Three-dimensional reciprocity of floral morphs in wild flax (*Linum suffruticosum*): a new twist on heterostyly

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Summary

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• Here, we studied the floral morphology and pollination of the distylous plant *Linum suffruticosum* (Linaceae) in southern Spain.
• We observed a previously unreported form of distyly that involved twisting and bending of styles and stamens during floral development to achieve three-dimensional reciprocity of anthers and stigmas in the long-styled (pin) and short-styled (thrum) morphs. This developmental pattern causes pin pollen to be placed on the underside of pollinating *Usia* flies (Bombyliidae), and thrum pollen to be placed on the top of the thorax and abdomen. The pin stigmas contact the flies on the dorsum, apparently picking up predominantly thrum pollen, and the thrum stigmas contact the flies on the ventral surface, apparently picking up predominantly pin pollen.
• This form of heterostyly would appear on morphological grounds to be far more efficient in dispersing pollen between compatible morphs than the typical pin–thrum system. If so, this plant fits Darwin's prediction of efficient pollen flow between heterostylous morphs more closely than anything Darwin himself reported.
• Molecular phylogenetic analyses indicate that this form of heterostyly evolved in a lineage that already had typical heterostyly. The analyses also indicate that there have been several independent origins of heterostyly in *Linum* and at least one reversal to stylar monomorphism.

Key words: disassortative pollen flow, distyly, flax, floral development, heterostyly, *Linum suffruticosum* (wild flax), pollination.

Introduction

Darwin (1862, 1864, 1877) was the first to provide a detailed explanation of the function and adaptive significance of two or more morphs of bisexual flowers occurring in the same population (called heterostyly or floral polymorphism). He determined that usually only intermorph pollinations result in seed set (intramorph incompatibility) in heterostylous plants. Darwin was introduced to heterostyly in *Primula* by his botanical mentor John Henslow in 1830 (Kohn *et al.*, 2005), and this introduction, together with his later detailed studies, contributed to the formulation and refinement of his

hypothesis of natural selection (Darwin, 1859, 1872; see Huxley, 1958). Indeed, in his autobiography, Darwin stated of his research on heterostyly: 'I do not think anything in my scientific life has given me so much satisfaction as making out the meaning of the structure of these plants' (F. Darwin, 1905, p. 74). Darwin (1877) and others subsequently (see review in Barrett *et al.*, 2000), described heterostyly in terms of variation between floral morphs along a linear axis: the height of fertile floral parts above the base of the flower. For example, in distyly, short-styled flowers have long stamens (thrums) and long-styled flowers have short stamens (pins). This arrangement may lead to some degree of spatial coordination of pollen deposition on

pollinators by anthers and pollen pickup by stigmas of the corresponding height. Thus pin flowers should tend to send pollen to thrum stigmas, and thrum flowers should tend to send pollen to pin stigmas. A similar situation may occur with tristyly, where there are three floral morphs with three stigma and anther heights, although the transfer dynamics are more complicated (Darwin, 1864; Barrett *et al.*, 2000).

Darwin's idea that reciprocal positioning of anthers and stigmas in heterostylous flowers results in (and possibly evolved for) improvement of segregated pollen flow between morphs has generated considerable debate. While some theory and some empirical studies support the idea of efficient pollen segregation (Ganders, 1974; Nicholls, 1985a; Lloyd & Webb, 1992a,b; Cesaro & Thompson, 2004), others do not (Olesen, 1979; Ornduff, 1979, 1980a,b; see review in Ganders, 1979). The mechanical efficiency of heterostyly is particularly critical to the operation of models explaining the evolution of heterostylous incompatibility systems (cf. Charlesworth & Charlesworth, 1979; Lloyd & Webb, 1992a,b).

One conceptual problem with the mechanical function of reciprocally placed stigmas and anthers in distylous floral morphs relates to how pollen is placed on pollinators by anthers and picked up from them by stigmas. In nearly all described heterostylous flowers, reciprocal placement of structures occurs in a linear dimension (height above the corolla base) in a narrow tube, and pollinators push their tongues, heads or bodies past the thrum anthers (long stamens, short style) and pin stigmas (long style, short stamens) as they obtain nectar. This means that thrum pollen may often be smeared along much of the length of the proboscis or body rather than placed at a discrete location, and the pin stigma is often also dragged along this surface. Pollen is thus not placed as precisely as in flowers in which pollinators are stationary on the flowers before pollen is deposited or the stigma is engaged (e.g. Armbruster *et al.*, 1994, 2002, 2004; Classen-Bockhoff *et al.*, 2004). There is also a potential difference in the precision of pollen placement and stigma contact between morphs: pin anthers and thrum stigmas, because of their basal positions, are likely to be somewhat more precise in their reciprocal placement on, and pickup of pollen from, pollinators, respectively, than are thrum anthers and pin stigmas, potentially resulting in asymmetrical pollen flow between morphs (Stone & Thomson, 1994; Lau & Bosque, 2003; Cesaro & Thompson, 2004). Thus it seems unsurprising that heterostyly may often fail to segregate pollen flow effectively (Kohn & Barrett, 1992; Stone & Thomson, 1994; Lau & Bosque, 2003).

In this paper we describe what appears to be a far more efficient form of distyly, one that has not been previously reported. In one species of wild flax (*Linum suffruticosum*), the pin and thrum anthers and stigmas show full reciprocity in three dimensions, placing pollen on either the dorsal or the ventral surface of the pollinators. We describe how the floral morphologies interact with pollinators and how they might influence pollen-transfer efficiencies, and reconstruct the origin of this feature and the evolution history of heterostyly in *Linum* sect. *Linastrum*.

Materials and Methods

Study system

We examined the morphology of, and pollinator behaviour on, heterostylous morphs in the wild flax *Linum suffruticosum* L. (Linaceae) in southern Spain. Observations on *Linum* were an important part of Darwin's seminal study of heterostyly (1864, 1877). He documented intramorph incompatibility and reciprocal placement of anthers and stigma (as height above the corolla base) in several species. He also described several morphological precursors of three-dimensional variation in anther and stigma positions, although he did not observe reciprocity. He reported divergence of the styles away from the central axis in the short-style morph of *Linum grandiflorum* Desf. (which has dimorphic styles but no variation in stamen length), *Linum flavum* L. and *Linum perenne* L. (the last two both have dimorphic pistils and stamens). This allows the stigmas to protrude from between the thick basal portions of the staminal filaments. Darwin (1877) also noted that the styles of the long-styled morph of *L. perenne* rotate so that the stigmas face outward, presumably promoting the uptake of pollen from pollinators carrying pollen on their dorsal surface. Darwin did not comment, however, on any corresponding rotation of anthers on the long stamens (short-styled form). Little else has been written on the pollination of *Linum*, except for studies by Heitz (1980), who reported pollination of *L. perenne* by unnamed flies (with dorsal pollen placement by both morphs) and bees (with ventral pollen placement by both morphs) in France, Kearns & Inouye (1994), who reported pollination of *Linum lewisii* Pursh. by a large variety of flies and bees in Colorado, USA, and Johnson & Dafni (1998), who reported pollination of *Linum pubescens* Banks & Solander by *Usia* flies (Bombyliidae) in Israel. Du Merle & Mazet (1978) also reported *Linum salsoloides* and *Linum narbonense* as the major host plants of adult *Usia* in southern France. These flies appeared to be phenologically synchronized with the flowering of *L. salsoloides* (Du Merle & Mazet, 1978).

Linum suffruticosum is described as heterostylous and intramorph-incompatible in eastern Spain (Rogers, 1979). Rogers (1979) also reported that pollen size is the same in the two morphs, but that the exine sculpturing differs; this has also been reported in populations in the region of our study (Candau, 1987).

Field and laboratory observations

We observed floral morphology, morph frequencies, and pollination of flowers in two populations of *L. suffruticosum* in Andalucía, southern Spain. One main study site was at Puerto del Viento, in the municipality of Ronda, Málaga Province

(36°47′36.0″ N, 4°59′28.7″ W; 972 m elevation), and the second was at Puerto de las Palomas, in the municipality of Grazalema, Cádiz Province (36°47′17.2″ N, 5°22′34.9″ W; 1200 m elevation). Seven additional study sites in these provinces were examined briefly to note whether they had pin and thrum flower morphologies generally consistent with those seen at the two intensively studied sites.

We observed floral visits on two days at each site, and noted whether and where each visitor contacted anthers and/or stigmas on each of the two morphs. At the flowering peak, we estimated morph frequencies at Grazalema and at a third site, Sierra de Líjar, in the municipality of Algodonales, Cádiz Province (36°54′16.8″ N, 5°24′18.3″ W; 1000 m elevation), recording the morphs of all plants in blossom along a random walking transect of approx. 500–1000 m. Fruit set was estimated separately for each morph by counting the number of flowers with fully developed capsules vs flowers with undeveloped ovaries at the end of the blooming period. It was possible to determine the morph of each fruit because styles remain attached to the ovary during maturation.

Ethanol (70%)-preserved flowers were placed under a dissecting microscope and flower parts measured with callipers. Stigmas of both morphs were measured with a micrometer to the nearest 0.01 mm under a dissecting microscope at ×25. Voucher insects were collected with a net, pinned, and provisionally identified using available manuals and published papers. Location of pollen on visitors was noted in the field and with a microscope.

DNA sequencing and phylogeny estimation

In order to ascertain the evolutionary antecedent of the unusual expression of heterostyly in *L. suffruticosum*, we estimated the phylogenetic relationships of 16 *Linum* accessions, plus one species of the related genus *Radiola*.

DNA extraction and internal transcribed spacer (ITS) sequencing A set of 11 individuals representing the diversity of *Linum* sect. *Linastrum* and five individuals representing four additional sections (*Cathartolinum, Syllinum, Dasylinum* and *Linum*) were sampled. *Radiola linoides* was used as the outgroup. This sample is part of a broader study in progress (J.M. Martínez, F. Muñoz & P. Vargas, unpublished). Total genomic DNA was extracted from material collected in the field and from some herbarium specimens. All the material used for Fig. 2 (see below) is labelled and deposited in the herbarium of the Royal Botanic Garden (Madrid, Spain). Field collections were dried in silica gel. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) and amplified using polymerase chain reaction (PCR) on a Perkin-Elmer PCR System 9700 (Perkin-Elmer, Foster City, CA, USA) or an MJ Research (Watertown, MA, USA) thermal cycler. After 4 min of pretreatment at 94°C, PCR conditions were: 24–35 cycles of 1 min at 94°C, 30 s to

1 min at 50–52°C, and 1–2 min at 72°C. Two external primers (17SE and 26SE) were used for amplification of the ITS region (White *et al.*, 1990). A volume of 1 μl of dimethyl-sulfoxide (DMSO) was included in each 25-μl reaction. Amplified products were cleaned using spin filter columns (PCR Clean-Up Kit; MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator version 2.0; Applied Biosystems, Little Chalfont, UK) following the manufacturer's protocols and run into polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism model 3700 automated sequencer. The ITS5 and ITS4 primers were used for cycle sequencing of the ITS region (Sun *et al.*, 1994). Sequenced data were assembled and edited using the program SEQED (Applied Biosystems, Foster City, CA, USA). The limits of the ITS region were determined by comparison with previous publications (Yokota *et al.*, 1989). International Union of Pure and Applied Chemistry (IUPAC) symbols were used to represent nucleotide ambiguities.

Molecular analysis and character reconstruction Sequences were aligned using CLUSTAL X 1.62b (Thompson *et al.*, 1997), with further adjustments by visual inspection. Insertion/deletion mutations (indels) were not used for the analysis. Maximum parsimony (MP) and Bayesian inference (BI) analyses were then performed. All parsimony analyses were conducted using Fitch parsimony (as implemented in PAUP*; Swofford, 1999) with equal weighting of all characters and of transitions/transversions. Branch and bound analyses were performed to obtain optimal trees in exhaustive searches following initial heuristic methods. Internal support was assessed using 1000 replicates of full bootstrapping. To determine the simplest model of sequence evolution that best fits the sequence data, the hierarchical likelihood ratio test (hLRT) and Akaike information criterion (AIC) were implemented using MRMODELTEST 1.1b (Nylander, 2002). A Bayesian inference analysis was conducted using MRBAYES 3.0b4 (Ronquist & Huelsenbeck, 2003) and sampling for one million generations (four Marker Chain Monte Carlo (MCMC), chain temperature = 0.2; sample frequency = 100; burn-in < 1000). A 50% majority-rule consensus tree was calculated from the pooled sample using the sumt command to yield the final Bayesian estimate of phylogeny. The distribution of the style polymorphism (heterostyly, three-dimensional reciprocity and stylar monomorphism) in the 15 taxa of *Linum* was based on the literature (Ockendon & Walters, 1968; Nicholls, 1985a,b) and personal observations. Patterns of evolution were explored using the character-state optimization function of MACCLADE 4.06 (Maddison & Maddison, 1999), assuming Fitch parsimony. Both ACCTRAN (maximizing the proportion of the homoplasy that is accounted for by parallelism) and DELTRAN (maximizing the proportion accounted for by reversals) optimizations were considered and analyzed. Characters

were traced initially onto the strict consensus of shortest trees obtained. To gain further insights into morphological character evolution, the MP tree displaying most congruence with the BI tree, under the simplest model of sequence evolution, was used for the final character-change reconstruction (see Results).

Results

Floral morphology

Pin (L) and thrum (S) morphs were found to be of nearly identical appearance, except for the length and orientation of the sexual parts. Style and stamen lengths differed significantly in the expected direction [$P < 0.001$; analysis of variance (ANOVA) on log-transformed data; $F_{1,38} = 105.8$, 13.86 for style and anther lengths, respectively; $n = 20$ for all measurements]; however, the differences were small compared with those in many other species of *Linum* and other heterostylous species. Pin morphs measured at Puerto Las Palomas, Grazalema, had a mean ($\pm$ SE) style length of 9.67 (0.20) mm and stamen length of 6.51 (0.21) mm. Thrum morphs had a mean ($\pm$ SE) style length of 6.96 (0.16) mm and stamen length of 7.56 (0.11) mm. These differences were, however, insufficient to generate accurate reciprocity, particularly between pin styles and thrum stamens. Reciprocity calculated as (style length$_{pin}$ – stamen length$_{thrum}$) was 2.11 mm, and that for (style length$_{thrum}$ – stamen length$_{pin}$) was 0.45 mm. There was no detectable difference in pollen size, as previously reported by other authors. However, stigma width differed significantly between morphs (mean $\pm$ SD: L, 0.51 $\pm$ 0.06 mm, $n = 17$; S, 0.36 $\pm$ 0.03 mm, $n = 16$; Mann–Whitney $Z = -4.2866$, $P \ll 0.001$). Pollen tends to be purple in pin flowers, and yellow in thrum flowers.

The main difference between styles of pin and thrum morphs was the erect posture of pin styles, which form a column in the center of the flower, with stigmas facing outward. In contrast, the thrum styles spread outward, nearly appressed to the corolla wall, with the tips extending about one-third of the way up the petals and the stigmas facing inwards (Fig. 1).

The stamens differed similarly between pin and thrum morphs. Pin stamens spread from near the base, appressed to the corolla wall, and extending one-third of the way up the petals; the openings of the dehiscing anthers face inwards. The thrum stamens are erect, forming a column in the center of the flower, and the anthers are rotated so that the openings of the dehiscing anthers face outwards (Fig. 1).

The anthers and stigmas of pin and thrum flowers are thus positioned reciprocally in three dimensions ('3D reciprocity'). The anthers and stigmas are not closely reciprocal in height, but rather in how the stamens and styles bend and twist. The result of this arrangement is that thrum stamens contact the back of any appropriately sized insect crawling down the petal to obtain nectar [resulting in dorsal placement of pollen (nototriby)]. Pin stigmas, in turn, contact insects in the same location. The pin stamens contact the underside of any insect crawling down the petal to obtain nectar and place pollen there (sternotriby). The thrum stigmas, in turn, contact such insects in the same location.

The minimum distances between the petals and stigmas of the pin morph and between the petals and anthers of the thrum morph (the 'gaps') establish the minimum standing height a nectar-foraging visitor must have in order to be a pollinator. The mean ($\pm$ SD) gaps at Ronda were 1.68 (0.37) mm for pin morphs ($n = 31$) and 1.54 (0.31) mm for thrum morphs ($n = 24$). The mean ($\pm$ SD) gaps at Grazalema were 1.83 (0.34) mm for pin morphs ($n = 14$) and 1.67 (0.42) mm for thrum morphs ($n = 14$). These values differed with marginal significance both between morphs and between sites (two-way factorial ANOVA; between morphs: $F_{1,79} = 3.25$, $P = 0.075$; between sites: $F_{1,79} = 2.93$, $P = 0.09$; interaction: $F_{1,79} = 0.03$, $P = 0.87$).

The flowers close by night and open in the morning between 10:00 and 11:30 h GMT, when the petals unfurl and spread away from the floral axis. The flowers last at least 2 d. The frequencies of the two morphs approached equality in

Fig. 1 Flowers of *Linum suffruticosum* (wild flax). Note reciprocal positions (in three dimensions) of anthers and stigmas in the two morphs. The slight difference in petal morphology between the two morphs reflects different floral ages (the pin is recently opened and the thrum is older), not a consistent morphological difference in morphs. (a) The pin (long-styled) morph, with *Usia* sp. 2 (large) (Bombyliidae) on its way into the flower to obtain nectar, contacting anthers with its ventral surface, and about to contact stigma with the dorsal side of thorax, which is pale with a thrum-pollen load. (b) The thrum (short-styled) morph, with *Usia* sp. at the corolla base drinking nectar. (c) The pin and thrum morphs compared, with petals removed.

Table 1 *Linum suffruticosum* (wild flax) fruit set in the field

Floral morph	Puerto de las Palomas (1988)	Puerto de las Palomas, subpopulation A (2005)	Puerto de las Palomas, subpopulation B (2005)	Sierra de Líjar (2005)
Pin (L morph)	0.103 (0.104) $n = 11$	0.446 (0.201) $n = 20$	0.378 (0.164) $n = 26$	0.501 (0.198) $n = 15$
Thrum (S morph)	0.204 (0.138) $n = 9$	0.462 (0.194) $n = 9$	0.424 (0.207) $n = 27$	0.535 (0.203) $n = 9$

Values are mean number of fruits per flower (standard deviations in parentheses). Although there was a significant site–year effect (two-way analysis of variance: $F_{3,118} = 15.60$, $P < 0.0001$), there was no detectable effect of morph on fruit set ($F_{1,118} = 15.60$, $P = 0.97$) or of the interaction between morph and site ($F_{3,118} = 15.60$, $P = 0.50$).

Table 2 Contact with stigmas and anthers by floral visitors at the Ronda site, 21–22 May 2005, during 13.75 person-hours of observation on several dozen flowers of *Linum suffruticosum* (wild flax)

Floral visitor	Reward collected	Pin			Thrum			Movement between morphs?
		Frequency of contact with stigmas	Frequency of contact with anthers	n	Frequency of contact with stigmas	Frequency of contact with anthers	n	
Usia sp. 1 (small)	Nectar	85% (dorsal surface only)	100% (ventral surface only)	26	98% (ventral surface only)	97% (dorsal surface only)	60	+
Usia sp. 2 (large)	Nectar	100% (dorsal surface only)	100% (ventral surface only)	1	100% (ventral surface only)	100% (dorsal surface only)	6	+
cf. *Megachile*	Pollen	100% (ventral surface only)	0%	6	0%	100% (ventral surface only)	49	+
Large orange Bombyliidae	Nectar	0%	0%	1	0%	0%	1	–

n, number of observed floral visits by each insect species.

the two measured populations (Grazalema: 1 : 0.920, $n = 169$; Sierra de Líjar: 1 : 1, $n = 124$). The two morphs set similar proportions of fruit, so there is apparently no tendency towards maleness or femaleness in either morph (Table 1).

Insect visitation

Insect visitation to *L. suffruticosum* started at *c.* 11:00 h GMT, when the flowers opened. Nectar is produced in small quantities at the base of the petals in what is effectively a narrow tube accessible only to insects with long proboscides. The nectar is presented in small amounts and was too viscous in our samples to be measured by conventional hand refractometers (perhaps because of high evaporation rates). The majority of visits at both sites were made by two species of bombyliid flies (Bombyliidae: *Usia* Latreille; Arabia Sánchez, National Museum of Natural Sciences, Madrid, Spain, personal communication), which crawled down the petals to feed on nectar at the base of the tube, and very occasionally approached the anthers to feed on pollen. The larger species (*Usia* sp. 2) contacted the stigmas and anthers of both morphs regularly, and the smaller species (*Usia* sp. 1) contacted fertile parts slightly less often, especially at the Grazalema site (Tables 2, 3), where the anther–petal (thrum morph) and stigma–petal (pin morph) gaps were larger. Nevertheless, nectar-seeking *Usia* were both common and effective, and hence the most important pollinators of *L. suffruticosum* at both sites.

Megachilid (Ronda site) and halictid (Grazalema site) bees were also quite common; they collected pollen, visiting thrum flowers more often than pin flowers (Tables 2, 3). The pollen-collecting bees only rarely contacted the stigmas on thrum flowers. On the few brief visits to pin flowers, these bees always landed on the stigmas, apparently mistaking them for anthers. Most of the observed pollen transfers by these bees were of orange *Cistus albidus* pollen to *Linum* stigmas. Thus these bees acted largely as pollen thieves rather than pollinators.

A species of sphecid wasp (Hymenoptera: Sphecidae) was another common floral visitor at the Grazalema site. Its visits were very brief, although the wasp commonly contacted thrum anthers and pin stigmas. It sought neither nectar nor pollen, however, but instead hunted *Usia* flies. It grabbed the flies with its legs and/or mandibles, stung them, and then flew off with its prey. The floral visits of the wasps probably resulted in reduced pollination, because the wasp removed effective pollinators from the population without doing much pollination itself. It is possible that these insects were

Table 3 Contact with stigmas and anthers by floral visitors at the Grazalema site, 22–23 May 2005, during 8 person-hours of observation on several dozen flowers of *Linum suffruticosum* (wild flax)

Floral visitor	Reward collected	Pin			Thrum			Movement between morphs?
		Frequency of contact with stigmas	Frequency of contact with anthers	*n*	Frequency of contact with stigmas	Frequency of contact with anthers	*n*	
Usia sp. 1 (small)	Nectar	38% (dorsal surface only)	96% (ventral surface only)	50	100% (ventral surface only)	69% (dorsal surface only)	48	+
Usia sp. 2 (large)	Nectar	61% (dorsal surface only)	100% (ventral surface only)	23	100% (ventral surface only)	100% (dorsal surface only)	25	+
cf. *Halictus*	Pollen	100% (ventral surface only)	50% (dorsal surface only)	8	11% (dorsal surface only)	100% (ventral surface only)	55	+
cf. *Lasioglossum*	Pollen	–	–	0	0%	100% (ventral surface only)	2	–
Bronze Halictidae	Pollen	–	–	0	0%	100% (ventral surface only)	5	–
cf. *Chelostema*	Pollen	100% (ventral surface only)	100% (dorsal surface only)	1	–	–	0	–
Sphecid wasp (hunting *Usia*)	–	91% (ventral surface only)	0%	11	0%	71% (ventral surface only)	7	+

n, number of floral visits by each insect species observed.

responsible for a small amount of asymmetrical pollen transfer (from thrum to pin).

Effects of floral morphology on pollen flow

Usia flies bore *L. suffruticosum* pollen on both the dorsal and ventral surfaces of their bodies. We saw clearly large amounts of thrum pollen being deposited on the top of the thorax and this pollen being transferred to pin stigmas (Fig. 1a). It was harder to tell from where the *Linum* pollen on the underside of the body came, but it is almost certain to have come from pin stamens, because this is the only part of the body of the fly that contacts the pin stamens (plus the tarsi occasionally). The pin pollen, in turn, was likely transferred to thrum stigmas, although this is inferred primarily from the part of the fly that touches the stigmas.

Phylogeny and evolution of heterostyly

Characteristics of ITS sequences The 16 ITS sequence lengths in *Linum* ranged from 607 bp in *Linum setaceum* Brot. to 631 bp in *Linum campanulatum* L., and in sect. *Linastrum* from 607 bp in *L. setaceum* to 623 in *Linum tenue* Desf. Within sect. *Linastrum*, the number of variable/potentially informative characters was 142/70. Corrected pairwise K-2-p divergences of the ITS region within sect. *Linastrum* range between 0.00% (between two accessions of *L. suffruticosum*) and 15.90% (*Linum strictum* L.–*Linum maritimum* L.), with 37.87% (*Linum viscosum* L.–*Linum catharticum* L.) being the

highest in *Linum*. The GTR + I + G model was selected as the simplest model of molecular evolution by MRMODELTEST.

Phylogenetic relationships and character reconstruction The MP analysis of ITS sequences resulted in five equally most parsimonious trees of 674 steps, a consistency index excluding uninformative characters (CI′) of 0.65 [consistency index including uninformative characters (CI) = 0.75], and a retention index (RI) of 0.64. The five trees differed in the relative positions of *Linum tenuifolium* L., *Linum carratracensis* (= *Linum suffruticosum* ssp. *carratracensis* Rivas Goday & Rivas Mart), *Linum appressum* Caball., and the two accessions of *L. suffruticosum* (results not shown). BI analyses yielded the identical topology to one of the MP trees, which was thus selected for character reconstruction (Fig. 2). MP and BI analyses recognize *Linum* sect. *Linastrum* as paraphyletic, given that *L. catharticum* (sect. *Cathartolinum*) is sister to one of the two subclades of *Linum* sect. *Linastrum*. The molecular phylogenetic analysis also showed that *L. suffruticosum* from Grazalema is closely related to five heterostylous taxa (Fig. 2), differing from the other *L. suffruticosum* accession in a nucleotide substitution at only one position (ITS-2).

MACCLADE reconstruction of character states indicated that heterostyly has originated several times (Fig. 2), not only within *Linum*, but also within sect. *Linastrum*, including one to three reversions to monomorphism (one of which is within the polymorphic species complex *L. tenuifolium*; not shown in Fig. 2). Our phylogenetic hypothesis suggests that heterostyly has evolved at least twice in the two *Linastrum* clades, although

Fig. 2 Hypothesis of character evolution for heterostyly based on one of the five most parsimonious trees obtained in the internal transcribed spacer (ITS) sequence analysis; the tree used is congruent with the Bayesian inference (see text). The character reconstruction was obtained by implementing the '*all most parsimonious states*' optimization in MacClade, which includes both ACCTRAN and DELTRAN optimizations (Maddison & Maddison, 1999). Bootstrap values above 50% are shown next to branch nodes. Population localities after plant names are Spanish locations, except as otherwise indicated. *Linum suffruticosum* accessions are indicated by collection locality. Our study populations are referred to *L. suffruticosum* Grazalema, from which they differ by 0–1 base-pair substitutions. Character-state coding was based on previous publications and our personal observations. *Linum tenuifolium* was scored as 'uncertain' because some populations are heterostylous and others monomorphic.
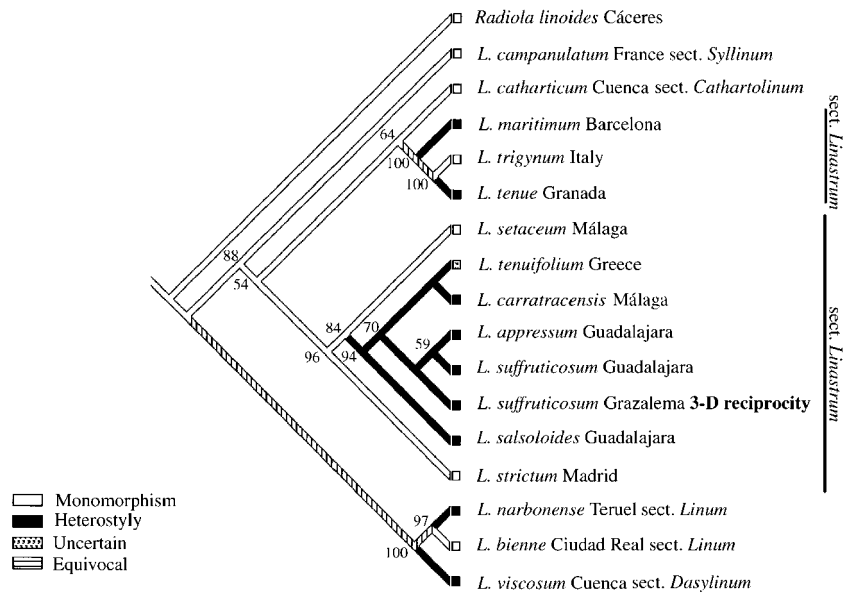
equivocal character transitions and the limited sample of taxa preclude inference of the exact number of shifts. The least restrictive optimization of heterostyly is consistent with at least three independent origins of heterostyly in *Linum*.

Discussion

Our observations of flower morphology and pollination of *L. suffruticosum* identify a new form of heterostyly, one that almost certainly greatly increases the amount of pollen flow between, rather than within, morphs (disassortative pollination). Instead of anthers and stigmas showing reciprocity in one dimension (height), they show it in three. The two floral morphs show only small differences in stigma width, pollen color, and style and stamen length. The last, although in the direction of reciprocity, would not alone lead to reciprocal positions of anthers and stigmas. Of greater significance are the intermorph differences in angles of divergence of styles and stamens from the central axis of the flower and degree of rotation of the styles and filaments. These latter differences result in dorsal (nototribic) pollen placement by short-styled (thrum) flowers and ventral (sternotribic) pollen placement by long-styled (pin) flowers. In turn, the stigmas of thrums contact the ventral side of the pollinator and the stigmas of pins contact the dorsal side.

The nearly 1 : 1 ratio of morphs that we observed is consistent with the S/L supergene incompatibility system with one morph being heterozygous and the other homozygous recessive (Lewis & Jones, 1992), further supporting the conclusion that the dimorphism we have observed is true distyly. Other genetic models cannot be ruled out, however; for example, equal morph ratios are also consistent with high disassortative pollen transfer, independently of the genetic system involved,

as in *Narcissus* (Baker *et al.*, 2000; Arroyo *et al.*, 2002) and *Anchusa* (Philipp & Schou, 1981; Schou & Philipp, 1984), where morph incompatibility is not present and isoplethy (equal morph ratios) sometimes occurs. In this context, isoplethy should be the result of very close reciprocity between sex organs and effective disassortative pollen transfer, as occurs in the study species. If so, this alternative would be more evidence for the efficacy of three-dimensional anther–stigma reciprocity in generating disassortative pollination.

Although Darwin studied *Linum* extensively (Darwin, 1864, 1877) and commented on both heterostylous and monomorphic species, he never observed any species showing three-dimensional heterostyly with dorsal/ventral reciprocity as we have described here. Ironically, had he seen this arrangement in *Linum*, his argument that selection for disassortative pollen flow has driven the evolution of heterostyly would have been greatly strengthened. Indeed, in *L. suffruticosum*, preliminary experiments with fluorescent dyes showed dye moving between morphs, with a predominance of intermorph movement (W. S. Armbruster, J. Arroyo, M. E. Edwards & R. Pérez-Barrales, unpublished data). Surprisingly, no one else appears to have documented any form of floral polymorphism involving dorso-ventral reciprocity in pollen placement and pickup.

A somewhat similar dimorphic system ('inversostyly') involving reciprocal placement of stigmas and anthers has recently been described in *Hemimeris racemosa* (Houtt.) Merrill (Scrophulariaceae, s.l.; Pauw, 2005). This differs from three-dimensional heterostyly firstly in that pollen of both morphs is placed on the ventral surface of pollinating bees (segregated into anterior and posterior patches) rather than on the dorsal and ventral surfaces, as in *Linum*. Secondly, the flowers are zygomorphic rather than actinomorphic, and only bees positioning themselves precisely will promote intermorph pollen

flow (Pauw, 2005; see also Armbruster *et al.*, 2004). This system appears not to have evolved from typical heterostyly, and there is apparently no intramorph incompatibility. It remains also to be established how well this system promotes disassortative pollen flow.

Another somewhat similar dimorphism ('flexistyly') has been reported in *Alpinia* spp. (Zingiberaceae), in which there are two morphs that differ in the position of the stigmas during the period in which pollen is arriving (Li *et al.*, 2001, 2002). Although this system appears to promote intermorph pollen flow (and outcrossing, as the plant is self-compatible), pollen of the two morphs is placed in the same location on pollinators. Intermorph pollen flow is promoted by the time of day at which the morphs are receptive and releasing pollen rather than where on pollinators pollen is placed.

The floral polymorphism reported here functionally resembles enantiostyly (mirror-image flowers), in that reciprocal morphs place pollen, and stigmas make contact, on opposite sides of the pollinator (Fenster, 1995; Barrett *et al.*, 2000; Jesson & Barrett, 2005). The *Linum* system differs from enantiostyly in that it is true heterostyly associated with differences in style and stamen length, pollen morphology, and intramorph incompatibility (Rogers, 1979). Three-dimensional heterostyly is also almost certainly derived from conventional, linear heterostyly with anther/stigma-height dimorphism, as is shown in the reconstruction of character evolution in Fig. 2. Another difference from enantiostyly is that the two morphs of *L. suffruticosum* place pollen and position stigma contact in reciprocal positions on the upper and lower surfaces of the pollinator, not on the left and right sides, as in enantiostyly.

Population differentiation?

It is interesting that the gaps between the stigmas and petals of pin morphs and between the anthers and petals of thrum morphs seem to vary between populations. One is tempted to explain this as local ecotypic differentiation, with the Ronda population being adapted to more efficient use of the smaller *Usia* sp. 1, which was by far the commonest visitor at that site. In contrast, the Grazalema population was visited abundantly by the larger *Usia* sp. 2, and may therefore have evolved slightly larger gaps. This interpretation requires more research, however, including determination of whether: (1) the difference in pollinating faunas persists throughout the flowering season and across years, (2) the morphological differences in flowers have a genetic basis, and (3) whether reciprocal transplants suffer from reduced pollination.

Macroevolutionary inferences

The molecular phylogenetic analysis indicated that *L. suffruticosum* is closely related to species with intramorph incompatibility and conventional one-dimensional heterostyly (where stigmas and anthers vary reciprocally in height above the base of the

flower, and pollen flow between morphs is not particularly efficient; e.g. *L. tenuifolium*; Nicholls, 1985a,b). Trait-change reconstruction indicates that three-dimensional reciprocity evolved after heterostyly and self and intramorph incompatibility, probably as a result of selection for increased efficiency of compatible pollination rather than increased outcrossing. This shows that selection may favor mechanisms promoting disassortative pollination even in the absence of an outcrossing advantage, hence providing very strong support for both Darwin's (1877) original hypothesis and for the Lloyd & Webb model of the evolution of heterostyly (Lloyd & Webb, 1992a,b).

The flowers of many *Linum* species, unlike in most groups of heterostylous plants, are broadly open rather than narrowly tubular. This may make variation only in height between morphs particularly inefficient in generating disassortative pollen flow, hence probably increasing the selective pressure for morphological improvements in this group. The origin of three-dimensional reciprocity in one or more distylous lineages may have been a key innovation, allowing the evolution of a greater variety of floral morphologies in *Linum* (e.g. open, campanulate, or chambered as in *L. suffruticosum*) in addition to the narrowly tubular morphology seen in many heterostylous species. Phylogenetic and morphological studies of many more *Linum* species (already in progress) are required to assess how many exhibit three-dimensional reciprocity and how many times it and other forms of heterostyly have originated.

In addition to these across-species patterns, there is also interesting variation in breeding system within species. Nicholls (1985a,b) reported both heterostyly with self-incompatibility and monomorphism with self-compatibility in *L. tenuifolium* from Italy, the latter apparently being derived from the former. The loss of heterostyly and self-incompatibility has also been reported in other groups, at both species (Kohn *et al.*, 1996; Schoen *et al.*, 1997; Pérez *et al.*, 2004; Pérez-Barrales, 2005) and population (Barrett *et al.*, 1989) levels. The observation that both monomorphic and heterostylous populations occur in *L. tenuifolium* further supports our conclusion that heterostyly is evolutionarily labile in *Linum*.

Final comments

The observations reported here strongly suggest that three-dimensional reciprocity may greatly enhance the efficiency of compatible (disassortative) pollination in heterostylous species. In retrospect, this seems an ideal way for heterostyly to work, as it is possible to achieve nearly perfect disassortative (intermorph) pollination. However, more data are clearly needed to supplement our preliminary experiments measuring efficiency of pollen and dye flow between morphs.

One fundamental question remains unanswered: if this system of heterostyly works so well, why has it not evolved in many distylous lineages and thus been reported previously? It is perhaps instructive to note that previous detailed studies of *L. suffruticosum* (Rogers, 1979) missed this feature, which

became obvious only after careful field observations of multiple populations. Further, interpretation of where flowers place pollen on pollinators can often be in error when pollinator behaviour is not observed (Keller & Armbruster, 1989). We thus predict that, with closer examination, we will find additional examples of heterostylous morphs that place pollen on the dorsal and ventral surfaces of the bodies of insects, and that this feature will be found in other heterostylous species that have open or campanulate, rather than narrowly tubular, flowers.

Acknowledgements

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