

Associations between sex-organ deployment and morph bias in related heterostylous taxa with different styler polymorphisms¹

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PREMISE OF THE STUDY: Populations of heterostylous species are characterized by two or three floral morphs with reciprocal positioning of stigmas and anthers. Theoretical models predict equal morph frequencies (isoplethy) when disassortative mating is prevalent in populations, but biased morph ratios may occur when variation in the expression of heterostyly causes deviations from intermorph mating.

METHODS: We explore the role of sex-organ deployment in governing morph ratios in two closely related genera of Boraginaceae, exhibiting striking variation in floral traits associated with the heterostylous syndrome. We sampled 66 populations of six species of *Glandora* and 39 populations of three species of *Lithodora* across their distributional range in the Mediterranean. In each population we estimated morph ratios and measured several floral traits. We used phylogenetically corrected and noncorrected regressions to test the hypothesis that differences in sex-organ reciprocity and herkogamy are associated with deviations from isoplethy.

KEY RESULTS: Biased morph ratios occurred in 24% of populations, particularly in *Lithodora*. Populations biased for the long-styled morph (L-morph) were more frequent than the short-styled morph (S-morph). Distylous species were less likely to exhibit biased ratios than species with stigma-height dimorphism. In *Lithodora fruticosa*, a species lacking reciprocity, decreased herkogamy in the S-morph was associated with increasing L-morph bias, perhaps resulting from self-interference.

CONCLUSION: Striking variation in the expression of heterostyly in *Glandora* and *Lithodora* is associated with biased morph ratios, which probably result from pollinator-mediated mating asymmetries within populations.

KEY WORDS disassortative mating; distyly; floral morph ratios; *Glandora*; heterostyly; *Lithodora*; Mediterranean plants; stigma-height dimorphism

Heterostyly is a genetic polymorphism in which populations are composed of two (distyly) or three (tristyly) floral morphs that differ in the reciprocal placement of stigmas and anthers within flowers

(Darwin, 1877; Barrett, 1992). The genetic control of the polymorphism in distylous plants usually involves a single Mendelian diallelic locus in which the long-styled morph is of genotype *ss* and the short-styled morph is of genotype *Ss*, although in several species the dominance relations at the *S*-locus are reversed (Lewis and Jones, 1992). The floral morphs are maintained in populations by negative frequency-dependent selection resulting from intermorph (disassortative) mating. With this genetic system and disassortative mating, a 1:1 morph ratio (isoplethy; Finney, 1953) is expected in equilibrium populations.

The classic textbook depiction of heterostyly as a balanced polymorphism (e.g., Roughgarden, 1979; Silvertown and Charlesworth, 2009; Charlesworth and Charlesworth, 2010) is largely based on knowledge of distyly in *Primula* and emphasizes how the reciprocal positioning of sex organs (reciprocal herkogamy) associated with a self and intramorph incompatibility system (heteromorphic incompatibility) promotes outcrossing in populations. The heterostylous

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syndrome has evolved on numerous occasions in unrelated animal-pollinated families of flowering plants (Ganders, 1979; Lloyd and Webb, 1992a; Barrett et al., 2000), and is perhaps the most well-studied discrete floral polymorphism.

Investigations of heterostyly have broadened since Darwin's seminal work on *Primula* and *Lythrum* (Darwin, 1877) to include many more families. It is now evident that reciprocal herkogamy may vary considerably in expression and can be associated with diverse compatibility systems, while still functioning to promote varying degrees of disassortative pollen transfer (Barrett and Richards, 1990; Dulberger, 1992; Lloyd and Webb, 1992a, b; Barrett and Cruzan, 1994; Ferrero et al., 2012; Zhou et al., 2015). Moreover, although the frequencies of style morphs in populations are governed by the aggregate patterns of mating in preceding generations, a variety of stochastic and deterministic processes can result in morph ratios that deviate significantly from equality. Founder events and genetic drift commonly result in biased morph ratios (anisoplethy), especially in species in which features of the life history (e.g., clonality and episodic sexual recruitment) slow progress to the isoplethic equilibrium (Ornduff, 1972; Morgan and Barrett, 1988; Eckert and Barrett, 1995). Although less commonly documented, morph-specific differences in reproductive fitness can also cause biased morph ratios in heterostylous populations (Barrett et al., 1983; 2004; Brys et al., 2008a; Weber et al., 2013). Thus, determining the causes of anisoplethic morph ratios in heterostylous populations is a complex problem that usually commences with a study of the reproductive correlates of morph-ratio variation, an approach we use here.

Haldane (1936) first recognized that distylous populations should proceed more rapidly than tristylous populations to an isoplethic equilibrium. He pointed out that in the absence of "illegitimate unions" (self and intramorph mating) in distylous populations, the frequencies of the L- and S-morphs should be fully restored to equality in one generation after any particular perturbation (Haldane, 1936, p. 396). This inference assumed a tight association between the stamen and style polymorphism and heteromorphic incompatibility. However, not all species with stylar polymorphism possess strong heteromorphic incompatibility, and in species in which self and intramorph mating are permitted, other morph ratio dynamics and equilibria are possible. For example, if style morphs in a population differ in rates of disassortative and assortative mating, various L-morph and S-morph biased anisoplethic equilibria are possible. Indeed, there is empirical evidence of biased morph ratios in species in which enforced disassortative mating does not occur because of the absence of heteromorphic incompatibility (Ganders, 1975; Ray and Chisaki, 1957; Barrett et al., 1996, 2004; Baker et al., 2000a, b; Pérez-Barrales and Arroyo, 2010; Simón-Porcar et al., 2015a). In such cases, floral morphology plays a more significant role in governing patterns of pollen transfer and mating than in the species envisioned by Haldane, in which illegitimate unions were prevented by physiological incompatibility.

Because of the functional link between floral morphology, pollen transfer, and mating in heteromorphic taxa, the degree of reciprocity of stigmas and anthers between the style morphs and their spatial separation within a flower (herkogamy) should play a key role in governing rates of disassortative mating with consequences for morph ratios (Barrett et al., 2004). In species with heteromorphic incompatibility, weak sex-organ reciprocity will be costly because pollen transferred to incompatible stigmas by animal pollinators involves gamete wastage. This loss in male outcrossed

siring opportunities through illegitimate pollen transfer should select for greater sex-organ reciprocity and greater herkogamy. In contrast, in species in which intramorph mating is permitted, because of the absence of heteromorphic incompatibility, pollen wastage does not occur and we might expect less stringent selection for reciprocal herkogamy and perhaps smaller herkogamy distances.

Recent investigations of the floral biology of several heteromorphic genera have revealed an unusual combination of reproductive features not evident in previously studied heterostylous taxa. In several species of *Anchusa*, *Lithodora*, *Glandora* (Boraginaceae), and *Narcissus* (Amaryllidaceae) the style morphs are self-incompatible but intramorph compatible—a pattern inconsistent with heteromorphic incompatibility and indicative that they possess distinct self-recognition systems (*Anchusa*—Dulberger, 1970; Philipp and Schou, 1981; Schou and Philipp, 1984; *Lithodora* and *Glandora*—Ferrero et al., 2011a, 2012; *Narcissus*—Dulberger, 1964; Barrett et al., 1997; Arroyo et al., 2002; Baker et al., 2000b; Simón-Porcar et al., 2015b). In *Anchusa* (Schou and Philipp, 1983) and *Narcissus* (Dulberger, 1964; Sage et al., 1999; Santos-Gally et al., 2015; Simón-Porcar et al., 2015b) self-rejection involves late-acting ovarian incompatibility. Significantly, sex-organ reciprocity is less well developed in these taxa despite clear polymorphism for style length. Anther heights are usually less well differentiated between the style morphs, and where this occurs in dimorphic taxa, the polymorphism is referred to as stigma-height dimorphism, with populations composed of L- and S-morphs (reviewed in Barrett et al., 2000).

Theoretical models of the evolution of distyly include stigma-height dimorphism as an intermediate stage in the transition from stylar monomorphism to distyly (Charlesworth and Charlesworth, 1979; Lloyd and Webb, 1992b). Comparative evidence in *Narcissus* and *Lithodora* involving phylogenetic reconstructions of the evolutionary history of stylar polymorphisms generally support these models (Graham and Barrett, 2004; Pérez-Barrales et al., 2006; Ferrero et al., 2009). However, stigma-height dimorphism is clearly a stable polymorphism in each of these taxa because it is reported from a significant number of species. Moreover, at least in *Narcissus*, there is experimental evidence that despite incomplete sex-organ reciprocity and intramorph compatibility, disassortative mating is promoted by stigma-height dimorphism (Simón-Porcar et al., 2014, 2015a), and the stylar morphs are subject to frequency-dependent selection (Thompson et al., 2003) in the same manner as in distylous populations.

Here, we investigate the reproductive correlates of variation in style morph frequencies in populations of 11 dimorphic taxa formerly belonging to *Lithodora* (recently split into *Lithodora* and *Glandora*; Thomas et al., 2008; Ferrero et al., 2009). We chose this group for several reasons. First, our earlier investigations revealed considerable variation in both sex-organ reciprocity and compatibility systems, including species with classical distyly with heteromorphic incompatibility, distyly with self and intramorph compatibility and, finally, stigma-height dimorphism with self-incompatibility and intramorph compatibility (Ferrero et al., 2009, 2011a, 2012). To our knowledge, this range of reproductive variation surpasses that found in any dimorphic group of closely related species. Second, our preliminary observations of natural populations indicated a wide range of style-morph ratios raising the possibility that their contrasting reproductive systems may play a role in causing this variation.

Our study addressed three main questions: (1) Are the patterns of morph frequency variation in populations of *Lithodora* and

Glandora associated with the type of stylar polymorphism of each species? We predicted greater deviation from isoplethy in taxa with stigma-height dimorphism compared to those that were distylous. (2) Can quantitative measures of the degree of sex-organ reciprocity predict variation in morph ratios? We predicted that greater reciprocity between the style morphs should increase rates of disassortative pollen transfer and mating leading to more even style morph ratios in populations. (3) Are differences between the style morphs in herkogamy (spatial separation of stigmas and anthers) associated with biased morph ratios? Because morph-specific differences in herkogamy have the potential to influence selfing and assortative mating in stylar dimorphic populations (see Baker et al., 2000a), we predicted increasing morph-ratio bias where differences in herkogamy between the morphs were most evident. To address these questions, we surveyed morph frequencies in 66 populations of seven taxa of *Glandora* and 39 populations of four taxa *Lithodora* over much of their geographical range. In each population, we sampled flowers for morphological characterization of each style morph. We tested our predictions using phylogenetic methods to take into account the nonindependence of data and we also analyzed each species independently.

MATERIALS AND METHODS

Study group—*Lithodora* and *Glandora* are primarily composed of small, insect-pollinated shrubs, distributed around the Mediterranean basin (Thomas et al., 2008). *Glandora* consists of six species, five occurring in the western Mediterranean region, which is the main center of diversity. *Lithodora* consists of three species; one distributed in the western Mediterranean (*L. fruticosa*) and the remaining two equally distributed between the central and eastern Mediterranean. Several subspecies are recognized: two in *G. prostrata* and three in *L. hispidula*. Some taxa are narrow endemics: *G. moroccana* in central Rif (N. Africa), *G. nitida* in the southeastern part of the Iberian Peninsula (Blanca et al., 2003), *G. oleifolia* in the eastern Pyrenees, and *L. zahnii* in southern Peloponnese (Fig. 1). We were not able to sample *L. hispidula* subsp. *cyrenaica*, which is endemic to Libya. Most species flower in April–May, although some have longer flowering periods from January–June (e.g., *G. prostrata*). The two genera exhibit a range of compatibility systems including fully self-compatible taxa to those that are self-incompatible, including some with typical dimorphic incompatibility and others that are intramorph compatible (Ferrero et al., 2012). Associated with this variation are different types of floral polymorphism: distyly, stigma-height dimorphism, and “relaxed stigma-height dimorphism” (see Ferrero et al., 2011a, b). In the latter condition, each anther within a flower occurs at a different height. Insect visitors to flowers of both genera include solitary bees (mainly *Anthophora*), species of Lepidoptera (principally *Macroglossum stellatarum*), and Diptera (mostly *Bombylius*), and these visitors have been shown to differ in pollination efficiency and the range of species they visit (Ferrero et al., 2011b).

Surveys of style-morph ratios—We surveyed 2–31 populations from four *Lithodora* taxa and 4–22 populations of seven *Glandora* taxa throughout the Mediterranean basin and the Atlantic coast of southwestern Europe (see Table 1 for taxa and sample sizes). Our sampling covered most of the distributional range of the taxa included in our survey (Fig. 1). In each population, we randomly

sampled one flower per plant from 100 individuals (where possible), and in smaller populations we sampled all individuals. Because taxa of *Lithodora* and *Glandora* do not appear to propagate by clonal reproduction, the sampling of genets was relatively unambiguous. We preserved flowers in 70% ethanol for later classification on the basis of style length and measurements of sex-organ reciprocity. In each population, we calculated an index of style morph bias as the absolute value of the difference in the number of individuals of the L- and S-morphs, divided by the total number of flowering individuals sampled (see Endels et al., 2002; Brys et al., 2008a). The index identifies deviations from isoplethy, with values ranging from 0, when both style morphs are present at equal frequency, to 1, when there is only one style morph present in the population.

We used G-tests to determine whether the style-morph ratios of individual populations differed significantly from isoplethy and used pooled goodness-of-fit G-tests to determine whether pooled morph ratios for individual taxa differed significantly from isoplethy. G-heterogeneity statistics were calculated to test for heterogeneous morph ratios among populations of taxa.

Sex-organ reciprocity and herkogamy—In the laboratory, we measured stigma and anther heights of the preserved flowers using digital photography and the image analysis software *analySIS* (version 5.0.), following procedures detailed in Ferrero et al. (2009, 2011a). We then applied the method of Sánchez et al. (2008, 2013) to calculate an index of reciprocity (*R*) between complementary sex organs in each population. This index is based on comparing the position of all anthers of each flower with the stigmas of all flowers of the opposite morph in a population. When reciprocity between anthers and stigmas is highest, the value of the index approaches one. Values that depart from one toward zero occur when reciprocity is low, and are modulated by the average standard deviation of all height measurements for each sex-organ level, so the greater the dispersion of values the greater departure from one. The computational software is licensed by Creative Commons Attribution 3.0 (see Sánchez et al., 2008, 2013). Herkogamy was calculated as the difference between the means of stigma and anther heights for each morph in all populations (Fig. 2). We averaged anther heights within a flower in these calculations. We used two-way ANOVA to compare the degree of herkogamy between morphs and species.

Inference of the time-calibrated phylogenetic tree—We estimated a time-calibrated phylogeny of taxa of Boraginales from DNA sequences downloaded from GenBank (Appendix S1, see the online Supplementary Data tab with this article). This was undertaken to test our hypotheses at the species level while accounting for the fact that lineages are not independent (Felsenstein, 1985). Most taxa in our trees belonged to Boraginaceae. Outgroup taxa consisted of members of Hydrophyllaceae, Heliotropiaceae, Cordiaceae, and Ehretiaceae (Mansion et al., 2009). The six DNA regions used to infer the trees were: *trnL* intron, *trnL-trnF* intergenic spacer, *ndhF* gene, the *rbcL* gene, part of the *trnK* intron, and the *matK* gene (both coded as the *matK* region). These analyses are described in detail in Appendix S2 and information on node calibration is specified in Appendix S3.

Morphological traits associated with style-morph ratios—We first examined the relation between style morph bias (Table 2) and the type of stylar polymorphism (Table 1) at the species (or subspecies)

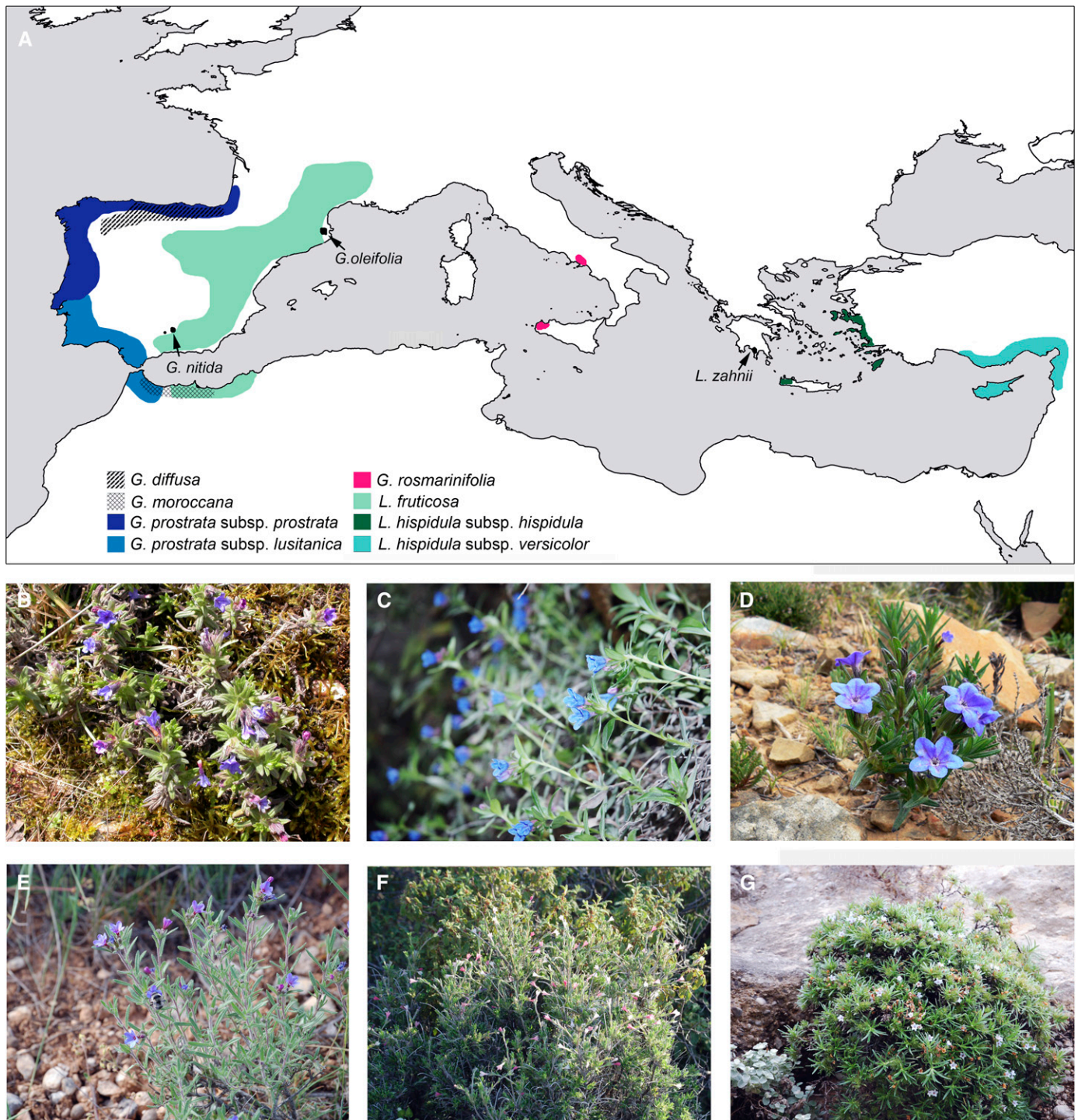


FIGURE 1 (A) Geographic range of *Lithodora* and *Glandora* species and images of three species each of *Glandora* and *Lithodora* species illustrating their diverse growth forms. (B) *G. diffusa*—prostrate to ascending dwarf shrub (C) *G. oleifolia*—dwarf shrub (D) *G. prostrata* subsp. *lusitanica*—erect or ascending dwarf shrub (E) *L. fruticosa* and (F) *L. hispidula* subsp. *versicolor*—much branched dwarf shrub (G) *L. zahni*—dwarf shrub. Distributions are based on Pignatti (1982), Valdés (2002), Greuter et al. (1989) and V. Ferrero, personal observation.

level. For this, we characterized the taxa into two groups based on their degree of reciprocity: distylous populations, which generally display greater levels of reciprocity, and nondistylous populations, including those with stigma-height dimorphism and relaxed stigma-height dimorphism, which exhibit weaker reciprocity (Ferrero

et al., 2011a). Then, we calculated a mean morph bias per taxon. We fitted a phylogenetic-corrected Linear Model using Generalized Least Squares: function ‘glS’ in the R package ‘nlme’ (R Development Core Team, 2014). We used the index of style morph bias as the dependent variable and the type of polymorphism (distylous vs.

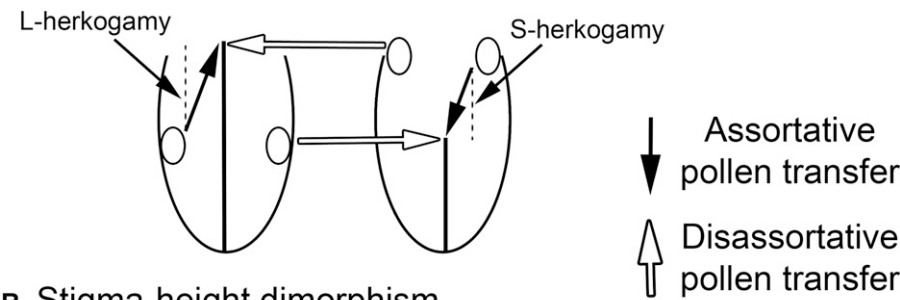
TABLE 1. Reproductive traits of *Glandora* and *Lithodora* species. N_p and N_f refer to the number of populations and flowers sampled, respectively, SC: self-compatible, SI: self-incompatible, IMC: intramorph compatible, IMI: intramorph incompatible. Mean and standard deviations are provided for corolla size, reciprocity index (R), and herkogamy distance for the L- and S-morph. ? = absence of information. Measurements are in millimeters.

| Species | N_p | N_f | Type of polymorphism | Incompatibility system | Range of distribution | Corolla size | R | L-morph herkogamy | S-morph herkogamy |
|--|-------|-------|----------------------------------|------------------------|-----------------------|--------------|-------------|-------------------|-------------------|
| <i>Glandora diffusa</i> | 12 | 1083 | Distyly | SC + IMC | Wide distribution | 16.22 ± 1.01 | 0.73 ± 0.08 | 3.65 ± 0.49 | 4.52 ± 0.66 |
| <i>G. moroccana</i> | 4 | 343 | Distyly | SC + IMC | Wide distribution | 18.44 ± 0.36 | 0.77 ± 0.02 | 5.12 ± 0.62 | 5.08 ± 0.17 |
| <i>G. nitida</i> | 3 | 170 | Distyly | SI + IMI | Narrow endemic | 18.11 ± 2.75 | 0.67 ± 0.15 | 5.75 ± 0.68 | 5.85 ± 0.74 |
| <i>G. oleifolia</i> | 2 | 114 | Distyly | SC + IMC | Narrow endemic | 18.32 ± 1.06 | 0.68 ± 0.08 | 6.29 ± 0.81 | 6.42 ± 0.35 |
| <i>G. prostrata</i> subsp. <i>lusitanica</i> | 10 | 939 | Relaxed stigma height dimorphism | SI + IMC | Wide distribution | 17.68 ± 0.66 | 0.43 ± 0.07 | 3.65 ± 0.57 | 3.53 ± 0.39 |
| <i>G. prostrata</i> subsp. <i>prostrata</i> | 31 | 3013 | Relaxed stigma height dimorphism | SI + IMC | Wide distribution | 18.27 ± 1.24 | 0.26 ± 0.19 | 3.83 ± 0.56 | 4.80 ± 0.87 |
| <i>G. rosmarinifolia</i> | 4 | 232 | Distyly | ? | Wide distribution | 18.92 ± 1.22 | 0.74 ± 0.12 | 5.89 ± 1.15 | 5.14 ± 0.14 |
| <i>Lithodora fruticosa</i> | 22 | 2091 | Stigma-height dimorphism | SI + IMC | Wide distribution | 14.15 ± 1.57 | 0.25 ± 0.22 | 2.55 ± 0.46 | 2.41 ± 0.53 |
| <i>L. hispidula</i> subsp. <i>hispidula</i> | 7 | 658 | Distyly | ? | Wide distribution | 12.42 ± 1.04 | 0.58 ± 0.08 | 3.36 ± 0.67 | 3.66 ± 0.24 |
| <i>L. hispidula</i> subsp. <i>versicolor</i> | 6 | 560 | Distyly | ? | Wide distribution | 12.99 ± 0.50 | 0.65 ± 0.08 | 3.26 ± 0.30 | 2.32 ± 0.34 |
| <i>L. zahni</i> | 4 | 200 | Stigma-height dimorphism | ? | Narrow endemic | 17.92 ± 0.26 | 0.58 ± 0.12 | 3.03 ± 1.01 | 3.65 ± 0.29 |

nondistylous) as the independent variable. The correlation structure of the data was given by the expected covariance of taxon traits, given the phylogenetic tree and evolutionary model. The phylogeny of Boraginales containing the taxa of *Glandora* and *Lithodora* was pruned accordingly. We performed three analyses, one in which the phylogenetic signal (Pagel’s λ) was estimated by maximum like-

lihood, and another two in which λ was forced to 0 (i.e., there is no influence of the evolutionary history of the species on the relationship between traits) and 1 (i.e., the phylogenetic signal is maximum, and the relation between variables has evolved according to a Brownian motion model of evolution). The fit of the models was assessed using the Akaike weights (AICw) (Burnham and Anderson, 2002).

A Distyly



B Stigma-height dimorphism

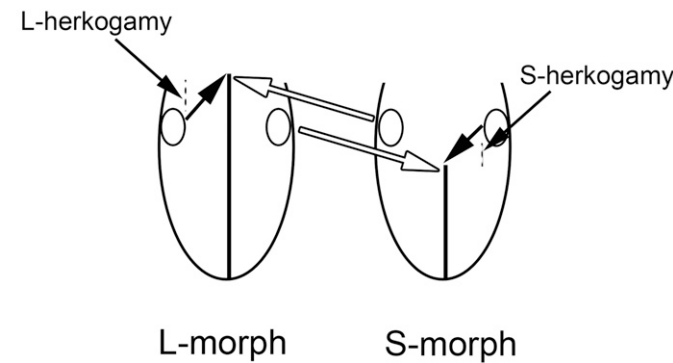


FIGURE 2 Expected influence of sex-organ deployment on pollen transfer, mating patterns, and morph ratios in species with contrasting stylar polymorphism. (A) distyly; (B) stigma-height dimorphism. Equality in the proportion of morphs (isoplethy) is expected to be maintained when: (1) sex-organ reciprocity favors disassortative pollen transfer and; (2) decreased herkogamy increases assortative pollen transfer and selfing.

The AICw of any particular model varies from zero (no support) to unity (complete support) relative to the entire set of models (Johnson and Omland, 2004).

Second, we tested whether the variability in morphological traits among population was related to deviations from isoplethy. If reciprocity and the degree of herkogamy play an important functional role in governing disassortative mating (Fig. 2) and thus promoting isoplethy, we would predict greater variation in morphological traits among populations associated with greater deviation from isoplethy. For this reason, we used the interquartile range (IQR) to standardize measurements of data dispersion allowing comparisons among species. For each taxon, we calculated the IQR of the morph-bias index, the IQR of reciprocity and the herkogamy distance, and we tested for the effect of the morphological measurements on the IQR of the morph-bias index. We used the function ‘gls’ in the R package ‘nlme’, following the same procedure as for the previous analysis.

Finally, we analyzed the relation between the morph-bias index and the reciprocity and herkogamy distances independently for each taxon. We carried out this analysis only in those taxa with more than four populations sampled and used General Linear Models. We constructed models for different subsets of factors and used the Akaike weights (AICw) to determine which candidate model best

TABLE 2. Morph frequency data collected from surveys of populations of *Glandora* and *Lithodora*. Average morph ratios per species and the number of isoplethic and anisoplethic populations (L-biased or S-biased) are shown for each species. Sample sizes for populations and flowers are given in Table 1; L = L-morph; S = S-morph. Significant results for departure from isoplethy after pooled and heterogeneity G-tests at $\alpha = 0.05$ are highlighted in bold.

| Species | Average morph ratio | | Number of isoplethic populations | Number of anisoplethic populations | | G_{pool} | G_{het} |
|--|---------------------|------|----------------------------------|------------------------------------|----------|-------------------|------------------|
| | L | S | | L-biased | S-biased | | |
| <i>Glandora diffusa</i> | 0.56 | 0.44 | 12 | | | 15.13 | 16.44 |
| <i>G. moroccana</i> | 0.50 | 0.50 | 4 | | | 0.08 | 0.81 |
| <i>G. nitida</i> | 0.48 | 0.52 | 3 | | | 0.21 | 1.84 |
| <i>G. oleifolia</i> | 0.62 | 0.38 | 1 | 1 | | 4.23 | 18.48 |
| <i>G. prostrata</i> subsp. <i>lusitanica</i> | 0.40 | 0.60 | 8 | | 2 | 39.30 | 25.87 |
| <i>G. prostrata</i> subsp. <i>prostrata</i> | 0.58 | 0.42 | 26 | 5 | | 72.58 | 69.35 |
| <i>G. rosmarinifolia</i> | 0.48 | 0.52 | 4 | | | 0.02 | 5.93 |
| <i>Lithodora fruticosa</i> | 0.70 | 0.30 | 8 | 13 | 1 | 298.75 | 539.03 |
| <i>L. hispidula</i> subsp. <i>hispidula</i> | 0.45 | 0.55 | 7 | | | 5.91 | 15.82 |
| <i>L. hispidula</i> subsp. <i>versicolor</i> | 0.39 | 0.61 | 5 | | 1 | 27.76 | 7.85 |
| <i>L. zahnii</i> | 0.28 | 0.72 | 2 | | 2 | 40.40 | 1.26 |

explained the data. In all analyses, we used 0.05 as the significance level.

RESULTS

Variation in style-morph ratios—Morph ratios varied to different degrees in each taxon across their geographical ranges (Figs. 3–4). Style-morph ratios departed from isoplethy (1L:1S) in 8 (12.1%) populations of *Glandora* and 17 populations (43.6%) of *Lithodora* (Table 2 and Appendix S4). For each taxon, the pooled G values indicated significant deviation from equal morph frequencies except for *Glandora moroccana*, *G. nitida*, and *G. rosmarinifolia* (Table 2). Heterogeneity in frequencies of style morphs among populations was statistically significant in *G. oleifolia*, *G. prostrata* subsp. *lusitanica*, *G. prostrata* subsp. *prostrata*, and *L. fruticosa* (Table 2). S-morph bias was detected in *G. prostrata* subsp. *lusitanica* (2 populations), *L. fruticosa* (1), *L. hispidula* subsp. *versicolor* (1), and *L. zahnii* (2). L-morph bias was found in *G. oleifolia* (1), *G. prostrata* subsp. *prostrata* (5), and *L. fruticosa* (13, but also one S-morph bias).

Sex-organ deployment—A total of 105 populations of seven taxa of *Glandora* and four of *Lithodora* were sampled. For each taxon, mean values for corolla length, herkogamy, and reciprocity are summarized in Table 1. In these taxa, style length ranged between 7.3–14.6 mm in the L-morph and 3.4–8.2 mm in the S-morph; whereas anther height ranged between 5.2–10.7 mm in the L-morph and 5.6–13.6 mm in the S-morph. Mean values for corolla length and sex-organ position are summarized in Fig. 5, and detailed information on measurements for each population is presented in Appendix S5. Reciprocity values varied from high reciprocity in populations of *G. moroccana* (0.77 ± 0.02) to low reciprocity in *L. fruticosa* (0.25 ± 0.22 ; Table 1). There were significant differences in the degree of herkogamy between morphs ($F_{1,187} = 14.68$, $P < 0.001$), species ($F_{10,187} = 52.39$, $P < 0.001$), and their interaction ($F_{10,187} = 5.14$, $P < 0.001$). Herkogamy distance in the L-morph was significantly greater than the S-morph in four out of the eight taxa we investigated (see Tables 1, 2 and Fig. 5). Herkogamy in the L-morph varied from 2.55 ± 0.46 mm in populations of *L. fruticosa*, to 6.29 ± 0.81 mm in *G. oleifolia*, whereas herkogamy in the S-morph varied from 2.32 ± 0.34 mm in popula-

tions of *L. hispidula* subsp. *versicolor*, to 6.42 ± 0.35 mm in *G. oleifolia* (Table 1).

Relations between morph ratios and floral traits—We compared the mean morph-bias index per taxon between distylous and non-distylous taxa (stigma-height dimorphic and relaxed style dimorphic). As predicted, distylous taxa were significantly more isoplethic than nondistylous species, which often had biased style-morph ratios ($t = -4.6$, $df = 10$, $P < 0.001$). There was evidence that phylogenetic relationships among taxa contributed toward morph-ratio variation, because the value of λ in our analysis was 1, indicating that the mean values for morph-bias index of related taxa were more similar to each other than random values drawn from the same tree. With regard to the relations between variation in deviations from isoplethy and sex-organ deployment at the species level, we found no significant associations between the IQR of morph-bias index (IQR_b) and the IQR of reciprocity (IQR_r), L-morph herkogamy (IQR_L), or S-morph herkogamy (IQR_S) (Appendix S6). Note that because we found differences in the herkogamy distance between morphs, we considered the herkogamy distance in the L-morph (IQR_L) and the S-morph (IQR_S) as independent factors in these analyses. The phylogenetic signal in the analysis had a value of $\lambda = 0$, indicating that values of IQR_b among closely related taxa were no more similar than among less-related taxa (Appendix S6).

When studying each taxon independently, seven subsets of factors were analyzed: (1) reciprocity + herkogamy in the L-morph (L-herkogamy) + herkogamy in the S-morph (S-herkogamy); (2) reciprocity + L-herkogamy; (3) reciprocity + S-herkogamy; (4) L-herkogamy + S-herkogamy; (5) reciprocity; (6) L-herkogamy; and (7) S-herkogamy. We detected a significant relationship between the morph bias index and reciprocity ($t = 2.8$; $df = 19$; $P = 0.012$) and S-herkogamy ($t = -2.9$; $df = 19$; $P = 0.011$) in *L. fruticosa* under the best-fit model for this species (Appendix S7). Smaller values of herkogamy in the S-morph were related to greater deviations from isoplethy in the direction of increasing L-bias. Moreover, unexpectedly, a higher degree of reciprocity was related to greater deviations from isoplethy (Fig. 6).

DISCUSSION

In this study, we investigated the relationships between variation in the position of female and male sex organs and population-style

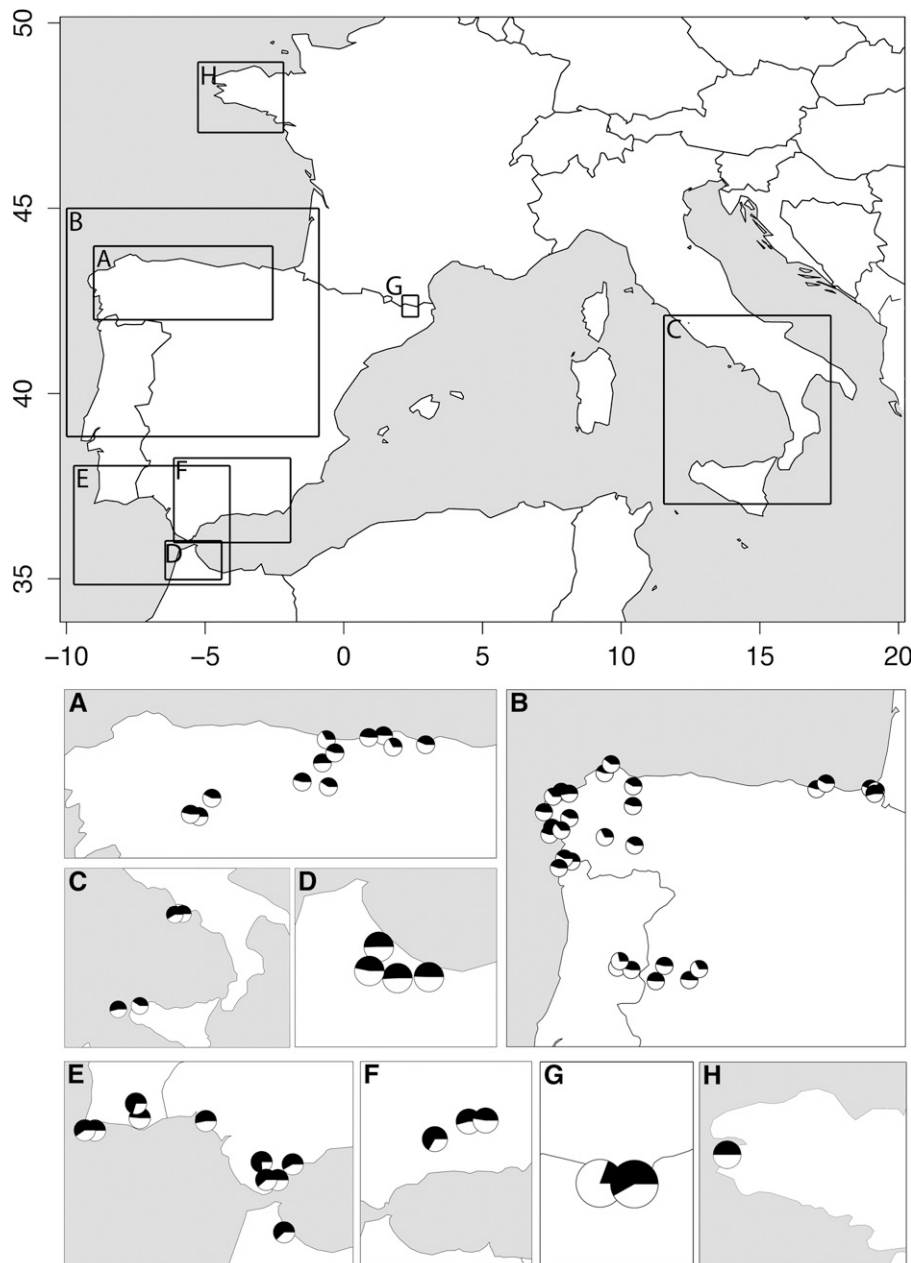


FIGURE 3 Geographical distribution of style-morph ratios in species of *Glandora* throughout its range in the Mediterranean basin and Atlantic coast of Europe. The frequency of the L-morph (white) and S-morph (black) are indicated for each population. The top map identifies the areas sampled. (A) *G. diffusa*, $N = 12$ populations; (B and H) *G. prostrata* subsp. *prostrata*, $N = 31$ populations; (C) *G. rosmarinifolia*, $N = 5$ populations; (D) *G. moroccana*, $N = 4$ populations; (E) *G. prostrata* subsp. *lusitanica*, $N = 10$ populations; (F) *G. nitida*, $N = 3$ populations; and (G) *G. oleifolia*, $N = 2$ populations.

morph ratios in a lineage of heteromorphic plants, which vary in stylar polymorphisms and incompatibility-compatibility systems. We examined this association independently in 11 taxa of *Glandora* and *Lithodora*, but also in a phylogenetic context because it has been shown that heteromorphic traits show numerous transitions during the evolutionary history of this lineage (Ferrero et al., 2009). As predicted, our results demonstrated that across all species, the type of sex-organ deployment found in a particular taxon was associated

with the amount of deviation from isoplethy. Distylous taxa collectively exhibited less deviation than those with stigma-height dimorphism and relaxed stigma-height dimorphism. However, within taxa there was little evidence for an association between intraspecific variation in sex-organ position and morph ratios. Only in *L. fruticosa*, a species with stigma-height dimorphism and weak reciprocity between stigma and anther heights, was there evidence that variation in sex-organ position may influence morph-ratio variation. Our discussion considers the reproductive, ecological, and genetic factors that may help to explain the striking variation in style-morph ratios exhibited by this unusual lineage of heteromorphic plants.

Causes of the general association between type of stylar polymorphism and morph-ratio bias

Diverse factors influence the relative frequency of the L- and S-morphs in dimorphic species of angiosperms. These include stochastic forces associated with finite population size, and deterministic forces resulting from differences among the style morphs in fertility and mating patterns. Although we did not estimate population size quantitatively in our survey, we grouped distylous and nondistylous populations into three classes (small, medium, and large, Appendix S4), but found no statistically significant relation between population size and morph-ratio variation (Generalized Linear model: $F_{2,99} = 0.8$, $P = 0.465$) or in the interaction: Size \times Type of polymorphism ($F_{2,99} = 1.3$, $P = 0.271$). However, there was a significant relation between the type of polymorphism and morph-ratio variation ($F_{1,99} = 11.4$, $P = 0.001$), confirming our results at the species level. Population size undoubtedly influenced variation in morph ratios in some of the very small populations in our sample; however, it does not appear to have played a significant role in causing the overall difference between distylous and nondistylous species in morph-ratio variation. Prolific clonal propagation or differences in the fertility or strength of incompatibility systems between morphs are known to affect their frequency in populations of some heteromorphic plants (e.g., Thompson et al., 2003; Wang et al., 2005; Brys et al., 2008b; Castro et al., 2013), but in the case of *Lithodora* and *Glandora*, the species appear to be nonclonal and we have no evidence for consistent fertility differences or variation in the type and strength of incompatibility between the style morphs within species.

We found equal proportions of the style morphs in 76% of the 105 populations that we surveyed in this study. In the more widespread taxa that were sampled more extensively, the opportunities

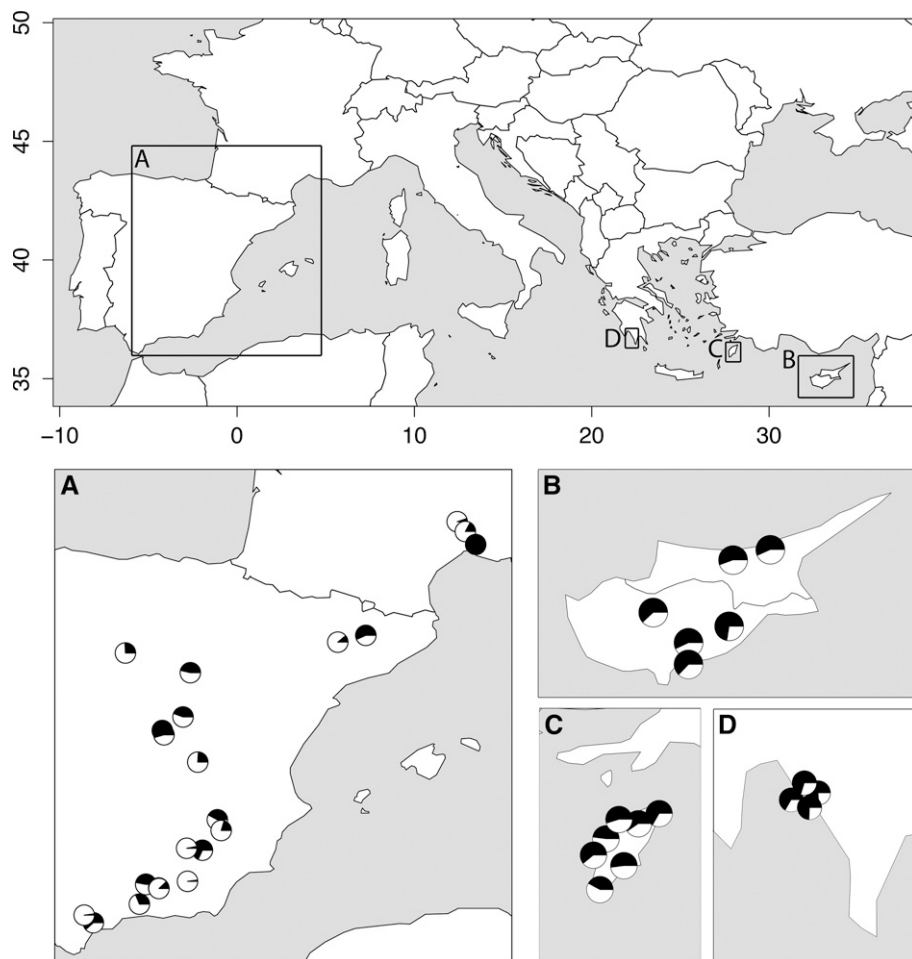


FIGURE 4 Geographical distribution of style-morph ratios in species of *Lithodora* throughout its range in the Mediterranean basin. The frequency of the L-morph (white) and S-morph (black) are indicated for each population. Image on the top shows the localization of the detailed areas. (A) *L. fruticosa*, $N = 22$ populations; (B) *L. hispidula* subsp. *versicolor*, $N = 6$ populations; (C) *L. hispidula* subsp. *hispidula*, $N = 7$ populations; and (D) *L. zahinii*, $N = 4$ populations.

for detecting bias increased because of the number of populations sampled. The most plausible hypothesis to explain the observed difference between the two major classes of stylar polymorphisms in the relation between sex-organ reciprocity and morph-ratio bias concerns the aggregate patterns of mating in populations. In those with a high level of disassortative mating, equal morph ratios are expected as a result of negative frequency-dependent selection. In species with a dimorphic incompatibility, as in *Glandora nitida*, this mating pattern is guaranteed and all three populations sampled were isoplethic. However, for the remaining three distylous taxa in our survey for which the compatibility system has been determined experimentally (Ferrero et al., 2012), none possess dimorphic incompatibility and all are self- and intramorph compatible. In these species sex-organ reciprocity is the primary mechanism promoting disassortative mating and indeed there is evidence that this may occur. *Glandora moroccana* and *G. diffusa* are fully self- and intramorph compatible with high sex-organ reciprocity (Ferrero et al., 2011a, 2012; see Fig. 5) and all populations sampled were isoplethic (Fig. 3G). Elsewhere among heteromorphic plants, high levels of disassortative mating resulting

in equal morph ratios have been reported in self-compatible, tristylous *Eichhornia paniculata* (Barrett et al., 1987; Kohn and Barrett, 1992) and in stylar dimorphic *Narcissus papyraceus* (Simón-Porcar et al., 2014, 2015a). We observed abundant pollinator visitation by long-tongued bees in distylous *Glandora* and *Lithodora* populations (Ferrero et al., 2011b) and these insects probably play a key role in promoting disassortative mating and causing isoplethic morph ratios.

In this study, we distinguished species of *Glandora* and *Lithodora* with stigma-height dimorphism and relaxed stigma-height dimorphism (sensu Ferrero et al., 2009) from those exhibiting distyly. We made this distinction because angiosperm species with these polymorphisms generally lack strong sex-organ reciprocity (reviewed in Barrett et al., 2000, and see Sánchez et al., 2008, 2013). In species with stigma-height dimorphism, anther levels are positioned similarly in the floral tubes of the L- and S-morphs, whereas with relaxed stigma-height dimorphism, they are scattered in their distribution within the corolla and show little reciprocity. Our measures of reciprocity (R , see Sánchez et al., 2008) generally supported the expectation that stigma-height dimorphic taxa exhibit less reciprocity of sex-organ positioning compared to those that were distylous (Table 1). Values of R ranged from 0.77–0.58 for distylous species and from 0.58–0.25 for stigma-height dimorphic species, including the relaxed form.

In three of the four species with stigma-height dimorphism, the style morphs, although self-incompatible, are intramorph compatible allowing assortative mating in populations. This type of incompatibility system

has been reported in other taxa of flowering plants with stigma-height dimorphism and can cause asymmetrical mating and biased morph ratios (Barrett and Cruzan, 1994; Baker et al., 2000a; Arroyo et al., 2002; Barrett and Hodgins, 2006). However, the extent to which the style morphs differ in levels of assortative mating depends on a variety of factors including the size of populations and their pollination biology, and the particular configurations of sex organs in the style morphs. For example, in stigma dimorphic *Narcissus assoanus*, both L-morph biased and isoplethic populations are reported in southern France and these differ in size and the quantity and quality of pollinator service (Baker et al., 2000a, c). Differences in the types of pollinators visiting populations of other stigma-height dimorphic *Narcissus* species have also been implicated in causing variation in morph ratios (Arroyo and Dafni, 1995; Simón-Porcar et al., 2014). A theoretical model of pollination and mating in stigma-height dimorphic populations, based on empirical data on the floral biology of *Narcissus* species, demonstrated that greater assortative mating in the L-morph, because of the reduced herkogamy of this morph compared to the S-morph, could explain the L-biased populations that are most commonly

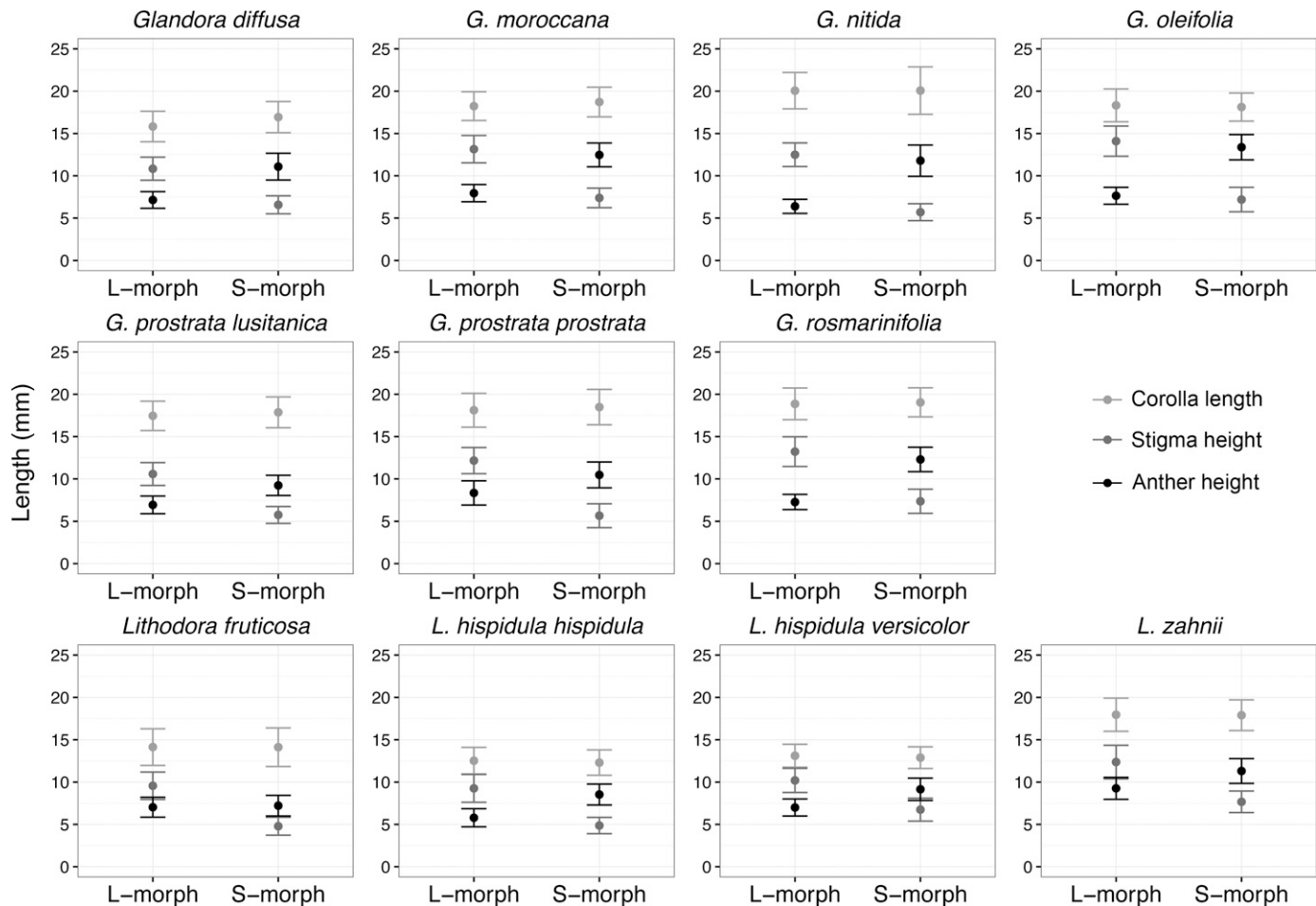


FIGURE 5 Variation in corolla length and sex-organ height (mean \pm SD in millimeters) in flowers of the L- and S-morph in *Glandora* (seven species) and *Lithodora* (four species). The number of populations per species that were used for calculations is summarized in Table 1.

encountered in this genus (Baker et al., 2000a). However, in *Glandora* and *Lithodora*, the degree of herkogamy does not differ between the style morphs in a consistent way (Table 1). Thus, it is unclear the extent to which herkogamy generally influences mating patterns and morph ratios in a consistent way across species.

In our survey of stigma dimorphic species, a wide range of morph ratios were encountered but overall, as expected, they exhibited a greater tendency to depart from isoplethy compared to distylous species. We found some exceptions to the general association between weak reciprocity and biased morph ratios. For example, *G. prostrata* subsp. *prostrata* possesses relaxed stigma-height dimorphism, intramorph compatibility, and a low reciprocity value of $R = 0.26$. Nevertheless, of the 31 populations we surveyed, 26 exhibited equal morph ratios and the remaining 5 were L-biased. In closely related *G. prostrata* subsp. *lusitanica*, which also has the same characteristics, among 10 populations sampled, eight were isoplethic and the remaining two were S-biased. It is unclear what reproductive mechanisms operate in populations of this species to promote disassortative pollination and why the direction of bias differs between the two subspecies.

Association between sex-organ positions and morph ratios within species—Our study of *Glandora* and *Lithodora* taxa was designed to investigate general patterns of morph-ratio variation among dif-

ferent classes of stylar polymorphism. As a result, the power to detect significant associations between sex-organ position and morph ratios was weak in many of the taxa. This was particularly the case for rare species, because of the limited number of populations that could be sampled. Indeed, in seven of the 11 taxa in our survey, less than 10 populations were sampled. However, where larger samples were obtained, several patterns were revealed—the most interesting of which involved *L. fruticosa*.

Among the 22 populations of *L. fruticosa*, a negative relation between the degree of herkogamy of the S-morph and deviations from isoplethy was evident (Fig. 6). As the distance between stigmas and anthers of the S-morph became smaller in populations, the frequency of this morph also declined. In contrast, there was no relation between the herkogamy of the L-morph and morph ratios (Appendix S7). Two potential mechanisms could explain this pattern. The reduction in frequency of the S-morph could arise if intramorph mating in this morph caused morph-specific inbreeding depression resulting from sheltered load at the S-locus (Strobeck, 1980; Barrett et al., 1989). A second possibility is that the smaller distance separating female and male sex organs within a flower may result in self-interference (Barrett, 2002; Cesaro et al., 2004; Valois-Cuesta et al., 2011). In self-incompatible species like *L. fruticosa*, self-interference may reduce female fitness because stamens physically impede pollen deposition on stigmas (Barrett and Glover,

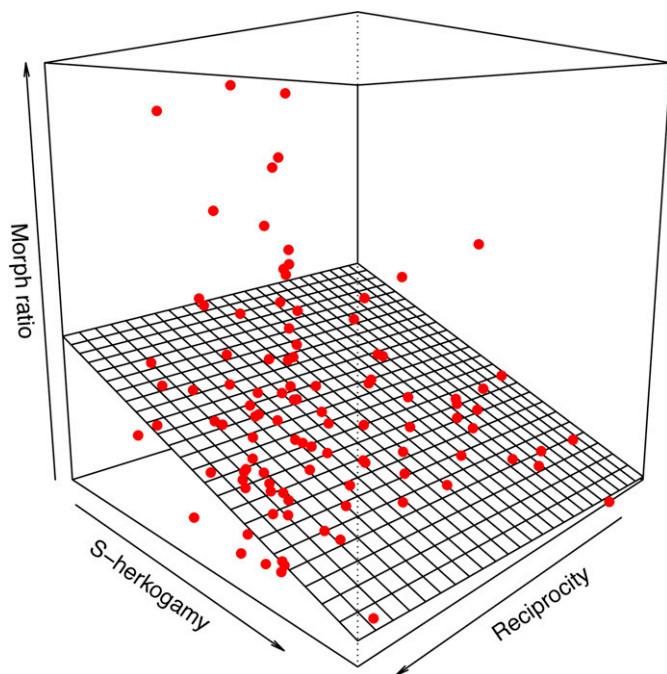


FIGURE 6 Plot of response surface illustrating the effect of reciprocity, and herkogamy in the S-morph, on the morph ratio of populations of *Lithodora fruticosa*. The morph-bias index was calculated as the absolute value of the difference in the number of individuals of the L- and S-morph, divided by the total number of flowering individuals sampled. Reciprocity index was calculated following Sánchez et al. (2013).

1985), and/or there is an increased likelihood that self-pollination causes stigma or stylar clogging (Howlett et al., 1975; Shore and Barrett, 1984; Webb and Lloyd, 1986; Scribailo and Barrett, 1994; Cesaro et al., 2004). In species with late-acting ovarian self-incompatibility, self-pollen tubes may disable ovules otherwise available for cross-fertilization, i.e., ovule discounting (Waser and Price, 1991; Barrett et al., 1996; Sage et al., 1999, 2006; Navarro et al., 2012). Whether *L. fruticosa* possesses a late-acting self-incompatibility system is currently unknown; however, it may be significant that this form of incompatibility has been previously reported in species with stigma-height dimorphism, including members of the Boraginaceae (Dulberger, 1964, 1970; Philipp and Schou, 1981; Schou and Philipp, 1984; Barrett et al., 1997; Simón-Porcar et al., 2015b). Thus, reduced herkogamy in the S-morph could conceivably result in lower fitness of this morph in populations driving its frequency downward.

Integration of phylogenetic and species level approaches—In this study, we used a phylogenetic approach in our statistical analyses. This was necessary for our comparative study because the two taxa we investigated were previously considered a single genus, although they are clearly differentiated based on phylogenetic evidence (Thomas et al., 2008; Ferrero et al., 2009). The primary rationale for the use of phylogenetically based statistical methods was the expected nonindependence among species trait values due to their phylogenetic relatedness (Felsenstein, 1985). The branching pattern and estimated times of divergence in our Bayesian analysis based on six DNA regions was largely consistent with previous work, although here we obtained stronger support for most clades (Ferrero

et al., 2009; Mansion et al., 2009). We found a phylogenetic signal when analyzing the influence of the type of polymorphism on morph-ratio bias. However, our analyses at the species (and subspecies) level revealed that variation among populations in sex-organ deployment was largely unrelated to morph-ratio variation. Considerable effort involving a large number of populations sampled over a broad geographical range is often necessary to detect significant associations between functional traits and style-morph ratios (e.g., Barrett et al., 2004). However, where this is not possible, comparative studies conducted within a phylogenetic framework can expose novel associations between morphological characters and style-morph ratios providing opportunities for future hypothesis testing at the population level.

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