

RESEARCH PAPER

Reciprocal style polymorphisms are not easily categorised: the case of heterostyly in *Lithodora* and *Glandora* (Boraginaceae)

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Ancillary character; disassortative mating; distyly; heterostyly; phenotypic integration; relaxed stilar dimorphism; stilar polymorphism.

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Editor

F. Roux

Received: 14 September 2009; Accepted: 20 November 2009

doi:10.1111/j.1438-8677.2009.00307.x

ABSTRACT

Interest in reciprocal floral polymorphisms, such as heterostyly, has increased in recent decades because they can be used as suitable model systems to study mechanisms of outbreeding and disassortative mating in plants. Heterostylous plants are characterised by the presence of discrete morphs that differ in sex organ position and in some other ancillary traits. As regards sex organ deployment, different types of polymorphisms have been described, depending on number and type of discrete classes present in populations and degree of reciprocity between them. However, a clear-cut characterisation of stilar polymorphisms does not appear to be the best approach when there is great variability among populations because of continuous variation of some of traits examined. A recent study in *Lithodora sensu lato* (recently split into two separate genera, *Lithodora* and *Glandora*) showed a wide variation in sex organ position across species in the genus, which warrants precise population analysis of stilar polymorphism and its reciprocity. We provide a detailed morphometric analysis of flower sexual traits and include those considered to be ancillary characters. We report a wide variation in these traits in populations of *Lithodora s.l.* and highlight the subjectivity of the former characterisation of style polymorphism based on visual inspection. Ancillary traits appear repeatedly in *Lithodora* and *Glandora*, particularly in the latter. The appearance of these traits seems to be related to greater reciprocity between sexual whorls in *Glandora*, with the exception of *G. prostrata*. These results agree with evolutionary steps proposed in the build-up of heterostyly according to some evolutionary models. We also examined variation in polymorphisms in light of current models for evolution of heterostyly, and, more specifically, we sought to verify the prediction that flower traits as a whole (*i.e.*, flower integration) respond to selective pressure to assure the exact location of pollen on the pollinator body. Most reciprocal populations and species, where between-morph pollen transfer is expected to be higher, would show greater integration. Our results confirm this hypothesis.

INTRODUCTION

Heterostyly has been traditionally described as a discrete floral polymorphism, primarily characterised by reciprocity in sex organ position between two (distyly) or three (tristyly) morphs in a population. Distyly is the most frequent type of heterostyly and has generated much more interest. Some authors (Lloyd & Webb 1992a) have also termed distyly 'reciprocal herkogamy', since it involves reciprocity and separation between anthers and stigmas, (*i.e.*, herkogamy; Lloyd & Webb 1986). Therefore, heterostyly, or reciprocal herkogamy, involves two morphs within a single population: approach herkogamous, where the stigma is placed above the anthers in a flower (also called long-styled or L-morph), and reverse herkogamous, with the stigma placed below the anthers (short-styled or S-morph). These arrangements are also frequently found in monomorphic, non-heterostylous popula-

tions and species (see Lloyd & Webb 1986 for review). As explained above, the distinction between these types is only morphological, but it has important functional consequences. The reciprocal positioning of sex organs between morphs has been considered a way of increasing legitimate pollinations (*i.e.*, between morphs) and reducing pollen wastage while avoiding interference between male and female functions within the flower (Darwin 1877; Lloyd & Webb 1992a,b; Barrett 2002). Reciprocal herkogamy is often accompanied by a diallelic incompatibility system that prevents self- and intra-morph cross-fertilisations; moreover, L- and S-morphs tend to show differences in other floral traits called ancillary characters (Ganders 1979; Dulberger 1992), which are defined below.

The presence of the two reciprocal style morphs has been regarded as a necessary and sufficient condition to characterise a species as distylous, provided that it is sufficient to

determine enough levels of legitimate pollination (Lloyd & Webb 1992b) due to the high reciprocity between morphs (Cesaro & Thompson 2004). However, in practical terms, most heterostylous species have been defined as such only if simple approach (L-morph) and reverse herkogamous (S-morph) flowers co-occur within a population (Webb & Lloyd 1986). Without taking into account the degree of reciprocity between sex organs (e.g., Baker 1964; O'Brien & Calder 1989; Goldblatt & Bernhardt 1990). While the presence of these two stylar morphs is easily observed by visual inspection, the extent of reciprocity between anthers and stigmas requires detailed measurements, which have been the subject of some controversy (Sánchez *et al.* 2008). Moreover, reciprocity is the key morphological trait that distinguishes distyly from other polymorphisms, such as style dimorphism (i.e., different morphs for style length but no variation in anther position between morphs, leading to low reciprocity between sex organs; Baker *et al.* 2000; Barrett *et al.* 2000; Arroyo *et al.* 2002).

The distinction between style dimorphism and distyly is critical because recent models for the evolution of distyly consider the former, which should support high levels of illegitimate (i.e., within-morph) cross-pollination, as the intermediate ancestral state to distyly (Charlesworth & Charlesworth 1979; Lloyd & Webb 1992a). Such a prediction has been tested in a few plant groups, but the distinction does not consider quantitative levels of reciprocity (Graham & Barrett 2004; Pérez *et al.* 2004; Pérez-Barrales *et al.* 2006; Ferrero *et al.* 2009), despite the fact that some experimental studies have demonstrated that only a certain degree of reciprocity – which is far from perfect – may account for high enough legitimate pollination (Cesaro & Thompson 2004; Cesaro *et al.* 2004). Thus, it is critical to quantitatively estimate levels of reciprocity when variation in sex organ position is observed across plants in a population or in populations within a species and in species within a lineage, to be able to provide a sound basis for further testing of hypotheses on the evolution of heterostyly.

An additional source of confusion is that flowers are considered modules with high phenotypic integration due to architectural constraints, genetic correlations or selective pressures for enhancing pollen delivery and receipt (Berg 1960; Hansen *et al.* 2007; Pérez-Barrales *et al.* 2007; Ordano *et al.* 2008). This may strongly constrain the independent evolution of sex organ position within a flower. Therefore, high reciprocity between morphs in heterostylous species should be related to a pollination mode that prompts the precise location of pollen on particular parts of pollinator bodies, which, in turn, should determine high flower integration, considering both flower sex organs and perianth traits.

Other features, called ancillary traits, have also commonly been related to heterostyly. Among these, differences in pollen size and production, exine sculpturing, size and shape of stigmas and their papillae have been widely reported in heterostylous taxa, usually linked to style morphs (see reviews by Ganders 1979; Dulberger 1992). The function of ancillary traits is generally associated with the promotion of legitimate pollination to a similar extent in both morphs and the prevention of selfing or fertilisation among plants of the same morph (Ganders 1979; Dulberger 1992). Although less frequently reported, any differences in flower size among

morphs are also considered to be ancillary traits, although their functional significance remains doubtful (Ganders 1979). However, this flower size dimorphism may affect primary heterostylous traits (i.e., sex organ position); thus, a functional connection would be expected, at least for this ancillary trait. Before sound testing of the evolutionary hypotheses of heterostyly can be carried out, it will be necessary to study and quantify in detail the morphological variation of all the traits assumed to be involved, and to establish the association between them.

The family Boraginaceae presents numerous cases of stylar polymorphism in diverse genera (see Harriman 2005 for references). Among them, *Lithodora* and *Glandora* (*Lithodora s.l.*) show a particularly high variation in stylar conditions, making it possible to test evolutionary hypotheses of heterostyly (Ferrero *et al.* 2009). All species of *Lithodora* and *Glandora* are apparently stylar polymorphic, since populations of all of these species were found to have two morphs for style length: one morph with styles above the anthers (approach herkogamous) and the other positioned below (reverse herkogamous). Preliminary studies in only a few populations have shown that sex organ reciprocity, in comparison with other taxa, is highly variable (Sánchez *et al.* 2008). This variability makes the group suitable for quantifying variation in all traits associated with heterostyly, whether they are primary or ancillary. In particular, the aims of this study were: (i) to study flower morphometrics in several populations of all species in *Lithodora s.l.* (three species of *Lithodora* and six of *Glandora*) to determine the level of reciprocity and its variation across morphs, populations and species; (ii) to estimate the level of flower phenotypic integration and relate it to the level of reciprocity; and (iii) to measure the flower traits that may show ancillary variation (perianth, pollen and stigma morphology). The general purpose of the study was to explore morphological variation in populations and species of a plant group that may provide further insight into the evolution of heterostyly.

MATERIAL AND METHODS

Study species

The genus *Lithodora*, which traditionally consisted of nine species distributed around the Mediterranean Basin, has recently been split into the genera *Glandora* and *Lithodora* (Thomas *et al.* 2008), according to molecular phylogenetic information. *Glandora* and *Lithodora* are perennial shrubs with flowers on top of the foliate branches. Flowers in both genera are actinomorphic and sympetalous, form a floral tube, and have five small stamens inserted in the corolla tube.

Flower morphometrics

We surveyed five populations of each species and subspecies (*Lithodora fruticosa*, *L. hispidula*, *L. zahnii* and *Glandora diffusa*, *G. moroccana*, *G. nitida*, *G. oleifolia*, *G. prostrata*, *G. ros-mariniifolia*) in the spring of 2005 or 2006. We included the two existing species of *G. prostrata* (*G. prostrata* subsp. *prostrata* and *G. prostrata* subsp. *lusitanica*) and two out of three of *L. hispidula* (*L. hispidula* subsp. *hispidula* and *L. hispidula*

subsp. *versicolor*). We were not able to sample *Lithodora hispidula* subsp. *cyrenaica* (endemic to Libya). For some narrow endemic species, the number of populations sampled was limited by the populations that are currently known (see Appendix S1). In each population, we collected one recently opened flower per plant in 100 individuals, where possible. We took special care to avoid repeatedly sampling ramets within genets, which sometimes occur in these plants. For this reason the individuals sampled were separated by at least 1 m. Flowers were preserved in 70% ethanol until measured in the laboratory. We also collected a voucher specimen for identification of the samples (deposited in SANT herbarium). Flowers were split longitudinally and measurements were taken from digital photos (on flowers before and after dissection) with the image analyser software *analySIS 5.0* (Soft Imaging System GmbH, Münster, Germany). The floral traits recorded were: (i) corolla length; (ii) tube width (on photo of the flower before splitting); (iii) style length, up to the stigmatic surface; (iv) height of each of the five stamens, up to the midpoint of each anther; (v) length of each anther. Measurements (i), (iii) and (iv) were taken from the bottom of the corolla tube (see Fig. 1A). The number of samples is shown in Table S1 (see Supporting information).

For characterisation of each population and species as distylous or stylar dimorphic, we followed criteria similar to the method used in Ferrero *et al.* (2009). The distinction between distylous and stylar dimorphic species was that the former has two morphs for both style length and anther height, whereas morphs of the latter lack anther height dimorphism and their stamens are approximately equal in height. When sexual whorls are perfectly reciprocal, the position of stigmas and complementary anthers in the opposite

morph should coincide. The overlapping area of this complementary positioning would include the dimensions of the whole anther. Thus, we considered that if the separation between the height of stigmas and corresponding stamens exceeded half of that of the anther dimension, there would be no overlap, thereby resulting in a lack of reciprocity (Fig. 1B). The following comparisons were made: half of the length of S anthers was measured against the difference in stigma–stamen heights for the higher whorl, and the opposite for the lower whorl. We used the term *mixed polymorphism* when upper and lower whorls presented different polymorphisms.

We also calculated the degree of reciprocity between sexual whorls for each population following Sánchez *et al.* (2008). The reciprocity index proposed by Sánchez *et al.* (2008) compares stigma–stamen height gaps for all potential crosses in the population. This index comprises stigma–stamen distance as well as dispersion, and is not skewed by favouring the more prevalent morph. These data may be meaningful when comparing populations.

To analyse whether the length of the stamens and styles experienced the same variation in each of the whorls [*i.e.*, styles in the L-morph and stamens of the short (higher whorl) and the opposite (lower whorl)] we plotted the stamen/style CV (coefficient of variation) ratio for both sexual level (higher and lower whorls) and populations. A ratio more than one means that stamen heights experience greater variation than style lengths at that level; a value < 1 indicates the opposite.

For each species, we compared corolla length, tube width, anther length, pollen production and pollen axis length between morphs and among populations using a two-way mixed model ANOVA, with ‘population’ as a random factor and ‘morph’ as a fixed factor. In the endangered and narrow endemic *G. nitida*, a *t*-test for independent samples was carried out for corolla length, tube width and anther length on the Sierra Mágina population only. The small sampling size in the other two populations studied ruled out other statistical analyses.

Phenotypic integration

To determine whether all flower traits respond together to selective pressures related to precise pollen delivery and deposition by pollinators, we estimated the flower phenotypic integration indices in morphs and populations of all species, including both sex organ and perianth traits shown in the morphometric analysis. Precise pollination would imply high reciprocity between sex organs of the morphs and also high correlation (*i.e.*, integration) among all other flower traits affecting the behaviour of the pollinator in the flower. Phenotypic integration was estimated through the eigenvalues of a correlation matrix and subsequent principal component analysis (PCA; Wagner 1984; Cheverud *et al.* 1989). The magnitude of phenotypic integration is represented by the integration index (variance of the eigenvalues of the correlation matrix of each population). Because sample size varied among populations, we used the corrected integration index, by subtracting the expected value of integration under the assumption of random covariation of traits (random integration = no. of characters – 1/no. of plants; Wagner 1984;

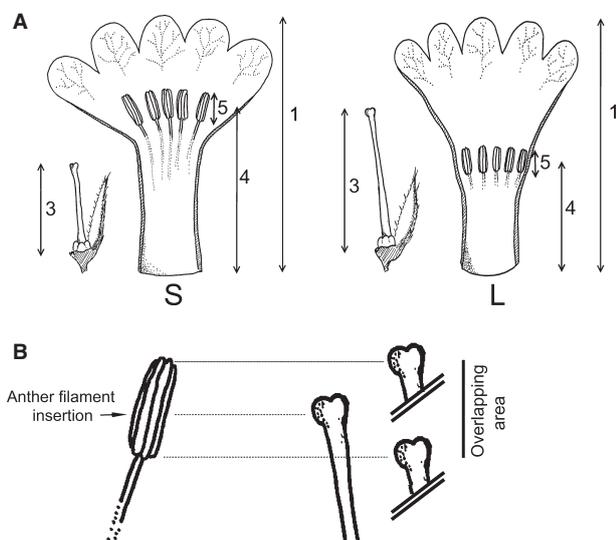


Fig. 1. A: Short-styled (S) and long-styled (L) flowers of a typical distylous *Glandora* species (*G. moroccona*). Numbers correspond to morphometric measurements of each flower: (i) corolla length; (ii) corolla tube width; (iii) style length; (iv) stamen height; and (v) anther length. Corolla tube width was measured on photos of the flowers before splitting (not shown). B: Detail of the overlapping area between an anther and three possible stigma heights for which it would be reciprocal. The overlapping area corresponds to the mean stamen height (corresponding to the filament insertion height) \pm half of anther length.

Herrera *et al.* 2002). The integration index was also expressed as the percentage of maximum possible value, which is the number of traits considered (Herrera *et al.* 2002). For each population, we elaborated two correlation matrices, one for each morph, including all the flower traits measured in each flower (one per plant) to allow for a comparison of integration between morphs, which was done by bootstrapping (20,000 runs) the observed values of integration for each morph across populations within a species (only species with data from at least four populations were used for comparisons). Also, averaged values of both morphs were used as the population value, which were regressed against population reciprocity values. Ancillary traits of stigmas and pollen were not included in the integration analysis since they were measured in different flowers.

Ancillary traits of pollen and stigmas

Pollen production

To evaluate the number of pollen grains produced in each flower morph, we selected one anther per bud in 10 individuals per morph (where possible) and kept them in 70% ethanol (Table S2 for number of samples, see Supporting information). Under a magnifying glass, we extracted all the pollen in the bud and placed it on a drop of isotonic solution, ISOTON II, on a microscope slide. Then, using more isotonic solution, we poured all the pollen carefully into a glass vial containing 20 ml ISOTON II. Pollen grains were counted from 0.1 ml of solution using a Z2™ Coulter Particle Counter® (ETL Testing Laboratories Inc., NY, USA). In order to keep the sample homogenised when counting, the protocol was as follows: 10 s in a bath sonicator (Ultrasonic Cleaner; Fungilab, Spain) – three measurements. We repeated this twice for each individual sample. To estimate total pollen per flower, the number of grains was multiplied by the number of anthers in a flower, *i.e.*, five in all cases. The L:S ratio in pollen grain production was then calculated.

Pollen size and morphology

For pollen size, samples were collected from buds preserved in 70% ethanol. We chose one anther per bud from 10 individuals of each morph. For some species, only one population was sampled because of the impossibility of collecting more floral buds. Pollen was mounted on slides in a drop of glycerol and photographed under an optical microscope ($\times 200$). Then, we measured the polar and equatorial axes in 50 grains from photos taken with *analySIS 5.0* software. We calculated the S:L ratio as the relation between pollen size in S- and L-morphs. For characterisation of pollen shape, samples of the L- and S-morph, kept in 70% ethanol, were prepared according to the acetolysis method (Erdtman 1960) and dropped onto Isopore membranes. Samples were mounted on metallic stubs and coated with a gold/palladium film at high vacuum in a sputtering chamber. Pollen was then observed with a Jeol JSM 6700 f scanning electron microscope (SEM), operating at 12 kV. The locations of the samples are indicated in Appendix S1. For pollen size, we first analysed whether both axes were correlated. For species with only one population sampled (*G. moroccana*, *G. nitida*, *L. hispidula* and *L. zahonii*), we carried out a *t*-test for independent samples.

Morphology of stigmatic papillae

In order to find differences in papillae size between morphs, measurements of maximum diameter of 15 papillae (when possible) were taken using photos from the SEM and the image analyser software *analySIS 5.0*. We chose one style per flower in 10 individuals of each morph. Flowers were the same as those used for characterisation of pollen exine sculpturing.

For the characterisation of stigma shape, styles from opened flowers were collected and preserved in 70% ethanol. Samples were dehydrated through successive aqueous ethanol solutions of increasing concentrations (70–100%), and then ethanol was replaced by successive amiloacetate:ethanol solutions (1:3, 2:2, 3:1). Finally, samples were critical point-dried, and the same protocol used in pollen shape was then followed. This methodology has already been used in Boraginaceae (Bigazzi & Selvi 2000). The sample locations are summarised in Appendix S1. For the difference between morphs in papillae size, a one-way ANOVA was carried out for each species.

RESULTS

Flower morphometrics

Average values of measurements of flower traits for each population studied are given in Table S1. Measurements of sexual whorls for each species are represented in comparative plots in Fig. 2 for *Glandora* species and in Fig. 3 for *Lithodora* species. For characterisation of populations according to their floral polymorphism, in some cases each sexual level displays a different degree of anther–stigma separation (*i.e.*, one sexual whorl shows a value of the separation above half-anther dimension, whereas the other is below it). Following our criteria to characterise the polymorphism, *Glandora prostrata* subsp. *prostrata* and subsp. *lusitanica*, *Lithodora fruticosa* and *L. zahonii* can be defined as stylar dimorphic, despite the fact that it becomes mixed polymorphic in some cases. *Glandora diffusa*, *G. moroccana*, *G. rosmarinifolia* and *L. hispidula* subsp. *hispidula* mainly present distylous populations. However, some populations also show mixed polymorphism, and in the case of *L. hispidula* subsp. *hispidula*, two of the populations are characterised as stylar dimorphic (Table S1). In *L. hispidula* subsp. *versicolor*, one of the populations is distylous and the others are mixed polymorphic. In *G. nitida*, one population is distylous whereas the other two are described as style dimorphic (but with a very small population size). In *G. oleifolia*, one population is defined as distylous and the other as mixed polymorphic. It is interesting to note that in mixed polymorphic populations the low sex organ level has greater stigma–anther reciprocity than the high sex organ level. Results for the index of reciprocity between sexual whorls are plotted in Fig. 4. The index values become smaller as the populations achieve perfect reciprocity. In the overall species set, index values for species of *Glandora* (except for *G. prostrata*) are lower (*i.e.*, they present higher reciprocity) than those for *Lithodora*. It is interesting to note that the index value is also quite variable, even among populations within a species, as in *G. prostrata* subsp. *prostrata*. Populations of *L. fruticosa* present, as a whole, the highest values in their genus.

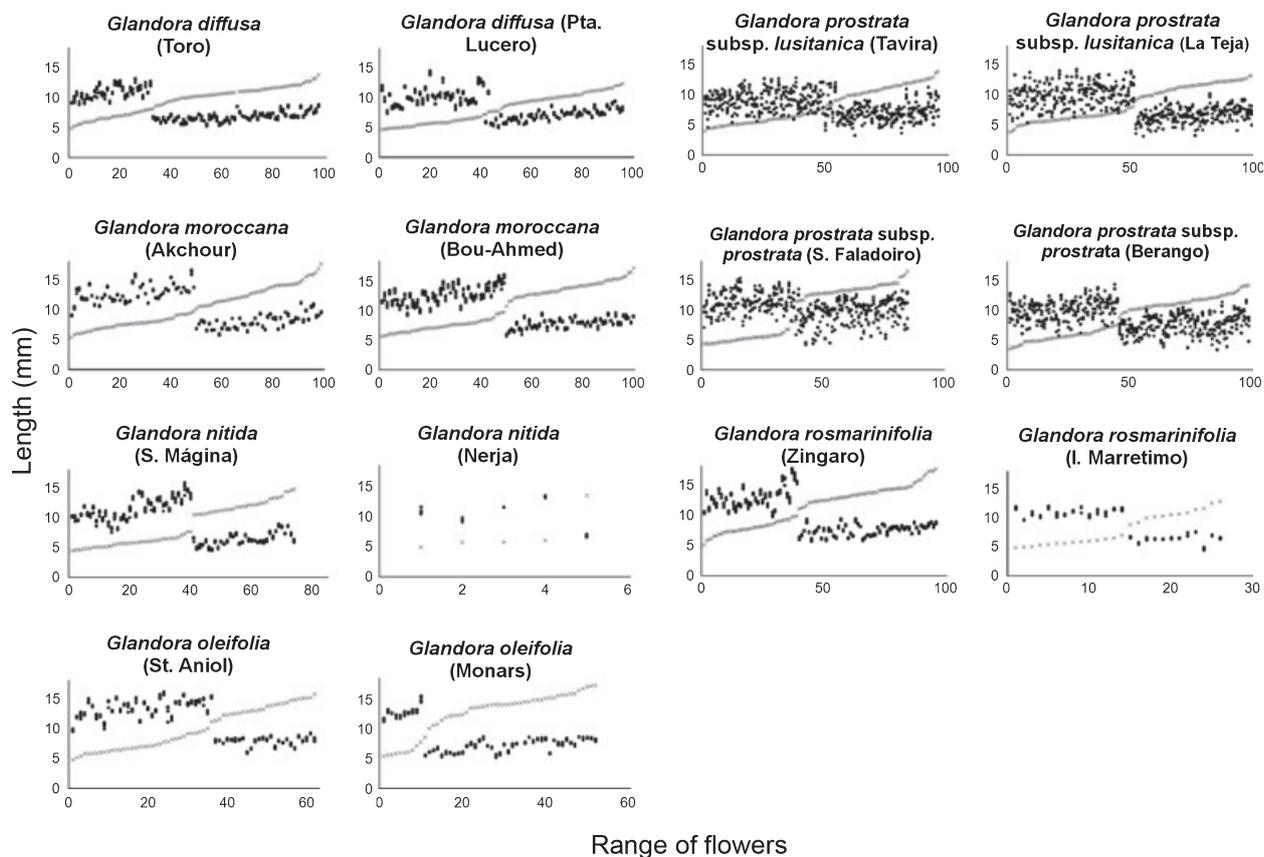


Fig. 2. Variation in style length (+) and every stamen height (●) in two contrasting populations of each species of *Glandora*. Plots summarise individuals in the populations sampled, which are arranged in order of increasing style length.

The results of the comparison between morphs and among populations for corolla length, tube width and anther length are summarised in Table 1. Differences were found in all species for both morph and population factors.

Figure 5 includes the ratio between the CV of stamens and styles for each population. Most populations present values close to one in both whorls, except the populations in both subspecies of *G. prostrata*, with maximum values for the higher level (*i.e.*, variation of stamen height is greater than that of styles).

Phenotypic integration

The average value of flower phenotypic integration for all the species and subspecies sampled was 50.4%, although variation across species was also high (range: 33.9% in *G. prostrata* subsp. *lusitanica* to 64.7% in *G. nitida*). Differences between morphs (examined only when at least four populations were sampled) were not significant ($P = 0.0883$ – 0.3233), except in *G. prostrata* subsp. *prostrata* ($P = 0.0173$) and *G. prostrata* subsp. *lusitanica* ($P = 0.0187$). In both cases, the S-morph showed higher phenotypic integration than the L-morph. There was a significant negative correlation ($R = -0.3504$, $df = 43$) between flower phenotypic integration and the sex organ reciprocity index (although the variance explained was low (12.3%); Fig. 6).

Note that reciprocity index values are lower for more reciprocal species, thus the correlation between integration and reciprocity is positive.

Ancillary characters

Pollen production

The results for pollen production are summarised in Table 1. Pollen production is highly variable among the species (ranging from 116,700 to 540,333 grains per flower). Significant differences among populations were found in *G. diffusa*. Differences between morphs in pollen production were found in *G. nitida* and *G. rosmarinifolia* (Table S2). In both cases, the L-morph produces more pollen than the S-morph. The S-morph pollen:L-morph pollen ratios in *G. nitida* and *G. rosmarinifolia* are close to two and greater than two, respectively.

Pollen size and morphology

The results for pollen size characterisation are summarised in Table S2. Correlation between the polar and equatorial axes was found to be high and significant ($r > 0.70$, df from 6 to 38, $P < 0.001$) in all the species. For this reason, we only carried out comparisons between morphs for the polar axis. Significant differences between morphs were found for *G. nitida* and *G. rosmarinifolia*. Differences among populations were

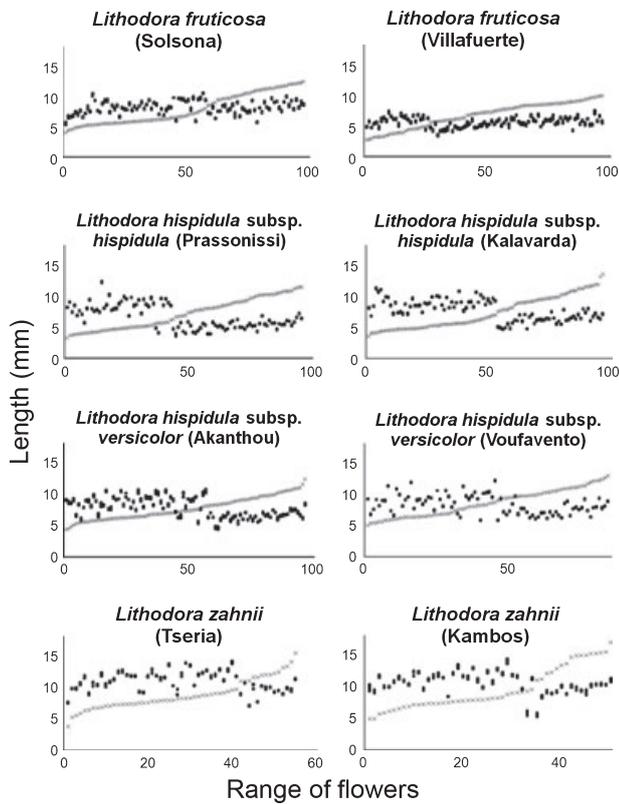


Fig. 3. Variation in style length (+) and each stamen height (●) in two contrasting populations of all species of *Lithodora*. Plots summarise individuals in the populations sampled, which are arranged in order of increasing style length.

found in *G. prostrata* subsp. *prostrata*. The ratio between morphs is close to one in most species, but it is >1.5 in *G. rosmarinifolia* and close to 1.4 in *G. nitida*.

With regard to pollen exine sculpturing, no differences were found between morphs in any species (Fig. 7). Pollen grains in *Glandora* species are 4–(5)–(7)–8–9–10-zonocolporate (variable even within species), from subprolate to prolate. Ectoapertures are peculiarly rhomboidal in shape, covered by conic processes. The tectum is diffusely psilate with small, scattered puncta. The only differentiation between *Glandora*

and *Lithodora* species is that, in the latter, pollen grains are 4–5 zonocolporate (also variable within species), and the tectum is psilate, but with scattered granules in the equatorial region.

Stigmatic papillae size and morphology

Stigmatic papillae size ranges from 10.25 to 21.54 μm (total number of individuals $N = 100$; data not shown). There were no significant differences between morphs in any species. L:S ratios for papillae size are close to one in all cases (not shown).

Moreover, there were no differences between morphs for the papillae shape. Papillae in *Lithodora* species are smooth but in the *Glandora* genus they are rugose (except for *G. nitida*). In most species, papillae are lageniform structured (flask-like), characterised by an apical plate-like cap with four to six or seven crenellated lobes. Papillae shape is highly variable among the species, within the species and also among different periods during the flower life span (V. Ferrero, personal observations). Papillae in *G. nitida* and *G. rosmarinifolia* virtually lack the distinct cap, so their upper part forms a smooth dome rising from a more or less striate neck (see Fig. 8 for some examples).

DISCUSSION

Most of the research carried out on heterostylous plants has relied on the discrete characterisation of polymorphic types in plant populations. While some of these types (e.g., reverse *versus* approach herkogamy, distylous *versus* tristylous conditions) can be easily determined through visual inspection of a large enough sample within each population, others are more subtle (style dimorphism, Barrett *et al.* 1996) or require detailed measurement because of the continuous nature of the traits (e.g., sex organ reciprocity). In recent years, there has been increasing evidence that continuous variation is common (Sánchez *et al.* 2008). It is essential to ascertain the exact polymorphism type in order to conduct further sound evolutionary or functional studies on sex polymorphisms. The present study demonstrates that strong variations may occur among closely related species and even among populations within a species. Moreover, the results obtained in our study evidence that the discrete characterisation of species as distylous or style dimorphic is complex in some plants.

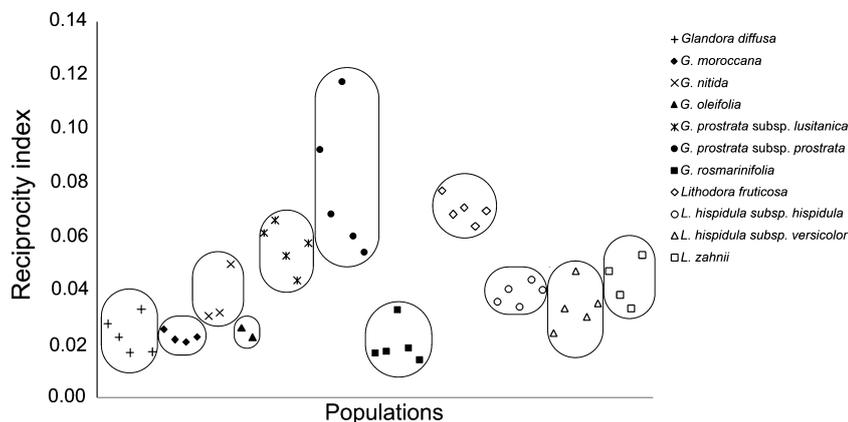


Fig. 4. Style–stamen reciprocity index values (calculated following Sánchez *et al.* 2008) for all the analysed populations of *Lithodora* and *Glandora*. Populations belonging to the same species are framed. The most reciprocal populations are those with an index closest to zero.

Table 1. Results of two-way mixed effect ANOVAS for the comparisons in corolla length, tube width and anther length in species of *Glandora* and *Lithodora*. Results for *Glandora nitida* correspond to *t*-test for independent samples. Values differ significantly at **P* < 0.05, ***P* < 0.01. n.s. = no significant difference.

taxon	corolla length			tube width		anther length	
	df	F	P	F	P	F	P
<i>Glandora diffusa</i>							
Morph	1,4	45.01	**	7.32	n.s.	4.31	n.s.
Population	4,4	28.78	**	21.19	**	22.63	**
Morph*population	4,478	1.86	n.s.	5.27	**	2.67	n.s.
<i>Glandora moroccana</i>							
Morph	1,3	9.00	*	40.21	**	2.37	n.s.
Population	3,3	4.93	n.s.	14.95	*	3.06	n.s.
Morph*population	3,338	0.82	n.s.	2.53	n.s.	0.66	n.s.
<i>Glandora nitida</i>							
Morph	71	0.73	n.s.	0.22	n.s.	1.41	n.s.
<i>Glandora oleifolia</i>							
Morph	1,1	26.89	n.s.	2.05	n.s.	1.01	n.s.
Population	1,1	326.46	*	149.19	n.s.	10.28	n.s.
Morph*population	1,112	0.07	n.s.	0.64	n.s.	0.23	n.s.
<i>Glandora prostrata</i> subsp. <i>lusitanica</i>							
Morph	1,4	2.99	n.s.	27.63	**	0.01	*
Population	4,4	8.63	*	9.43	*	56.37	**
Morph*population	4,476	1.50	n.s.	1.25	n.s.	0.92	n.s.
<i>Glandora prostrata</i> subsp. <i>prostrata</i>							
Morph	1,4	3.36	n.s.	2.83	n.s.	6.04	n.s.
Population	4,4	29.65	**	40.25	**	912.9	**
Morph*population	4,469	2.77	*	1.92	n.s.	0.42	n.s.
<i>Glandora rosmarinifolia</i>							
Morph	1,4	3.20	n.s.	9.92	*	18.11	**
Population	4,4	20.83	**	8.08	*	9.54	*
Morph*population	4,238	0.60	n.s.	1.97	n.s.	2.08	n.s.
<i>Lithodora fruticosa</i>							
Morph	1,4	1.57	n.s.	0.44	n.s.	0.56	n.s.
Population	4,4	105.91	**	161.44	**	10.34	*
Morph*population	4,480	0.32	n.s.	0.52	n.s.	2.16	n.s.
<i>Lithodora hispidula</i> subsp. <i>hispidula</i>							
Morph	1,4	1.91	n.s.	141.19	**	12.51	*
Population	4,4	36.32	**	153.53	**	21.82	**
Morph*population	4,480	2.29	n.s.	0.569	n.s.	2.19	n.s.
<i>Lithodora hispidula</i> subsp. <i>versicolor</i>							
Morph	1,4	1.73	n.s.	11.17	*	4.77	n.s.
Population	4,4	6.17	n.s.	6.65	*	6.49	*
Morph*population	4,477	3.01	*	4.51	**	1.65	n.s.
<i>Lithodora zahnii</i>							
Morph	1,3	0.02	n.s.	19.86	*	13.43	*
Population	3,3	1.74	n.s.	58.74	**	12.47	*
Morph*population	3,204	1.45	n.s.	0.34	n.s.	0.74	n.s.

Different approaches have been considered to characterise distyly, such as equal separation between anthers and styles in both morphs (Richards & Koptur 1993; Paillet & Thompson 1997; Ree 1997; Faivre & McDade 2001); separation between closer reciprocal sexual whorls (Pérez *et al.* 2004 in species with more than one stamen whorl); graphical representation (Passos & Sazima 1995); or, in many cases, significant differences in position of reciprocal organs between morphs (*e.g.*, Baker *et al.* 2000; Arroyo *et al.* 2002).

According to our criteria, of all the possible types of *style polymorphism*, *style dimorphic* populations are defined whenever the difference between reciprocal stigmas and stamen heights (at each level) is greater than half the length of the anther dimension, which implies a lack of reciprocity (loss of stigma–anther overlap in position). By contrast, they are classified as *distylous* when the positions of stigma and anthers overlap. Both polymorphisms occur in our study group (*Lithodora s.l.*).

Previous descriptions of style dimorphism in the genera *Lithodora* and *Glandora* have considered these genera in different ways. The opinion of Johnston (1953) regarding the style dimorphic state of *L. fruticosa* has never been questioned, and is confirmed here according to our criteria. The same happens with classification of most of the other species as distylous. In a previous study (Ferrero *et al.* 2009), *L. zahnii* and *G. prostrata* were described as style dimorphic following the same criteria as used here (although differences in anther position in both subspecies of *G. prostrata* were also reported). After morphological characterisation of the species, the great variation in stamen position as compared to stigma position in both subspecies of *G. prostrata* would suggest a new type of polymorphism, called *relaxed stylar dimorphism*, which is characterised primarily by wide amplitude in the height of anthers, causing low reciprocity (Figs 4 and 5). We use the term ‘relaxed’ because anthers within a flower do not present a uniform height, as in typical style dimorphic and distylous species (*e.g.*, Riveros *et al.* 1987; Paillet & Thompson 1997). Additionally, a recent study by Ferrero *et al.* (2009) on evolutionary transitions of style polymorphisms in the tribe Lithospermeae reported that species with *relaxed stylar dimorphism* (*G. prostrata*) present a condition derived from distyly. We hypothesise that this derived condition may be a consequence of the relaxation of stabilising selective processes that maintain the precise positioning of the anthers in distylous species. Pollinators in most *Glandora* and *Lithodora* species are mainly solitary bees, *Anthophora* sp. (V. Ferrero, unpublished data), which are thought to be very efficient pollinators in probing the flowers. Variable stamen lengths could be selected if they have higher delivery rates of pollen to the opposite morph, perhaps by a wide array of pollinators. The species show many isoplethic (equal morph ratios) populations, which would be a consequence of high disassortative pollen transfer, given the lack of heteromorphic incompatibility (V. Ferrero, unpublished data, see also Aigner 2001). Future studies analysing pollinator effectiveness (in delivery/deposition of pollen grains) in plants with different polymorphisms would be of major interest to determine whether such relaxation has this functional significance.

Our results clearly indicate that stigma–anther reciprocity of populations is also a variable condition among both species and populations (Fig. 4). Therefore, reciprocity indexes that quantify this condition (Richards & Koptur 1993; Eckert & Barrett 1994; Sánchez *et al.* 2008) must be considered instead of discrete characterisation. Reciprocity is also diverse between higher and lower whorls within flowers in the populations. There is usually less reciprocity (according to our criterion of overlap) between stamen and styles in the higher sex organ level than in the lower one (Table S1). Differences in reciprocity between stamen whorls have also been found

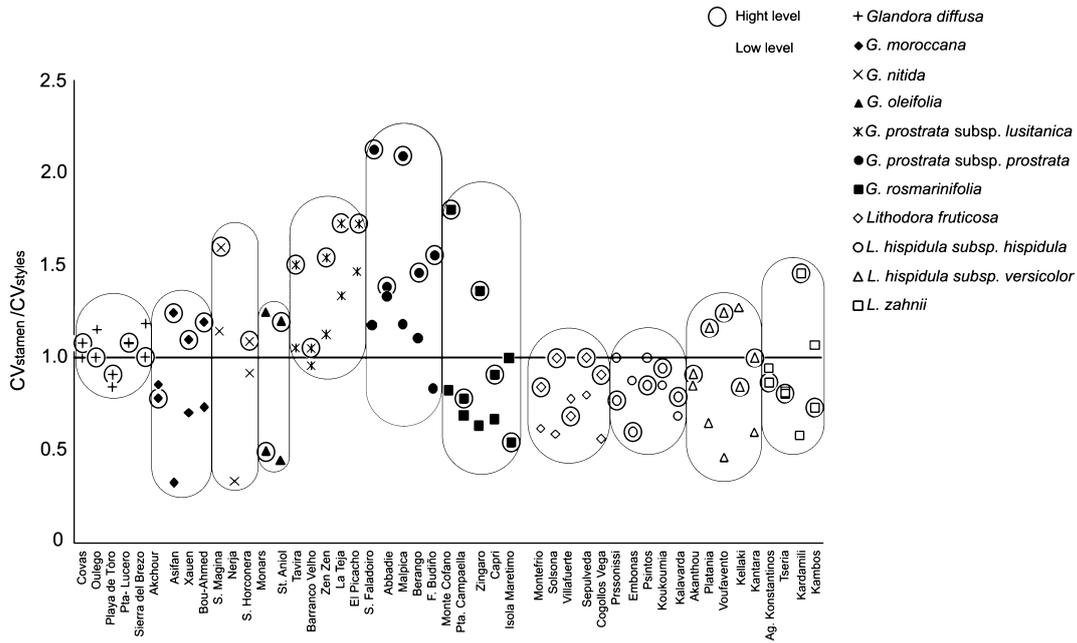


Fig. 5. Ratio of the coefficient of variation of stamens and styles for the higher level (styles of the L-morph and anthers of the S-morph) and the lower level (styles of the S-morph and anthers of the L-morph) in species of *Glandora* and *Lithodora*. Values for the higher-level whorls are circled; populations belonging to the same species are framed.

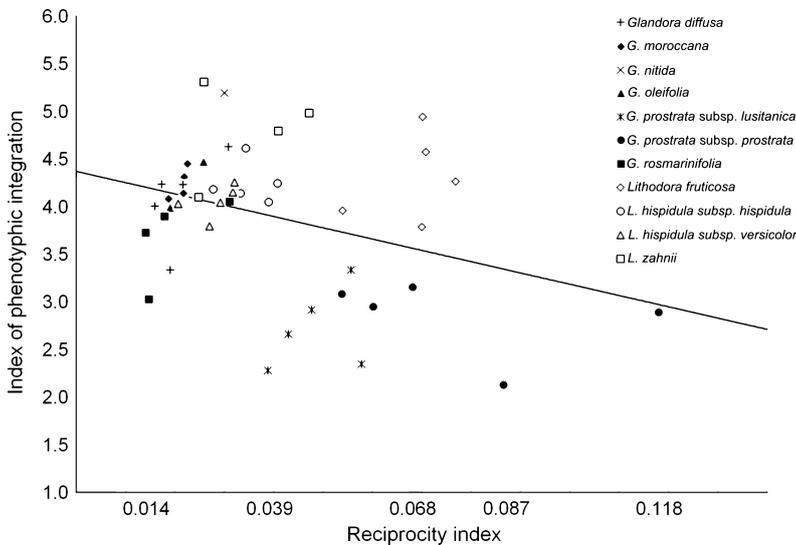


Fig. 6. Plot of the index of phenotypic integration (see Materials and Methods) as a function of the reciprocity index (Sánchez *et al.* 2008). Lower values for the reciprocity index mean greater reciprocity.

in species of *Narcissus*, but the upper whorl was more reciprocal than the lower one (Baker *et al.* 2000; Cesaro *et al.* 2004). Access to stigma for pollinators in the L- and S-morph is different. Pollen and stigmas at the lower level are mainly reached by long-tongued insects and have restricted access due to the narrow corolla width, which could promote the more efficient deposition of pollen on particular parts of the visitor body. Reciprocity is considered to be directly related to efficient pollen transfer between morphs. The exact positioning of pollen on the insect's body may be favoured by very precise adjustment between perianth traits and pollinators.

In all species analysed, phenotypic flower integration showed high values (33.9–64.7%) compared with the database examined by Ordano *et al.* (2008) across angiosperms (21.5% in their sample of 36 species). There is some controversy about the magnitude and pattern of phenotypic integration being determined by a selective process associated with pollination (Ordano *et al.* 2008; Fornoni *et al.* 2009; Harder 2009 and references therein). In the species studied, it is plausible to expect high values of phenotypic integration because style polymorphic species are usually pollinated by insects that are very precise in delivering and depositing pollen in these flowers. There are several reasons that would

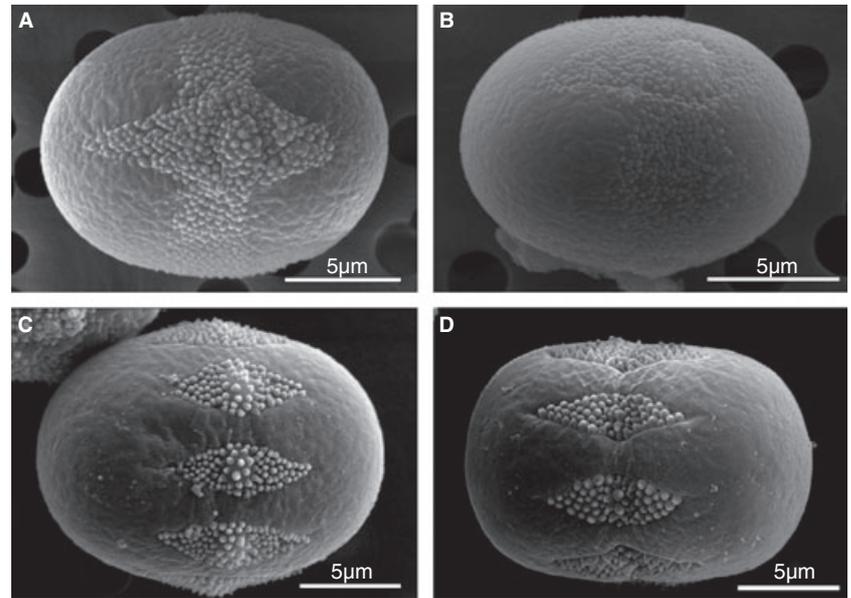


Fig. 7. Pollen grains of *Glandora* and *Lithodora* species under an SEM. A: *L. hispidula* subsp. *versicolor* S-styled, equatorial view. B: *L. hispidula* subsp. *versicolor* L-styled, equatorial view. C: *G. prostrata* subsp. *prostrata* S-styled, equatorial view. D: *G. prostrata* subsp. *prostrata* L-styled, equatorial view.

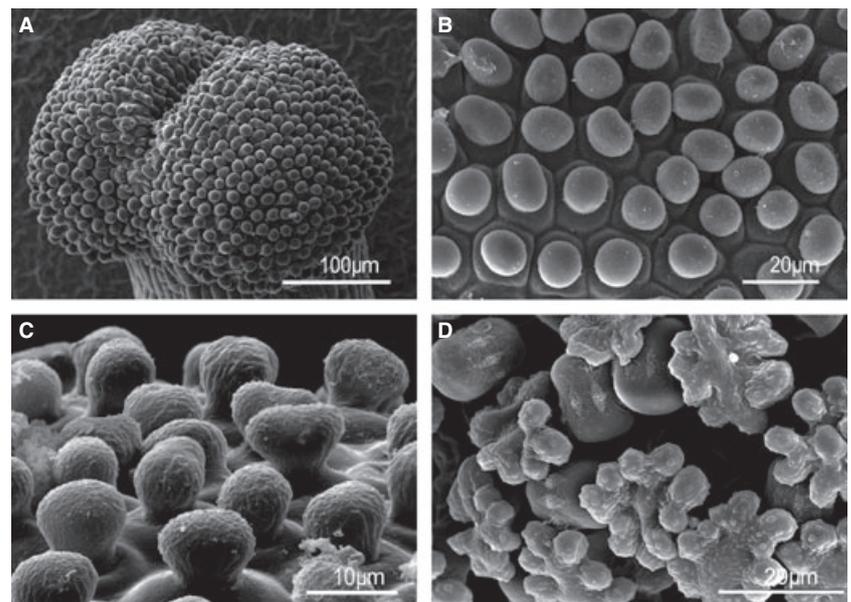


Fig. 8. Stigma and papillae morphology in *Glandora* and *Lithodora* species under an SEM. A and B: *G. nitida* stigma and papillae in detail, respectively. C: *G. rosmarinifolia* papillae detail. D: *G. prostrata* subsp. *prostrata* papillae detail.

indicate this to be the case in *Glandora* and *Lithodora* species. First, in the two taxa with between-morph differences in integration (*G. prostrata* subsp. *prostrata* and subsp. *lusitanica*), the S-morph showed greater integration. This morph has a concealed stigma that is pollinated only by long-tongued insects (V. Ferrero, unpublished data), which in general fit the flower more tightly and hence may prompt higher integration than in L-morph flowers, where pollinator fit is not so precise (Pérez-Barrales *et al.* 2007). Second, and more importantly, the positive correlation between sex organ reciprocity and integration clearly supports the hypothesis that precise pollination typical of reciprocal style polymorphic species also determines high intercorrelation (*i.e.*, integration) among other flower traits due to the tight fit between the pollinators and these flowers (Berg 1959; Armbruster *et al.* 2009). It is worth noting that taxa showing the lowest popu-

lation values of phenotypic integration (*G. prostrata* subsp. *prostrata* and subsp. *lusitanica*; Fig. 6) were categorised as relaxed stylar dimorphic. This low integration is consistent with the hypothesised relaxation of selective pressures exerted by pollinators from a distylous, more reciprocal ancestor (Ferrero *et al.* 2009), as some general models of evolution of heterostyly implicitly assume (Lloyd & Webb 1992a). We must admit, however, that other factors may also affect much of the unexplained variance in phenotypic integration, such as the effect of phylogenetic/phylogeographic relatedness of species and populations, respectively (Eble 2004). The effect of allometric relationships on phenotypic integration could also be argued, although it seems to be negligible in our sample ($R = 0.269$, $P = 0.073$ for the L-morph and $R = 0.158$, $P = 0.299$ for the S-morph, analysis not shown). The only taxa with clear between-morph differences in flower

size (as surrogated by flower tube length: *G. diffusa* and *G. moroccana*, Table 1) do not suffer reduced correlations among their flower traits (integration of 51.03% and 53.07%, respectively) as a consequence of variation in corolla size.

In this study, there were differences between morphs in ancillary traits in almost all species; however these are only marginal compared to other distylous species. In Boraginaceae, larger flowers in the S-morph appear in species of *Lithospermum* (Johnston 1952; Levin 1968), and some *Amsinckia* (Ganders 1975; Ornduff 1976). Variation in corolla length has been associated with differences in reciprocity (Thompson & Dommée 2000). Theoretically, an increase in corolla length in the S-morph, in species with stamen affixed, may promote reciprocity at the high level because it helps anthers reach the height of the reciprocal stigmas in the L-morph when protruding out of the corolla. Accordingly, these differences might compensate for asymmetrical pollen flow in the populations (Ganders 1979). Differences between morphs as a by-product of developmental constraints have also been repeatedly suggested (see Dulberger 1992; Richards & Barrett 1992; Faivre 2000), although this does not rule out an adaptive role. In our species group, the more reciprocal species tend to be those with larger between-morph difference in corolla size, like *G. diffusa* or *G. moroccana* (see Fig. 4 and Table S1). In contrast, L-corollas are, in general, wider than S-corollas (Table S1). Because of this, it is difficult to assume that between-morph differences are a result of allometric relationships. It is likely that the deeply inserted stamens of L-flowers require a wider tube, which would only be the result of an architectural constraint, in comparison with the exerted stamens of S-flowers.

As regards pollen dimorphisms, Ganders (1979) showed that the ratio of S:L pollen volume was positively correlated with the ratio of L:S pollen grain number. Here, our results also show this inverse relation between pollen size and production, particularly in *G. rosmarinifolia* and *G. nitida*. Variation in pollen size has been linked to the larger storage reserves needed in S-pollen to grow along longer L-styles (Darwin 1877), whereas varying pollen production has been related to the lower efficiency of S-styles in capturing pollen as compared with L-styles (e.g., Ganders 1975). In other cases, variation in pollen size and production has been related to heteromorphic incompatibility systems (see references in the review of Dulberger 1992), which could be the case of *G. nitida* (V. Ferrero, unpublished data). In Boraginaceae, there are several combinations of pollen size variation and incompatibility reactions: pollen dimorphism and heteromorphic incompatibility in some *Amsinckia*, *Lithospermum* and *Pulmonaria* species (Weller & Ornduff 1977; Ganders 1979; Casper *et al.* 1988; Richards & Mitchell 1990); pollen monomorphism and self-compatibility in *Cryptantha* species (Casper 1983, 1985), and pollen dimorphism and homomorphic incompatibility in *Anchusa* (Philipp & Schou 1981), or even no relation to the incompatibility system (*Pulmonaria officinalis*, Olesen 1979; Brys *et al.* 2008), which point to independent evolution of both features in Boraginaceae. In contrast, stigmatic papillae were not different between morphs in any of the species studied, although differences have been reported in other distylous Boraginaceae (*Amsinckia grandiflora*, *Anchusa officinalis*; Ganders 1979; Philipp & Schou 1981; Dulberger 1992). It has also been suggested that

differences in pollen and papillae sizes between morphs could be the result of evolution of a lock-and-key system for both complementary traits (see Dulberger 1992).

In general, it is worth noting that among-population differences were found for all flower traits analyses, irrespective of between-morph differences. This stresses the importance of conducting detailed population sampling when reporting morphometric data of heterostylous species. This opens up the possibility that these traits, whether they act individually or as an ensemble (i.e., integration), are subjected to different selective pressures (e.g., by different pollinator arrays) that may determine local adaptation processes.

In conclusion, our results show that discrete stylar polymorphism, although discontinuous within populations by definition, may present a gradient across populations and species, which may pose special problems when qualifying species. Nevertheless, we are aware that discrete characterisation of species is still necessary to facilitate comprehension; to simplify comparisons with other heterostylous groups; and to test models on the evolution of heterostyly. In this sense, our discrete classification of types in *Lithodora s.l.* species confirms a previous proposal (Ferrero *et al.* 2009): species would be characterised according to the majority of populations. Moreover, future phylogeographical studies on species with high variation among populations would be particularly valuable to ascertain the direction of these subtle, continuous changes in flower polymorphisms, as evidenced by some of the spatial structuring of populations across the range (V. Ferrero, unpublished data). Our data support the idea that agents for promoting changes in style polymorphic conditions are pollinators and their precise behaviour within the flower, and the concomitant variation in phenotypic integration would seem to confirm this. Finally, the relationship between these stylar polymorphisms and other minor flower traits, called ancillary traits, also shows variable expression in populations and species, which supports the idea that they are not so critically linked to the polymorphism, and that the functional significance of each could be either promotion of equal pollen flow in both morphs or prevention of selfing.

ACKNOWLEDGEMENTS

The authors thank T. García, A. Pérez, I. Stanescu, S. Castro, R. Ajbilou, X. Oliver, E. Triano, U. Osuna, C. Muñoz, J.M. Gómez, B. Valdés and J. D. Thompson for their help in locating or collecting the samples; D. Rojas, A. Vale, J.M. Sánchez and J.M. Gómez for comments and help with the statistical analysis; J. Méndez and I. Pazos for the technical assistance in SEM. We are also grateful to C. García and C. Teed for the language revision and three anonymous reviewers for their constructive criticisms. This research was supported by the Spanish Dirección General de Investigación, Ciencia y Tecnología (DGICYT) through the Project CGL2006-13847-CO2-01-02, the Agencia Española de Cooperación Internacional (AECI) through the Project A/6962/06 and the Xunta de Galicia through the Project PGIDT04-PXIC31003PN. The Ministerio de Educación y Ciencia (MEC) financed the work of V. Ferrero through a PhD scholarship (AP-2004-6394) and the Fondo Social Europeo and the Xunta de Galicia financed the work of I. Chapela.

SUPPORTING INFORMATION

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

Appendix S1. Taxa and populations used in the morphometric analysis, with information on population location, geographical coordinates, elevation, number of flowers collected, use of collected flowers (abbreviated as m = flowers used for the morphometric analysis; P = flowers collected for pollen size characterisation and s = flowers collected for pollen and papillae characterisation), and voucher information (SANT = Herbarium of the University of Santiago de Compostela, Spain).

Table S1. Population sample sizes, corolla length, tube width, style length, stamen height and anther length for both morphs in populations of *Lithodora* and *Glandora*.

Table S2. Pollen production per flower, ratio between morphs in pollen production, pollen size characterisation and ratio between morphs in pollen size; short-styled/long-styled ratios of flowers of *Glandora* and *Lithodora*.

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Conflicts of interest added after online publication: The authors have declared no potential conflicts.