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Stigma–anther reciprocity, pollinators, and pollen transfer efficiency in populations of heterostylous species of *Lithodora* and *Glandora* (Boraginaceae)

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Abstract According to Darwin, the reciprocal position of sexual whorls in heterostylous plants enhances disassortative pollen transfer between different floral morphs. It is believed that greater reciprocity between morphs will promote more efficient transfer of pollen. Additionally, efficient pollination will act as a selective force in achieving greater reciprocity between floral morphs. In this study we test whether variation in reciprocity of sexual organs between morphs is related to the efficiency of pollinators in transferring pollen between them. To do this, we first describe the pollinator's array in several populations of species of the genus formerly known as *Lithodora*, which have different types of stylar polymorphism and degrees of reciprocity, and determine their abundance, plant visitation rate, number of flowers visited per plant and handling time in the population. We estimate the efficiency of the pollinator arrays by use of an approximation based on qualitative (location of pollen loads on different areas of insect bodies) and quantitative (plant visitation rate) measurements. Our results show a correlation between the degree of reciprocity and the efficiency of pollinators associated with the populations. These observations suggest that pollinators are a possible selective force driving the evolution of heterostyly.

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Introduction

Pollination efficiency has often been defined as the amount of conspecific pollen deposited on a stigma per visit (Inouye et al. 1994); however, the true effectiveness of a pollinator is also determined by the proportion of pollen grains picked up from each flower (Rademaker et al. 1997). Models predicting floral evolution mediated by pollinators rely on the quantification of the relationships between floral traits and pollen delivery or receipt accomplished by each pollinator species (Aigner 2001). Because heterostylous plants are characterized by the presence of discrete morphs that differ in sexual organ position, it could be expected that this relationship would have special importance in this kind of system, because the differences in anther and stigma position can cause dissimilar capability of accession to stigmas and anthers by pollinators in both morphs. The most common approach followed in studies of heterostylous plants assumes the same probability of pollen removal for both morphs and focuses exclusively in the success through female function, for example pollen deposition on stigmas, fruit production, and seed set (Ornduff 1975, 1978; Ganders 1976; Weller 1980; Nicholls 1985; Pailler et al. 2002; Brys et al. 2008). Few studies have considered differences in the probability of pollen exportation between morphs (Wolfe and Barrett 1989; Harder and Barrett 1993; Lau and Bosque 2003; Ornelas et al. 2004) or addressed the relationship between pollen removal and pollen deposition (Ornelas et al. 2004; Adler and Irwin 2006). Moreover, the intensity of an interaction between two species (e.g., a plant and its pollinator) is proportional to the frequency of

its occurrence (quantity) multiplied by the magnitude of its consequences on fitness when it is present (quality) (Herrera 1987).

Recent studies of species of the genus formerly known as *Lithodora* (Ferrero et al. 2009) support the Darwinian hypothesis proposed by Lloyd and Webb (1992), because of the observed succession in types of style polymorphisms occurring within the tribe Lithospermeae. According to Lloyd and Webb's (1992) proposal, pollinators are the selective forces behind the evolutionary step that leads to reciprocal herkogamy, which implies an increase in the degree of reciprocity between floral morphs. However, what remains to be tested in this system is whether there is actually a positive relationship among pollinator assemblage efficiency and the degree of sexual reciprocity between floral morphs at the population level, as expected if this prediction is correct. In this study we characterize the reciprocity of sexual whorls between floral morphs, and investigate differences in pollinator assemblage, abundance of each visitor, frequency of interactions, and efficiency in pollen transfer of the whole pollinator assemblage in several populations of the genus formerly known as *Lithodora*. As a preliminary approach to infer if the pollinators are involved in the evolutionary change we hypothesized that differences in reciprocity levels would be related to dissimilarities in:

1 pollinator array;
2 pollinator abundance; or
3 efficiency of pollen transport of the pollinator array when visiting the different floral morphs.

To assess the efficiency of pollinator array as a whole, we used an approximation by combining an estimate of pollination success, by measurement of pollen loads on insects captured within the populations, with the frequency of their visits to the plants (i.e., combining both the quantity and quality components of each insect species as pollinator).

Materials and methods

Species studied

Species of the former *Lithodora* (Boraginaceae), recently split into the genera *Glandora* D.C. Thomas, Weigend and Hilger and *Lithodora* Griseb. (Thomas et al. 2008), are Mediterranean shrubs, with bluish tubular flowers. Wide variation in style polymorphism—stylar dimorphism, distyly, or relaxed stylar dimorphism—and in the degree of reciprocity of sexual whorls between morphs has been reported for both genera (Ferrero et al. 2011). Different types of incompatibility systems, ranging from typical

heteromorphic incompatibility to complete lack of incompatibility, have also been described, but flowers always depend on pollinators to produce fruits (Ferrero 2009). Nectar is produced and accumulated at the bottom of the corolla tube and, therefore, nectarivorous pollinators should access this site. Floral lifespan is 3–5 days, with both pollen being released and the stigma being receptive from the first day of anthesis (Ferrero 2009). This study was conducted on seven taxa from the genera *Glandora* and *Lithodora*. Eleven populations, listed in Table 1, were selected to depict the extent of variation in the degree of reciprocity found in both genera. Most of the populations selected were close to isoplethy, i.e., had equal proportion of long (L) and short (S) morphs (details are given in Table 1).

Degree of reciprocity between morphs at the population level

One flower per plant from 100 shrubs, when available, was collected in the populations studied and kept in 70% ethanol until measurement. Flowers were slit longitudinally and two morphological characteristics were measured:

1 style length, from the base of the corolla to the stigma surface; and
2 every stamen height, from the base of the corolla to the midpoint of every anther (more details are given by Ferrero et al. 2009).

With these morphological measurements we calculated the index of reciprocity between sexual whorls in the morphs, following Sánchez et al. (2008). This index compares stigma–stamen height gaps for all potential crosses in the populations. Because this index combines stigma–stamen distance and their dispersion, it can be used as a meaningful measurement to characterize and compare reciprocity between populations and species.

Floral visitors assemblage

We characterized the floral visitors assemblage in each population by direct observations. The observations were performed in one flowering peak between 2006 and 2008 in one to three populations of each species (Table 1), in randomly selected areas of approximately 2–3 m². The observer was positioned approximately 1 m from the selected area, being able to monitor all the floral visitors without disturbing their foraging behavior. Visits were recorded during series of 15 min of surveillance sessions at different hours of the day (from 0800 to 1900 h, GMT + 1). We grouped the visitors in "morphotypes" (e.g., *Bombylius* sp., *Anthophora* sp.), because of the occasional impossibility of discerning at the species level in

Table 1 Taxa and populations analyzed in the study with information on locality, geographical coordinates, elevation, proportion of morphs (L, long morph; S, short morph), reciprocity index (following Sánchez et al. 2008), type of stylar polymorphism (following Ferrero et al. 2009), and effort, for insect characterization (total number of visits and total handling time, min)

Taxon	Locality	Coordinates	Elevation (m)	Proportion of morphs L:S	Reciprocity index (type of polymorphism)	Effort	
						Number of visits	Handling time (min)
Glandora diffusa (Lag.) D.C. Thomas	Spain: Suances	43°26′30″N 04°02′50″W	11	1.1:1	0.018 (distyly)	240	240
G. diffusa (Lag.) D.C. Thomas	Spain: Ventanilla (Palencia)	42°52′40″N 04°31′59″W	1,103	1.2:1	0.017 (distyly)	1,380	–
G. moroccana (I.M. Johnst.) D.C. Thomas	Morocco: Akchour	35°06′69″N 04°58′58″W	467	1:1	0.023 (distyly)	1,680	840
G. nitida (Ern) D.C. Thomas	Spain: S. Magina	37°42′06″N 03°27′53″W	1,591	0.9:1	0.030 (distyly)	2,940	2,940
G. oleifolia (Lapeyr.) D.C. Thomas	Spain: St. Aniol	42°19′34″N 02°35′10″W	–	0.7:1	0.019 (distyly)	2,700	1,320
G. prostrata (Loisel.) D.C. Thomas subsp. *lusitanica* D.C. Thomas	Spain: S. Bermeja	36°29′14″N 05°10′24″W	551	0.8:1	0.045 (relaxed stylar dimorphism)	540	540
G. prostrata (Loisel.) D.C. Thomas subsp. *lusitanica* (Samp.) D.C. Thomas	Spain: La Alcaidesa	36°15′17″N 05°21′58″W	157	0.6:1	0.055 (relaxed stylar dimorphism)	1,200	1,200
G. prostrata (Loisel.) D.C. Thomas subsp. *lusitanica* D.C. Thomas	Portugal: El Picacho	36°31′19″N 05°39′05″W	332	0.3:1	0.056 (relaxed stylar dimorphism)	1,620	1,620
G. prostrata (Loisel.) D.C. Thomas subsp. *prostrata*	Spain: Corrubedo	42°34′36″N 09°05′21″W	5	1.2:1	0.094 (relaxed stylar dimorphism)	2,220	1,800
Lithodora fruticosa Griseb.	Spain: Hueva	37°20′28″N 03°51′31″W	836	1.1:1	0.073 (stylar dimorphism)	3,060	1,800
Lithodora fruticosa Griseb.	Spain: S. Baza	37°23′54″N 02°50′29″W	1,602	48.5:1	0.051 (stylar dimorphism)	1,320	1,320

the field, and assuming that each taxon included in a morphotype had similar behavior when visiting the flower and similar efficiency in transport of the pollen to suitable parts to fertilize the flower (hereinafter referred to as "pollen transport"). When it was possible to identify the species, we considered separate "morphotypes" whenever their behavior and/or type of interaction with the flower differed from that of other closely related species. We considered all diurnal Lepidoptera within the same group ("other Lepidoptera") because of the scarcity of each species in most populations. These butterflies belong to the families Pieridae and Nymphalidae and have similar approaches to the flowers, only touching the sexual organs with the proboscis.

A total of 1,368 censuses were carried out, corresponding to 342 h of observation. During each session the visitor species or morphotype and the total number of plants and flowers visited were recorded. At the end of each session, visitors were captured to assess both the pollen loads carried on their bodies and the length of their proboscis.

We calculated the average abundance as the mean number of individuals per count (Herrera 1989). Differences in abundance of each pollinator species among populations were analyzed by use of generalized linear models (GLZ) in SPSS software (version 16, SPSS, Chicago, USA) with abundance adjusted to a Poisson distribution and a logarithmic link function for model responses.

To assess whether different pollinator arrays and pollinator abundance depended on plant species, permutational multivariate analysis of variance using distance matrices (ADONIS) was used with the total number of appearances and the abundance as the dependent variables. ADONIS enables the use of any semimetric (e.g., Bray-Curtis, aka Steinhaus, Czekanowski and Sørensen) or metric (e.g., Euclidean) distance matrix (McArdle and Anderson 2001). It uses a permutation test with pseudo-F ratios to assess significance that postulates only that the observation units are exchangeable under a true null hypothesis and thus does not rely on the assumption of multivariate normality. ADONIS

Fig. 1 Pollinators and differences in their behavior when entering the flowers of *Lithodora* and *Glandora*. **a** *Anthophora* sp.; **b** *Macroglossum stellatarum*; **c** *Bombylius* sp.; **d** *Gonepteryx rhamni*; **e** *Bombus pascuorum*; **f** *Autographa bractea* (abdomen (*a*) head (*h*), legs (*l*), proboscis (*p*), thorax (*t*), wings (*w*)). Hymenoptera (**a**, **e**) are capable of contacting L-morph stigmas with the proboscis (*p*) and the head (*h*). Lepidoptera (**b**, **d**, **f**) and Bombyliidae (**c**) heads do not contact stigmas. No other parts make contact with the sexual organs of the flowers

was performed in the Vegan package (Oksanen et al. 2008) of R (version 2.8.1, R Development Core Team 2008).

The number of flowers visited per plant and the handling time were compared among the most common insect species in each population; to do this we transformed the data to achieve normality and carried out one-way ANOVA followed by Tukey's test. When normality was not achieved even after transformation, we conducted a Kruskal–Wallis test for several independent samples followed by Mann–Whitney pairwise comparisons. For comparisons between two groups a Mann–Whitney pairwise comparison was carried out. We excluded from the analysis insect morphotypes with less than ten visits and the population of *G. prostrata* subsp. *lusitanica* at S. Bermeja and *G. diffusa* at Ventanilla (for handling time) because of data scarcity. This analysis was carried out with the SPSS software package.

Foraging efficiency

We here propose an approach to measure the population visitor assemblage efficiency based on the efficiency of each individual visitor in pollen transport. To this end we use pollen loads on the insect body and the frequency of interaction. In addition, measurements of proboscis length were performed under a stereo microscope using a digital

caliper. To calculate the amount of pollen carried on each zone of the pollinator's body, the insects captured were taken to the laboratory where pollen on each body part (abdomen (a), head (h), legs (l), proboscis (p), thorax (t) and wings (w)) was categorized under a stereo microscope, trying to distinguish *Lithodora* pollen from that of the co-flowering plants. *Lithodora* was the main resource for the pollinators in the studied populations and the pollinators show great fidelity for the plant species. Four quantitative classes were considered: class 0 when 0 grains were found on the examined body part; class 1 when up to 100 grains were found on the analyzed part; class 2 when 100–1,000 grains were found; and class 3 when more than 1,000 grains of pollen were found. Based on all the individuals captured we calculated a "mean pollen load" (P) for each part of the body of each morphotype or species. Total population visitor assemblage efficiency in pollen transport was calculated as the sum of the efficiency of each visitor in crosses delivering pollen in L-morph stigmas plus those on the S-morph. We consider that for *Lithodora* and *Glandora* species the pollen deposited on all observed insect proboscises and heads is efficient (hereinafter referred as E) for L-morph pollinations, because the heads of the insects can touch the stigma in the L-morph (Fig. 1). However, for the S-morphs only the pollen on the

proboscis (p) can be regarded as efficient (i.e., it can be delivered to S-stigmas). Pollen deposited in the remaining parts of the body was regarded as inefficient (hereinafter referred as In) because of its low or null probability of making contact with the stigmas of these plant species. Note that while foraging many bees place the pollen on areas of their bodies that do not make contact with sexual structures of the plant. Moreover, for Lepidoptera and *Bombylius* sp., we considered only proboscis (p) as efficient for both morphs, because the head never touches the stigma of any flower (V. Ferrero, field observations; Fig. 1). For a detailed identification of body parts see Fig. 1. Thus, we calculate total efficiency (TE) of each pollinator species as follows:

$$\text{TE} = \text{TEL} + \text{TES} = (\text{EL} - \text{InL}) + (\text{ES} - \text{InS})$$
$$\text{TEL} = \left[(P_p + P_h)/2\right] - \left[(P_t + P_a + P_l + P_w)/4\right]$$
$$\text{TES} = P_p - \left[(P_h + P_t + P_a + P_l + P_w)/5\right]$$

where TEL = TE in L-morph plants; TES = TE in S-morph plants; EL = efficiency in L-morph plants; InL = inefficiency in L-morph plants; ES = efficiency in S-morph plants; InS = inefficiency in S-morph plants; P_p = amount of pollen on the proboscis; P_h = amount of pollen on the head; P_t = amount of pollen on the thorax; P_a = amount of pollen on the abdomen; P_l = amount of pollen on the legs; P_w = amount of pollen on the wings.

Finally, we calculated the total visitor assemblage efficiency (efficiency of the whole pollinator array—TVAE) for each population (hence TE) by multiplying the TE by the percentage of flowers visited by each insect in each population.

Association between pollen transport efficiency and reciprocity

In order to check if higher efficiencies in pollen transfer were associated with greater reciprocity, we carried out a Pearson correlation analysis between the TE of the pollinator arrays and the reciprocity index values.

Results

Degree of reciprocity between morphs at the population level

Results for the extent of reciprocity between the sexual whorls of both morphs in each population are summarized in Table 1. The population presenting more reciprocity between morphs was Ventanilla (*G. diffusa*) whereas the less reciprocal was Corrubedo (*G. prostrata* spp. *prostrata*).

Floral visitor's assemblage

The observed number of pollinator interactions in the populations of *Lithodora* and *Glandora* studied are shown in Table 2, with flowers visited per plant and handling times per flower, average proboscis lengths, and the pollinator efficiencies. A total of 3,377 visits of 13 morphotypes or species belonging to three insect orders (Hymenoptera, Diptera, and Lepidoptera) were recorded (Table 2).

Overall, the flower-visitor assemblage was composed mostly of species with long proboscises. Most of the floral visitors were Hymenoptera (nine taxa), in particular *Anthophora* sp. which was present in all populations (see Fig. 2 for each species of pollinator abundance within populations). *Bombus pascuorum* was the most common species in Suances and Corrubedo (although it was also important in Ventanilla). *Eucera* sp. was the main pollinator in El Picacho and *Bombylius* sp. in La Alcaidesa and Ventanilla (Fig. 2).

The mean number of flowers visited per plant ranged from 1 to 20 (*Macroglossum stellatarum* in S. Mágina). The mean handling time ranged from 0.4 to 0.5 s (*M. stellatarum* in El Picacho and S. Mágina) to 5.5–5.9 s (*Apis* sp. in La Alcaidesa and "other Lepidoptera" in S. Mágina) (Table 2). Differences among insects in number of flowers visited per plant and handling time are summarized in Table 3.

With regard to pollinator abundance, significant differences were found among plant populations for all visitor species except for *Apis mellifera* and *Anthidium* sp. (the latter only present in one population) (see results of the analysis in Fig. 2). Results from ADONIS showed that visitor arrays during the spring of 2006–2008 did not differ between plant species ($F_{1,9} = 1.07$, $P = 0.390$ using the Bray–Curtis index and $F_{1,9} = -0.08$, $P = 0.980$ when using the Horn–Morisita index) more than could be expected from random behavior. When looking to the abundance, we found significant differences when comparing among plant species using the Bray–Curtis index ($F_{1,9} = 5.84$, $P < 0.005$) but marginally no significant differences were obtained when using the Horn–Morisita index ($F_{1,9} = 4.49$, $P = 0.060$).

Visitor's foraging efficiency

Most pollinators in species of *Lithodora* and *Glandora* are nectar collectors and approach the flowers frontally (Fig. 1). According to our proposal to measure efficiency of pollen transport, the most efficient pollinator belongs to Lepidoptera; first *M. stellatarum* with a TE value of 2.5 (ranging from 1.50 to 2.50) and the longest proboscis (mean 19.08 ± 5.52 mm). The group "other Lepidoptera" is the

Table 2 From top to bottom in each cell: number of interactions, number of flowers visited per plant, and handling time (in seconds); with proboscis length (mm), pollinator total efficiency in pollen transfer (TE) (see Materials and methods), and floral reward obtained (where n is nectar and p is pollen) of the pollinators of *Lithodora* and *Glandora* in the studied populations

Plant species	Population	Order Hymenoptera									Order Diptera	Order Lepidoptera			TVAE
		Anthophora sp.	*Andrena* sp.	*Anthidium* sp.	*Apis mellifera*	*Bombus pascuorum*	*Bombus terrestris*	*Eucera* sp.	*Thyreus* sp.	Sphecidae	*Bombylius* sp.	*Macroglossum stellatarum*	*Autographa bractea*	Other Lepidoptera	
G. diffusa	Suances	120 (38.8) 6.6 ± 7.3a 0.8 ± 0.4a				177 (57.3) 5.8 ± 5.3a 1.4 ± 1.0b	1 (0.3) 10– 2.0 ± 1.0		11 (3.6) 7.0 ± 4.2a 1.1 ± 0.9a						−0.25
	Ventanilla	83 (26.7) 5.1 ± 5.0a 0.6 ± 0.9	22 (7.1) 4.2 ± 3.1a			92 (29.7) 11.1 ± 16.6a 1.5 ± 0.8	5 (1.6) 4.0 ± 1.0– 1.1			8 (2.6) 7.0 ± 5.9–	96 (31.0) 3.8 ± 2.9a 1.7 ± 0.7			4 (1.3) 1.5 ± 0.6–	−0.30
G. moroccana	Akchour	893 (89.2) 6.3 ± 8.2a 0.6 ± 0.9				1 (0.1) 3.0– 1.5 ± 0.8	3 (0.3) 3.3 ± 1.5– 0.9 ± 0.1	4 (0.4) 3.3 ± 3.3– 0.7 ± 0.3			95 (9.5) 4.4 ± 4.0b 1.7 ± 0.7	5 (0.5) 4.0 ± 1.6–			0.70
G. nitida	S. Magina	465 (66.1) 15.5 ± 18.9a 0.6 ± 1.0a		5 (7.1) 2.2 ± 1.3– 0.9 ± 0.7	1 (0.7) 1.0– 0.7		1 (0.1) 1.0– 1.1	7 (0.9) 10.0 ± 6.1– 1.8 ± 1.6			28 (3.9) 6.3 ± 6.1b 3.4 ± 4.2b	158 (22.5) 19.6 ± 24.6a 0.5 ± 0.4c	30 (4.3) 15.0 ± 26.6ab 2.2 ± 2.2d	8 (1.1) 2.5 ± 2.3– 4.4 ± 9.0	1.11
G. oleifolia	St. Aniol	570 (75.2) 4.9 ± 6.0a 1.1 ± 1.2a				6 (0.8) 4.8 ± 4.4–					153 (20.2) 3.1 ± 2.8b 1.9 ± 0.5b	3 (0.4) 2.3 ± 0.68–		26 (3.4) 2.1 ± 1.6b	0.84
G. prostrata subsp. *lusitanica*	S. Bermeja	98 (100) 2.4 ± 1.7 0.5 ± 0.4													0.81
	Alcaidesa	150 (38.1) 5.7 ± 5.5a 1.1 ± 2.5a			1 (0.3) 2.0– 5.9 ± 6.8			31 (7.9) 4.3 ± 4.3a 1.4 ± 3.0a			212 (53.8) 4.7 ± 4.8a 2.5 ± 4.9b				0.18
	El Picacho				8 (0.7) 2.1 ± 1.6– 2.3 ± 3.0			1039 (91.1) 6.2 ± 5.9b 1.4 ± 1.7b			68 (5.9) 4.5 ± 4.1a 1.2 ± 0.9a	1 (0.1) 3.0– 0.4 ± 0.1			0.32
G. prostrata subsp. *prostrata*	Corrubedo	69 (9.1) 9.1 ± 11.6a 1.4 ± 1.5a				334 (43.9) 7.4 ± 9.2a 1.5 ± 1.1b	61 (8.0) 9.0 ± 8.9a		2 (0.3) 4.5 ± 0.7–		100 (13.2) 7.9 ± 6.9a 2.6 ± 2.1c		37 (4.9) 7.2 ± 7.8a 2.6 ± 2.0c	157 (20.7) 3.4 ± 3.7b 5.5 ± 4.4d	−0.11
L. fruticosa	Hueva	378 (35.5) 11.4 ± 15.0a 0.7 ± 0.8a	135 (12.7) 3.2 ± 3.2b 1.7 ± 1.5bc						13 (1.2) 13.7 ± 11.3a	52 (4.9) 5.3 ± 4.7ab 2.5 ± 2.6d	346 (32.5) 8.5 ± 10.1a 1.5 ± 1.1b	33 (3.1) 11.3 ± 12.4a 0.5 ± 0.3c	91 (8.5) 9.8 ± 12.1a 1.8 ± 1.4e	18 (1.7) 4.3 ± 3.9ab 2.9 ± 2.2f	0.07
	Baza	303 (65.3) 4.5 ± 4.5a 0.9 ± 4.2a	73 (15.7) 2.5 ± 4.3b 2.1 ± 4.4b					8 (1.7) 2.0 ± 1.9– 5.6 ± 4.1			70 (15.1) 4.7 ± 4.3a 1.6 ± 1.9b	4 (0.9) 4.0 ± 0.0– 0.4 ± 0.2	6 (1.3) 6.0 ± 5.6– 3.1 ± 2.6		0.21
Proboscis length (mm)/(n)		4.6 ± 0.8 (41)	3.5 ± 1.4 (8)		2.4 ± 0.3 (4)	4.7 ± 0.8 (5)	3.9 ± 0.4 (4)	4.1 ± 0.6 (6)	3.8 (1)		6.2 ± 1.9 (18)	19.1 ± 5.5 (4)	11.7 ± 0.4 (2)	9.2 ± 2.00 (4)	
Pollinators efficiency (TE)		0.81	−1.90		−0.26	−0.99	−1.50	0.35	0.15		−0.29	2.50	0	2.00	
Floral reward		n	p	p	p	n	n	n	n	p	n	n	n	n	

Total visitor assemblage efficiency (TVAE) in each population is shown in the last column. Values are given as total number of plants visited followed in parenthesis by the proportion of the interaction in each plant population (%), and mean ± SD for number of flowers visited and for handling time. For these last two, differences among insects were analyzed by use of one-way ANOVA followed by a Turkey test. Means with the same letter do not differ significantly at the α = 5% level
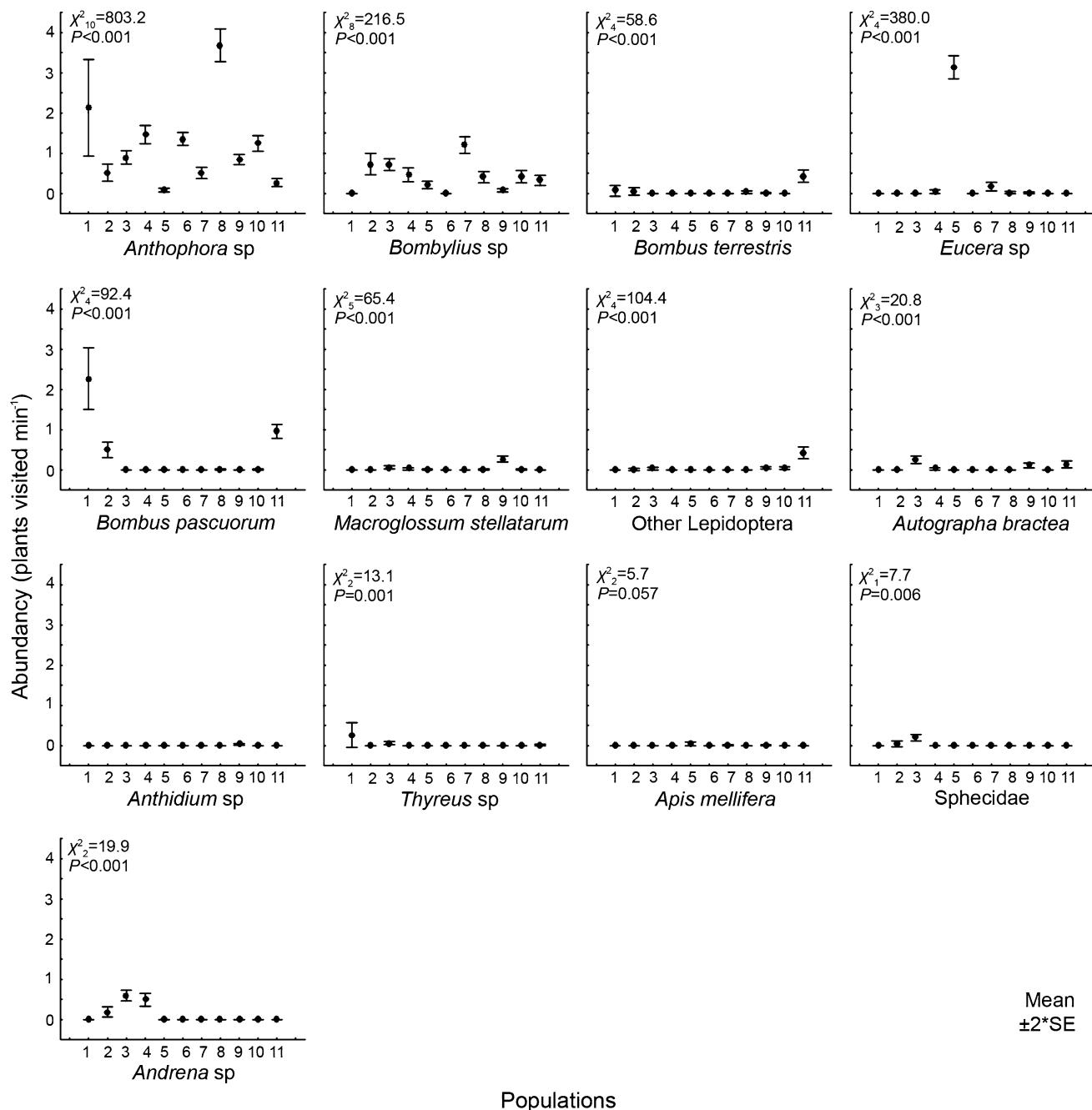
Fig. 2 Abundance of each pollinator morphotype in the populations. Results of the Wald χ^2 test for comparisons among each pollinator abundance in the populations are indicated on the *top left*. Analysis was not carried for *Anthidium* sp. because it was present only in one population. Populations are as follows: *1* Suances, *2* Ventanilla, *3* Akchour, *4* S. Mágina, *5* St. Aniol, *6* S. Bermeja, *7* La Alcaidesa, *8* El Picacho, *9* Corrubedo, *10* Hueva, *11* S. Baza. Values are given as mean ± 2SE

second most efficient pollinator morphotype, also having long proboscises (mean 9.20 ± 1.99 mm). Because pollen in "Lepidoptera" was deposited on the proboscis and other parts do not contact the sexual organs, there is no apparent inefficiency in this group. Within the Hymenoptera, the most efficient was *Anthophora* sp. with a positive TE value of 0.81, i.e., efficiency is greater than inefficiency (Table 2).

Association between efficiency and reciprocity

TE results for pollen transport of the whole pollinator arrays in the populations are summarized in Table 2. No significant correlation was found when all the populations were included ($r = -0.277$, $P = 0.409$). However, when the populations of *G. diffusa* were excluded from the

Table 3 Results from use of one-way ANOVA to compare the number of flowers visited per plant and the handling time among the different insect morphotypes for species of *Lithodora* and *Glandora*

	Population	Number of flowers visited			Handling time		
		F	*df*	*P*	*F*	*df*	*P*
G. diffusa	Suances	0.626	2, 305	0.535	340.28	2, 1,883	**<0.001**
	Ventanilla	2.060	3, 289	0.106	–	–	–
G. moroccana	Akchour	3.686	1, 989	0.055	−14.395	1 3,707	**<0.001**
G. nitida	S. Mágina	3.963	3, 677	**<0.008**	1,269.19	4, 9,451	**<0.001**
G. oleifolia	St. Aniol	8.993	2, 745	**<0.001**	1.948	1, 743	0.163
G. prostrata subsp. *lusitanica*	La Alcaidesa	2.930	2, 390	0.055	190.02	2, 1,953	**<0.001**
	El Picacho	3.017	2, 1,128	**0.049**	8.93	2, 6,908	**0.012**
G. prostrata subsp. *prostrata*	Corrubedo	18.401	5, 849	**<0.001**	1,182	4, 4,756	**<0.001**
L. fruticosa	Hueva	14.102	7, 1,058	**<0.001**	2,832.34	6, 7,502	**<0.001**
	Baza	6.614	2, 442	**0.001**	390.14	3, 2,163	**<0.001**

A Kruskal–Wallis test was performed when normality was not achieved. In the Akchour population, Mann–Whitney pairwise comparison was performed for handling time. Significant differences are highlighted in bold
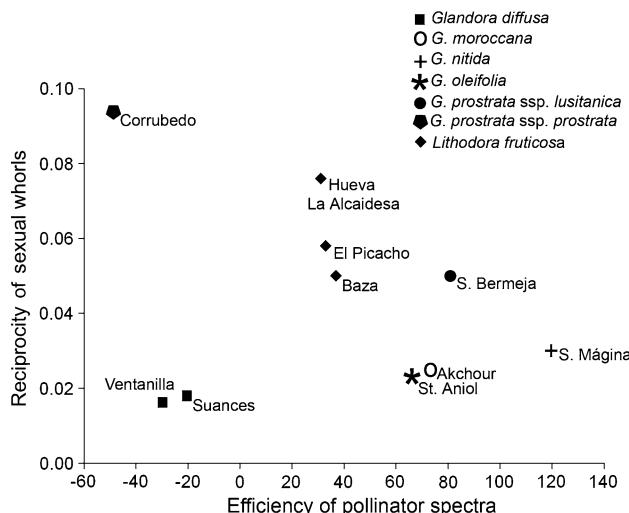

Fig. 3 Relationship between the degree of reciprocity among sexual whorls (following Sánchez et al. 2008) and pollinator efficiency in pollen transport (see Material and methods for an explanation of the calculation) at the population level. Low values for the reciprocity index mean greater reciprocity. Note that pollinator efficiency can reach negative values when high quantities of pollen are wasted

acquired via an intermediate unstable stage of stigma height dimorphism (populations with two style morphs, long and short-styled, but anthers remaining at the same level). This evolutionary hypothesis implies that the process should be enhanced, or at the least modulated, by the efficiency of pollinators which, in turn, can be affected by the degree of reciprocal herkogamy between morphs (Lloyd and Webb 1992; Ferrero et al. 2009, 2011). Previous studies (Ferrero et al. 2009; 2011) have shown that distyly, stylar dimorphism (Table 1), and relaxed stylar dimorphism, a derived new polymorphic state described by Ferrero et al. (2011) characterized by wide variation in the height of the anthers on both morphs, causing low reciprocity between them, are present in species of the former genus *Lithodora*. Considering that floral visitors with different morphology and behavior usually differ in their ability to transfer pollen between plants (Schemske and Horvitz 1984; Herrera 1987; Young 1988; Castellanos et al. 2003), estimates of both pollinator visit rates and efficiency are of great importance to address the actual implication of floral visitors in the process of selection on sexual traits.

The populations analyzed in this study were shown to have a diverse array of pollinators, composed of Hymenoptera, Lepidoptera, and Diptera. Pollinator species differ greatly, not only in morphological traits, but also in behavioral features, depending on their foraging strategy. Some pollinators made many rapid visits to the same plant (e.g., *Anthophora* sp.), enhancing geitonogamous pollen transfer, whereas others made few visits per plant staying longer periods per flower (e.g., Lepidoptera) promoting higher rates of xenogamous pollen transfer. The differences in handling times between them are mainly a consequence

analysis, a significant and strong negative correlation was found ($r = -0.850$, $P = 0.004$) (Fig. 3).

Discussion

Lloyd and Webb (1992) proposed that the driving force leading to heterostyly is the promotion of accurate pollination between morphs. They hypothesized that, from an ancestral condition of approach herkogamy (flowers with stigmas above anthers), reciprocal herkogamy would be

of their different nectar-extraction techniques, i.e., *Anthophora* sp. suck the nectar more quickly by lapping with their glossae (Harder 1982), whereas some species of Lepidoptera are slower, collecting the nectar by suction (Kingsolver and Daniel 1983). These behavioral differences together with morphological features may be some of the factors leading to the different efficiencies in pollen transport observed among insect species. *Anthophora* sp. occurred in all the studied populations and, overall, is the main pollinator of *Lithodora* and *Glandora*.

In previous studies with other heterostylous groups it was found that different flower morphologies and types of stylar polymorphism were associated with different pollinator fauna, which may be involved in shifts from one type of floral polymorphism to another (Arroyo and Dafni 1995; Barrett et al. 1996; Arroyo et al. 2002; Pérez-Barrales et al. 2007). In *Narcissus* a shift from style dimorphism to distyly was associated with a change to bee pollination, whereas pollination by butterflies and flies was associated with stable style dimorphism and monomorphism (Pérez-Barrales et al. 2006). In contrast, in *Lithodora* and *Glandora* there were no differences among species in the identity of pollinator arrays, meaning that all the studied species (and types of polymorphism) have similar pollinator fauna associated to them. Instead, there were different abundances of every insect species and pollinator array among populations. This implies that although having the same pollinators, each visitor species has a different relative abundance within the array of pollinators depending on the population and, consequently, a different impact on the overall efficiency of the pollinator array.

Pollinator efficiency should be measured as pollen removed from anthers and deposited on stigmas, also taking into account the quality of transferred pollen. But in the process of pollen collection and deposition, much of the pollen is lost by the floral visitors (Rademaker et al. 1997; Castro et al. 2008). For this reason, when assessing the efficiency of pollinators, it is also important to consider their inefficiency (e.g., as pollen loads on body parts that are not likely to contact the stigmas). Because flowers of *Lithodora* and *Glandora* have a narrow corolla tube, the proboscis is the only part of the insect able to touch the S-stigmas and L-anthers. However, L-styles and S-anthers protrude from the floral tube make contact with the insect head also. In our study this type of flower–visitor interaction was observed for most Hymenoptera (Fig. 1). However, bees also carried a large amount of pollen on parts of their body with low probability of making contact with the stigmas. Thus, the most efficient pollinators are insects with long proboscises capable of reaching low sexual whorls (i.e., S-stigmas and L-anthers) and causing minimum pollen wastage.

Integrating both approaches in measurement of pollinator efficiency, the most efficient pollinators according to our criteria are Lepidoptera (excluding *Autographa bractea*), which is also the group with longer proboscises. Among these, efficiency values are highest for *M. stellatarum*, a species also very common in one of the more reciprocal species (*G. nitida*, S. Mágina, Table 2). Adler and Irwin (2006) have already reported that visitors carrying the largest amounts of pollen on their bodies may not always be the most efficient at depositing pollen on stigmas. The greater efficiency found in Lepidoptera and the negative values for pollinator efficiency within Hymenoptera in our study are because of the pollen carried on inefficient parts of the insect body and could be interpreted as an inefficiency component in pollination by these insects, i.e., pollen that will not achieve fertilization.

Our approximation to measure pollinator efficiency as a whole furnished surprising results. Although preliminary, the positive correlation found between the degree of reciprocity and pollinator efficiency (note that high values in the index of reciprocity indicate low reciprocity and vice versa) points to greater efficiency of pollinator arrays (as a whole) in those populations in which the sexual whorls are placed more reciprocally. This seems to imply that pollinator arrays are the selective pressures driving the reciprocal position of sexual whorls. The only exception was the narrow endemic *G. diffusa*, for which results are puzzling and suggest that other forces are conditioning the floral polymorphism.

Several studies have considered pollinators as the main evolutionary force in the process of evolution of distyly. This study shows that sometimes discrimination of pollinator fauna is not a simple task based on description of the functional groups appearing in the populations, but that it should include a detailed analysis of pollinator arrays and their overall efficiency. When this is done, we can perceive a relationship between the degree of reciprocity and the efficiency of pollinator arrays that has not been noticed previously. Furthermore, the relationship observed in our study supports Darwin's (1877) predictions in respect of selective forces driving evolution of heterostyly. This idea encourages future studies focused at the individual level to test the specific pressures exerted on reciprocal herkogamy by pollinators in heterostylous genera such as *Lithodora* and *Glandora*.

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