ789844
<i>E. wildpretii</i>	Approach herkogamy	Spain (Dupont et al. 2004)	–	L43314	L43316
<i>Glandora diffusa</i>	Distyly	Spain: Suances (Ferrero et al., 2009)	43°26'30"N 04°02'50"W	FJ789863	FJ789845
<i>G. moroccana</i>	Distyly	Morocco: Akchour (Ferrero et al., 2009)	35°14'12"N 05°10'19"W	FJ789867	FJ789849
<i>G. nitida</i>	Distyly	Spain: S. Mágina (Ferrero et al., 2009)	37°42'06"N 03°27'53"W	FJ789868	FJ789850
<i>G. oleifolia</i>	Distyly	Spain: St. Aniol (Ferrero et al., 2009)	42°19'01"N 02°35'13"W	FJ789869	FJ789851
<i>G. prostrata</i>	Relaxed style dimorphism	Spain: Corrubedo (Ferrero et al., 2009)	42°34'35"N 09°05'20"W	FJ789869	FJ789853
<i>Lithodora fruticosa</i>	Stylar dimorphism	Spain: Hueva (Ferrero et al., 2009)	40°28'9"N 02°057'2"W	FJ789864	FJ789846
<i>Lithospermum caroliniense</i>	Distyly	USA (Levin, 1968)	–	EU044876	EU044914
<i>Macromeria viridiflora</i>	Approach herkogamy	USA (Boyd, 2004)	–	EU044870	EU044908

*Reported in Böhle et al. (1996); Hilger et al. (2004); Thomas et al. (2008); Ferrero et al. (2009).

the stamens are spread along the flower tube and display a distribution of different heights (Ferrero et al., 2009, 2011a).

Hand pollination experiments

To determine the types of incompatibility systems present in the studied species, controlled pollination experiments were conducted in the field during the spring of 2007 and 2008. On each plant, we marked and bagged four flower buds that were randomly assigned to one of the following treatments: (1) obligatory self-pollination; (2) intra-morph cross-pollination (L × L or S × S); (3) inter-morph cross-pollination (L × S or S × L); or (4) spontaneous self-pollination. For treatments (2) and (3), flower buds were emasculated. To study the extent to which the amount of pollen deposited on stigmas may limit fruit and seed production, on each plant, we marked two additional flowers that were not bagged (natural pollination was allowed), to which we applied supplementary manual inter-morph cross-pollination (treatment 5). Unmanipulated flowers (treatment 6) were also studied as controls (natural pollination was allowed). The six treatments were carried out on each plant, with at least 11 plants treated for each morph (sample sizes are summarized in Table 2). For inter- and intra-morph cross-pollinations, we used a mixture of pollen from the anthers of the recently opened flowers of several plants located 15–30 m away from the experimental plants.

Statistical comparisons between treatments were based on the seed to ovule ratio (S/O). We conducted two independent analyses, one for the incompatibility system and another for

the pollen limitation experiment. In both, the effects of pollination treatment, morph and species were considered fixed factors and explored by Generalized Estimating Equation (GEE) models and an 'independent' correlation matrix structure. We chose this matrix because it was the one best tuned for our data. The S/O ratio was modelled as a binomial distribution, with logit used as the link function. A backward stepwise elimination procedure was used to determine the minimal adequate model.

Reproductive indices

In order to characterize the breeding system of each species quantitatively, we calculated the following indices, using the S/O values obtained in the manual pollination experiments.

Self- and morph compatibility and self-fertilization indices. The self-compatibility index (SCI) (Lloyd and Schoen, 1992) was calculated as the ratio of S/O values from self-pollination and inter-morph crosses. We extended this index for calculating the degree of within-morph compatibility (MCI) in style polymorphic species, which was calculated as the ratio of S/O values from intra-morph crosses and inter-morph crosses. The same criteria used for SCI were used for MCI to differentiate the types of morph compatibility. An SCI or MCI ratio of 0.75 was used as the threshold value (Lloyd and Schoen, 1992). A value <0.75 indicates that the species are self-incompatible (SCI) or incompatible with others of the same morph (MCI). A value >0.75 for the SCI or MCI

TABLE 2. Number of treated flowers [n (total number of L plants treated, total number of S plants treated)], fruit set and seed to ovule ratio (S/O) for *Glandora* and *Lithodora* studied species under different hand pollination treatments

Pollination treatment	Morph	<i>G. diffusa</i>			<i>G. moroccana</i>			<i>G. nitida</i>			<i>G. oleifolia</i>			<i>G. prostrata</i>			<i>L. fruticosa</i>		
		n (15, 16)	Fruit set	S/O	n (16, 18)	Fruit set	S/O	n (20, 21)	Fruit set	S/O	n (12, 11)	Fruit set	S/O	n (29, 29)	Fruit set	S/O	n (16, 17)	Fruit set	S/O
Self-pollination	L	13	0.23	0.08	16	0.47	0.12	14	0.07	0.02	9	0.22	0.06	28	0.18	0.08	10	0.1	0.03
	S	15	0.40	0.15	18	0.22	0.06	13	0.23	0.06	10	0.10	0.03	27	0.22	0.09	15	0	0
	Total	28	0.32	0.12	35	0.34	0.09	27	0.14	0.04	19	0.16	0.04	55	0.20	0.09	25	0.04	0.01
Intra-morph ($L \times L$, $S \times S$)	L	14	0.36	0.13	15	0.53	0.13	18	0.22	0.06	8	0.38	0.09	26	0.46	0.23	11	0.45	0.11
	S	15	0.47	0.17	18	0.56	0.14	17	0.06	0.01	9	0.11	0.03	26	0.46	0.29	13	0.31	0.12
	Total	29	0.41	0.14	33	0.55	0.14	35	0.14	0.04	17	0.24	0.06	52	0.46	0.26	24	0.38	0.11
Inter-morph ($L \times S$, $S \times L$)	L	15	0.47	0.15	16	0.5	0.13	18	0.50	0.14	8	0.25	0.06	27	0.41	0.24	14	0.57	0.18
	S	14	0.36	0.21	15	0.2	0.06	19	0.42	0.11	9	0.33	0.08	25	0.52	0.24	14	0.21	0.09
	Total	29	0.41	0.18	31	0.35	0.10	37	0.46	0.12	17	0.29	0.07	52	0.46	0.24	28	0.39	0.13
Spontaneous self-pollination	L	15	0	0	16	0	0	17	0	0	11	0	0	27	0	0	14	0	0
	S	14	0	0	18	0	0	17	0	0	11	0	0	24	0	0	13	0	0
	Total	30	0	0	35	0	0	34	0	0	22	0	0	51	0	0	27	0	0
Supplementary pollination	L	9	0.89	0.44	16	0.65	0.19	15	0.60	0.15	11	0.55	0.14	11	0.73	0.23	8	0.63	0.14
	S	13	0.46	0.29	17	0.59	0.15	14	0.50	0.12	10	0.40	0.10	16	0.44	0.17	9	0.56	0.17
	Total	22	0.64	0.35	34	0.62	0.15	29	0.55	0.14	21	0.48	0.12	27	0.56	0.19	17	0.59	0.16
Control	L	12	0.58	0.25	15	0.47	0.12	13	0.23	0.06	11	0.27	0.07	9	0.67	0.30	10	0.50	0.16
	S	12	0.67	0.29	17	0.76	0.19	17	0.24	0.06	10	0.30	0.08	9	0.44	0.19	8	0.13	0.06
	Total	24	0.63	0.27	32	0.63	0.16	30	0.23	0.06	21	0.29	0.07	18	0.55	0.26	18	0.33	0.13

Floral morphs: L, long styled; S, short styled. Results are presented for each floral morph and the total for each species.

indicates self-compatibility or intra-morph compatibility, respectively.

Percentage of pollen limitation. Limitation of reproductive success due to low pollen delivery was measured as the percentage of pollen limitation (PPL), calculated as follows:

$$\text{PPL} = [100 \times (\text{PS} - \text{C})] / \text{PS}$$

In this equation, PS represents the S/O value of pollen-supplemented flowers, and C represents the S/O value of control flowers (Jules and Rathcke, 1999).

Molecular analysis

A matrix of two DNA regions (ITS; *trnL_{UAA}* intron) was constructed using sequences from GenBank for *Glandora*, *Lithodora* and other members of Lithospermeae, as well as from some members of the Anchuseae for which incompatibility system information is available. We chose *Anchusa officinalis* and *Borago officinalis* as outgroups, following Thomas *et al.* (2008). The sequences from the *Glandora* and *Lithodora* species correspond to the same populations used in the experiments described above, and they have been phylogenetically analysed by others (Ferrero *et al.*, 2009). Information about taxa and accession numbers is summarized in Table 1. Alignment of the two concatenated DNA regions was carried out with ClustalW (Thompson *et al.*, 1994) as implemented in MEGA 4 (Tamura *et al.*, 2007). Maximum parsimony (MP) and Bayesian inference reconstructions were carried out. Parsimony analyses were run using Fitch parsimony with PAUP* (Swofford, 1999), operated in a Windows environment using the PAUPup graphical interface (Calendini and Martin, 2005). Equal weighting of all characters and of transitions/transversions was used. Heuristic searches were replicated 100 times under the random taxon addition sequences option. Support for monophyly of groups was assessed by ‘full’ bootstrapping (1000 replicates) using the heuristic search strategy mentioned above. The Hierarchical Likelihood Ratio Test (hLRT) and Akaike Information Criterion (AIC) were implemented for each of the two partitions independently using MrModeltest 3.7 (Posada and Crandall, 1998; Nylander, 2002) to determine the simplest model of sequence evolution that best fits the sequence data. A Bayesian inference of phylogeny with Markov chain Monte Carlo sampling was conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). 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 = 10000). A general time-reversible model of DNA substitution and a shape parameter of the gamma distribution model (GTR + G) were used, with different parameter settings for the two genes. We estimated the 50 % majority-rule consensus of the remaining trees and used posterior probability (PP) as an alternative estimate of robustness.

Ancestral character reconstruction

The evolution of heterostyly and incompatibility systems assuming MP was reconstructed using the 50 % majority-rule

consensus tree recovered from the Bayesian analysis of the combined nuclear and plastid data sets. Three states of sexual polymorphism were considered, which are the three present in *Lithodora* and *Glandora* (Ferrero *et al.*, 2009, 2011a), and two types of monomorphism were considered: 0, non-herkogamous monomorphism (one morph with equal heights of stigma and anthers); 1, approach herkogamy (one morph with the stigma above the anthers); 2, stylar dimorphism (two morphs differing in stigma height but with a similar anther position among morphs); 3, distyly (two morphs with a reciprocal position of anthers and stigmas); 4, relaxed style dimorphism (similar to distyly, but with anthers spread along the corolla tube at different heights; see Ferrero *et al.* 2011a for further detail); and three states for the incompatibility system characterization on the basis of the results obtained from the experimental manipulations: 0, self- and intra-morph compatibility (self-compatibility in monomorphism); 1, self-incompatibility and intra-morph compatibility; and 2, self- and intra-morph incompatibility. For the characterization of the taxa added from GenBank, we consulted Ferrero *et al.* (2009, 2011a) for descriptions of their polymorphism, and Levin (1968), Philipp and Schou (1981), Corbet (1978), Montaner *et al.* (2000), Dupont *et al.* (2004) and Boyd (2004) for information about their incompatibility systems.

Stylar polymorphisms and incompatibility systems were reconstructed by MP using the ‘Unordered’ model in Mesquite 2.01 (Maddison and Maddison, 2007). According to this model, the cost of changing state is 1, and all changes among polymorphism states are equally probable. We also used a likelihood-based reconstruction method, which maximize the probability that the states would evolve under a stochastic model of evolution (Schluter *et al.*, 1997; Pagel, 1999). Maximum likelihood (ML) methods find the state that maximizes the probability of arriving at the observed states in the terminal taxa, given a particular model of evolution for each node. Maximum likelihood reconstruction was carried out based on an Mk1 model in which any particular change is equally probable (‘Markov k-state 1 parameter model’).

Tests for correlated trait evolution were conducted using Bayesian mutation mapping (Nielsen and Huelsenbeck, 2002), as implemented in SIMMAP Version 1.5 (Bollback, 2006), on the final 101 sampled post-burn-in trees of the Bayesian reconstruction. The method accounts not only for phylogenetic uncertainty, but also for mapping uncertainty by allowing the rate of evolution of the character to vary. SIMMAP uses a Gamma prior on the overall substitution rate of a character, in which both parameters of the Γ (Gamma) distribution can be defined. To calculate the priors, an MCMC analysis of 100 000 cycles was first performed on the Bayesian consensus tree to sample the overall rate values (Gamma prior). The samples from the posterior distribution of these parameters were then used to find the best-fitting Gamma distribution using the MASS and TeachingDemos packages (Venables and Ripley, 2002; Snow, 2005) for the R statistical computing platform (version 2.8.1, R Development Core Team, 2008).

The overall evolutionary rate prior had the parameters $\alpha = 4.5$, $\beta = 0.11$ and $\kappa = 60$ for the stylar polymorphism and $\alpha = 5.079$, $\beta = 0.012$ and $\kappa = 60$ for the incompatibility system.

The *D* statistic in SIMMAP was used to calculate the correlation between the states of each character (stylar polymorphism

and incompatibility system), and the M statistic was used to evaluate the correlations between character histories along the phylogeny for the two characters, where f is the fraction of time one state is associated with another in a character history. We let the program take ten stochastic draws from the prior distribution, and ten realizations were sampled for each tree.

RESULTS

Floral polymorphism and reciprocity

The reciprocity index ranged from 0.018 in *G. diffusa* to 0.094 in *G. prostrata* (Table 4), with low index values indicating high levels of reciprocity, and vice versa.

Hand pollination experiments and reproductive indices

The results of hand pollination experiments in terms of the S/O are presented in Fig. 1 and Table 2. There were no progeny after spontaneous selfing, as no unpollinated bagged flower produced fruit, indicating that all of the studied species depend on pollinators to achieve fertilization. For this reason, the spontaneous self-pollination treatment was

excluded in the statistical analysis. For the hand pollination treatment effect, we found significant differences in S/O between species and between treatments, as well as interactive effects in the simplest model (Table 3, Fig. 1). There were no significant differences between morphs in any case ($P > 0.280$). In *G. diffusa*, *G. moroccana* and *G. oleifolia*, there were no significant differences in the S/O between any treatments (Fig. 1). Moreover, S/O values in both self-pollination and intra-morph treatments were greater than zero, indicating that these species are equally self- and intra-morph compatible (Fig. 1). For these species, the values for SCI ranged between 0.54 and 0.91, and MCI values were >0.80 (Table 4). Significant differences in S/O ratio between treatments were found for *G. nitida*, *G. prostrata* and *L. fruticosa* (Fig. 1). However, whereas *G. prostrata* and *L. fruticosa* presented similar S/O values in intra- and inter-morph crosses, thus indicating that they are morph-compatible species (SCI < 0.36 and MCI > 0.86), *G. nitida* showed similar low levels of the S/O between both intra-morph and self-pollinations (SCI = 0.29 and MCI = 0.29), clearly displaying a heteromorphic incompatibility system (Table 4).

For the pollen limitation experiment, significant differences were found between species ($P < 0.001$) and treatments

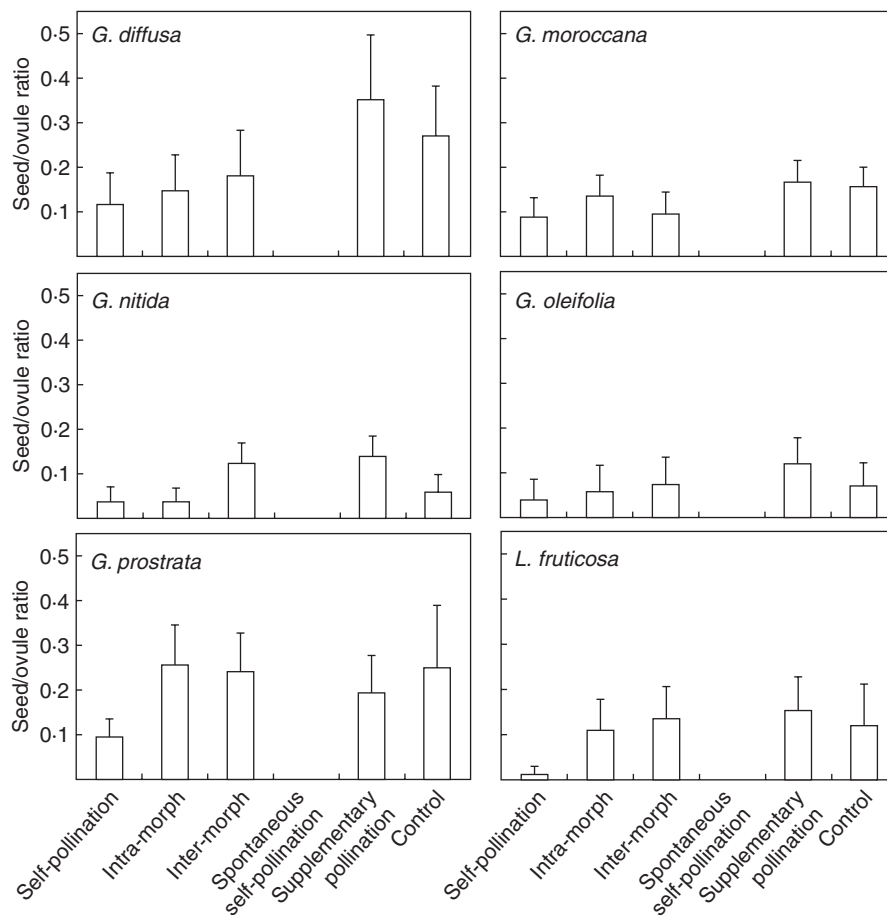


FIG. 1. Mean (with 95 % confidence interval) of the seed-to-ovule ratio in the studied species of *Lithodora* and *Glandora* following hand-pollination treatments. Treatments included self-pollination, intra-morph cross-pollination (L \times L, S \times S), inter-morph cross-pollination (L \times S, S \times L), spontaneous self-pollination, natural pollination plus supplementary pollination and control (natural) pollination. Because there were no statistical differences between floral morphs within species, the results for both morphs are plotted together for each taxon.

TABLE 3. Results of the Generalized Estimating Equations analysis for the comparisons of the seed/ovule ratios after different pollination treatments

Source	Self-pollination, intra-morph, inter-morph crosses			Control supplementary pollination		
	Wald χ^2	d.f.	P	Wald χ^2	d.f.	P
Species	34.35	5	0.000	34.81	5	0.000
Treatment	17.90	2	0.000	4.06	1	0.044
Species \times treatment	18.64	10	0.045	7.46	5	0.188

Analyses were carried out independently for the self-pollination, intra-morph crosses and inter-morph crosses on one hand and the control supplementary pollination on the other. Spontaneous self-pollination was not included in the analysis as it was nearly zero in most cases.

TABLE 4. Type of polymorphism, reciprocity index (following Sánchez et al., 2008), self-compatibility index (SCI, following Lloyd and Schoen, 1992), morph compatibility index (MCI, see details in the Materials and methods) and percentage of pollination limitation (PPL, following Jules and Rathcke, 1999) for the studied species

Species	Stylar polymorphism	Reciprocity index	SCI	MCI	PPL
<i>Glandora diffusa</i>	Distyly	0.0181	0.64	0.81	23.12
<i>G. moroccana</i>	Distyly	0.0254	0.91	1.13	9.09
<i>G. nitida</i>	Distyly	0.0302	0.29	0.29	57.71
<i>G. oleifolia</i>	Distyly	0.0225	0.54	0.80	40.0
<i>G. prostrata</i> subsp. <i>prostrata</i>	Relaxed stylar dimorphism	0.0945	0.36	1.08	-35.71
<i>Lithodora</i> <i>fruticosa</i>	Stylar dimorphism	0.0762	0.07	0.86	22.73

($P < 0.044$) in the simplest model (Table 3, Fig. 1). Significant differences between treatments were only found for *G. nitida* (Fig. 1). The PPL was revealed to be relatively high for *G. nitida* (57.7%) and *G. oleifolia* (40.0% Table 4).

Molecular analysis

For the ITS and *trnL_{UAA}* intron regions, the aligned sequences were 237 and 465 bp in length, respectively. In the MP analysis, 557 characters are constant, 82 are parsimony uninformative and 63 are parsimony informative. In the phylogenetic reconstruction, one polytomy is depicted in the outgroup, and the relationship between *G. nitida* and *G. oleifolia* is not well supported (0.70 PP). Bootstrap values and PP are quite high for the remaining reconstruction (77% and 0.96 PP as minimum values; Fig. 2).

Ancestral character reconstruction

Reconstruction of stylar polymorphism using MP methods indicates that distyly is the most likely ancestral condition in *Glandora* and that relaxed style dimorphism is a derived condition (Fig. 2). According to the MP reconstruction, self- and

morph compatibility is the ancestral condition in the genus *Glandora*, while heteromorphic incompatibility and self-incompatibility are derived conditions. However, following this method, the condition ancestral to the ingroup (i.e. including also *Lithodora*, *Echium*, *Lithospermum* and *Macromeria*) cannot be resolved (Fig. 2). The ML reconstruction method does not clarify any of the more ancestral stages for style polymorphism and does not resolve any ancestral state for the incompatibility systems. *M* and *D* statistics (Huelsenbeck et al., 2003) for measuring the correlation of stylar polymorphism and incompatibility system were not significant ($M = 0.014$, $P = 0.326$; $D = 0.111$, $P = 0.285$).

DISCUSSION

A wide variety of style polymorphisms has been recently reported for *Lithodora*, *Glandora* and other members of the tribe Lithospermeae. This level of variation is one of the highest described thus far at the family level (Ferrero et al., 2009). Here we report that there is also extensive variation in incompatibility systems, mostly within *Glandora*. Both style polymorphism and incompatibility are critical features for testing the alternative hypotheses regarding the evolution of heterostyly (Charlesworth and Charlesworth, 1979; Lloyd and Webb, 1992a). This taxonomic group presents an opportunity to determine which of these two hypotheses is best supported by phylogenetic evidence. Our results indicate that stylar polymorphism is not necessarily coupled with the incompatibility system.

Implications for the evolution of distyly

The great variety of incompatibility systems in the study group and the relatively small number of species precluded any sound ML reconstruction of evolutionary transitions of the relevant traits. Nevertheless, the parsimony analysis indicates that self-incompatibility evolved independently in *Lithospermum carolinense*, *G. nitida* and *G. prostrata*, although the latter is of a contrasted type (morph compatible). This is similar to what was previously found in other related Boraginaceae (*L. fruticosa*, this study; *Anchusa officinalis*, Phillip and Schou, 1981). At least within *Glandora*, incompatibility systems both linked and unlinked to style polymorphism have evolved in different species. Such a contrasting pattern within a single genus is reported here for the first time. In this respect, it is noteworthy that within Boraginaceae, some *Anchusa* species show an incompatibility system that is also unlinked to style length variation (Phillip and Schou, 1981; Schou and Phillip, 1983, 1984), whereas other style polymorphic species of the *Lithospermum* or *Amsinckia* genera typically show self-incompatibility that is linked to style morphs (Levin, 1968; Ganders, 1979). Thus, Boraginaceae is a very good candidate for further work exploring the genetic, physiological and evolutionary relationships between homomorphic and heteromorphic incompatibility systems (Lloyd and Webb, 1992a; Barrett and Shore, 2008).

In a previous study of character reconstruction in Lithospermeae (Ferrero et al., 2009), we found two striking results: that style dimorphism is the ancestral condition to distyly in the genus *Lithodora sensu lato* (including

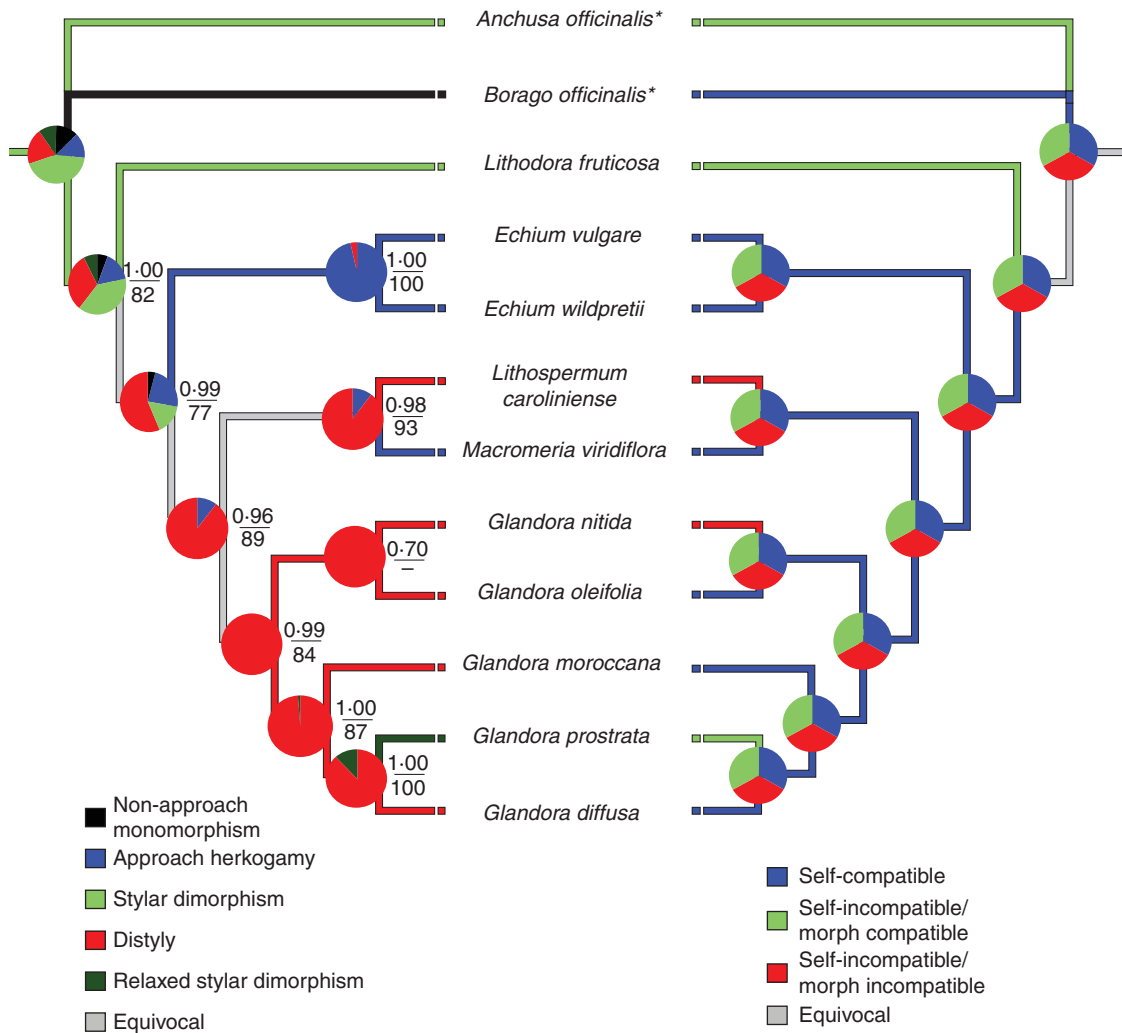


FIG. 2. Evolution of sexual polymorphisms and incompatibility systems within some genera of Boraginaceae, based on the majority-rule consensus tree recovered from the Bayesian analysis of the combined ITS and *trnL_{UAA}* intron data set. Numbers above branches on the left tree indicate posterior probabilities from the Bayesian analysis under the GTR + G model. Numbers below branches are bootstrap values from the MP analysis. The most-parsimonious states are shown under the unordered model (coloured lines of the tree). For the ML character reconstruction, probabilities at each node are reported as proportional likelihoods for each character (sectors of the pie charts).

Glandora), supporting current models for the evolution of distyly (Charlesworth and Charlesworth, 1979; Lloyd and Webb's 1992a), and that a newly described styler polymorphism, termed relaxed styler dimorphism, is derived from distyly in the genus *Glandora* (*G. prostrata*, with variable anther height within a single flower). The results presented here for the reconstruction of incompatibility system evolution can be considered additional support for the Darwinian hypothesis of Lloyd and Webb (1992a). We observed strong variation within the group and an apparent lack of association between incompatibility system and style length variation, and the Darwinian hypothesis predicts no such association between these two reproductive traits.

The sequence of evolutionary events that occurred in the studied taxonomic group is best represented by a model of heterostyly evolution that is partially in keeping with some of the premises of Lloyd and Webb (1992a, b). In particular, the acquisition of a full heteromorphic self-incompatible distylous

condition as a final step (*G. nitida*) of evolution from a self-compatible distylous ancestor fits well with the Lloyd and Webb model. Thus, *Glandora* represents a challenging system to study the physiological, molecular, genetic and ecological (pollen transfer dynamics) basis for the acquisition of the supergene coding for reciprocal herkogamy and heteromorphic incompatibility. It is also worth noting that even ancillary traits, such as the pollen size dimorphism or pollen production traits thought to be associated with the same supergene (Dulberger, 1992), are only found in *G. nitida*. *Glandora nitida* is the only studied species that displays all of the traits usually associated with heterostyly (Ferrero et al., 2011a; this study).

Incompatibility system variation

Incompatibility systems are diverse in their genetic basis and physiological expression, although they are all thought

to be mechanisms for avoiding self-crossing (Barrett, 2002). Whereas homomorphic (without morphological variation within the population) incompatibility systems are variable in their genetic control and site of reaction (Allen and Hiscock, 2008), heteromorphic incompatibility systems are frequently sporophytic and diallelic. The latter usually present two or three crossing groups and a linkage between the incompatibility locus and loci involved in discrete, reciprocal morph variation in stamen and stigma heights (Gibbs, 1986). It is commonly thought that homomorphic and heteromorphic incompatibility systems evolved independently and have distinct modes of action (Gibbs, 1986; Barrett and Shore, 2008). There is no available information on the genetic basis and the physiological mechanisms of the incompatibility system operating in our studied species, but our crossing experiments provide some information about the possible mechanisms involved.

In general, we observed low S/O values in all experimental pollination treatments; the values were all <0.44. Lower values of seed set have been reported previously for the Boraginaceae (Casper and Wiens, 1981; Goulson et al., 1998), which might be related to intrinsic limitations on the development of the four ovules in the flower. In light of our results, we are able to report full self-compatibility, self-incompatibility with intra-morph compatibility and self-incompatibility with intra-morph incompatibility. To our knowledge, this is the first report of such a wide-ranging set of incompatibility systems found within such a small group of species, and we believe that this observation casts doubt on their supposedly independent evolutionary origins. Full self-compatibility is a widespread feature in the studied group (*G. diffusa*, *G. moroccana* and *G. oleifolia*), and it is even found in some related species, such as *Echium vulgare*, *E. wildpretii* and *Macromeria viridiflora* (Corbet et al., 1978; Boyd, 2004; Dupont et al., 2004). Despite the lack of strong support for self-compatibility as a derived state in our reconstruction (see parsimony analysis in Fig. 2), it seems reasonable to assume that self-compatibility in the three species in our group was selected for reproductive assurance, as has been shown in other heterostylous groups (Schoen et al., 1997; Pannell and Barrett, 1998). It is worth noting that two of the three self-compatible species (*G. oleifolia* and *G. moroccana*) are narrow endemic species with very small and isolated populations, such that reproductive assurance is more likely to apply due to the scarcity of mates. However, these species still rely on insects for pollination (see results of supplemental and autonomous hand pollination experiments), which probably indicates a mixture of self-geitonogamous and cross-pollination that would result in a typical mixed-mating system for these species.

The three narrow endemic species, comprising a few small and sparse populations, show the lowest values of S/O in all the hand cross-pollination treatments (<10%), irrespective of their being self-compatible (*G. moroccana* in N Morocco and *G. oleifolia* in the E Pyrenees, NE Spain) or self-incompatible (*G. nitida* in S Spain). There is no information on their population genetic structure, although the restricted range and small population size may have led to low genetic variation and high biparental inbreeding levels in their populations (Allee effect; Courchamp et al., 1999; Stephens

et al., 1999). Moreover, the two narrowest and most threatened endemics (*G. nitida* and *G. oleifolia*; Bañares et al., 2003) suffer to some extent from the effects of pollen limitation (high PPL), an additional cause of low reproductive output in small populations and narrow distribution ranges (Knight et al., 2005).

In this study, we found that the three cases of style polymorphism in which there is a lack of perfect reciprocity share a particular incompatibility system: self-incompatibility with intra-morph compatibility. This association has been reported in other species, such as *Mirabilis froebelii* (Bateman, 1968), *Quinchamalium santalense* (Riveros et al., 1987), several species of *Narcissus* (Dulberger, 1964; Barrett et al., 1997; Sage et al., 1999; Baker et al., 2000; Arroyo et al., 2002; Pérez-Barrales et al., 2006) and *Anchusa hybrida* (Dulberger, 1970). Perhaps the presence of this incompatibility system, which is unlinked to style polymorphism (probably gametophytic and multiallelic; Dulberger, 1964), is only a by-product of the historical (phylogenetic) effects in these particular plant groups, but this claim requires a wider sampling of taxa to be conclusive. In this respect, it is worth noting that a recent paper by Cohen (2011) reports a variability of floral morphology in the phylogeny of *Lithospermum*, with several independent gains of heterostyly. It is desirable to have data on the breeding systems of these species in order to have a larger sample size for estimating phylogenetic correlations between these critical traits.

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