

Experimental tests of the function of mirror-image flowers

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Enantiostyly, the reciprocal deflection of the style to the left or right side of the floral axis has evolved independently in at least a dozen angiosperm families. Unlike other plant sexual polymorphisms, the adaptive significance of these mirror-image flowers remains unclear. Most authors have interpreted enantiostyly as a floral mechanism that promotes cross-pollination. However, any functional interpretation is complicated by the fact that enantiostyly occurs in two forms. In monomorphic enantiostyly there are left- and right-styled flowers on the same plant, while in dimorphic enantiostyly they are on separate individuals. In this paper we develop a model of pollen transfer which indicates that monomorphic enantiostyly should reduce geitonogamous pollination compared to a non-enantiostylous condition, and that the lowest levels of geitonogamous pollination should occur in dimorphic enantiostyly. We tested these predictions using floral manipulations of bee-pollinated *Solanum rostratum* in garden arrays. We compared mating patterns and fertility in five array types: non-enantiostylous and straight-styled, monomorphic enantiostylous, dimorphic enantiostylous, and arrays uniform for either left or right stylar deflection. Outcrossing rates in non-enantiostylous arrays ($t = 0.33 \pm 0.04$) were significantly lower than all other arrays, while monomorphic enantiostylous arrays ($t = 0.74 \pm 0.06$) had significantly lower outcrossing rates than dimorphic enantiostylous arrays ($t = 0.88 \pm 0.04$) and those uniform for stylar deflection ($t = 0.84 \pm 0.04$). In dimorphic enantiostylous arrays, intermorph pollen transfer accounted for 75% of all outcrossing events. In pollen-limited situations, both types of enantiostylous arrays had significantly higher female fertility than arrays fixed for one direction, demonstrating that enantiostyly promotes pollen transfer between flowers of opposite stylar orientation. Our results provide support for the hypothesis that enantiostyly functions to increase the precision of cross-pollination in bee-pollinated plants by reducing geitonogamy. © 2005 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2005, 85, 167–179.

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INTRODUCTION

Two important features of the biology of flowers influence their function. First, most plants produce hermaphroditic flowers and therefore reproduce as both female and male parents (Horovitz & Harding, 1972; Bell, 1985). Second, offspring arising from cross-fertilization are generally fitter than those from self-fertilization (Darwin, 1876; Charlesworth & Charlesworth, 1987). The fitness consequences of cross- and self-fertilization have led to the interpreta-

tion that most floral mechanisms function to promote cross-pollination (Richards, 1996). However, cross-pollination can be achieved in diverse ways. For example, the reciprocal positioning of sex organs in heterostylous plants actively promotes pollen transfer between plants and hence outcrossing (Darwin, 1877; Lloyd & Webb, 1992). In contrast, cross-pollination can also be promoted passively by discouraging self-fertilization and reducing ‘sexual interference’ between stigmas and anthers, either within a flower or between flowers on the same individual (van der Pijl, 1978; Lloyd & Yates, 1982; Lloyd & Webb, 1986; Webb & Lloyd, 1986; Bertin & Newman, 1993; Fetscher, 2001; Barrett, 2002a). Mechanisms that limit self-pollination by reducing conflict between

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female and male reproductive function may provide greater opportunities for outcrossing, without altering the basic process of cross-pollination (Lloyd, 1992; Barrett, 2002b).

Enantiostyly, the reciprocal deflection of the style either to the left (left-styled) or right (right-styled) side of a flower has been documented since the 19th century (Todd, 1882; Wilson, 1887; Robertson, 1890). Indeed, 9 days before his death, in what appears to have been his last scientific correspondence, Charles Darwin wrote to Todd requesting seeds of enantiostylous *Solanum rostratum* so that 'he may have the pleasure of experimenting with them' (Darwin, 1882, reprinted 1946). Only recently has it been explicitly noted that there are two fundamentally different types of enantiostyly (Barrett, Baker & Jesson, 2000a; Jesson & Barrett, 2003). Plants can be either fixed for stylar direction with populations containing both left and right-styled plants (dimorphic enantiostyly) or they can produce both left- and right-styled flowers on the same individual (monomorphic enantiostyly). The occurrence of these mirror-image flowers in at least a dozen unrelated families indicates that enantiostyly has originated independently on numerous occasions in flowering plants (Jesson & Barrett, 2003). However, despite the widespread distribution of enantiostyly, and sporadic interest by botanists for over a century, the functional significance of mirror-image flowers has remained unclear.

The most common interpretation of the adaptive significance of enantiostyly is that it functions to promote cross-pollination in animal-pollinated species (reviewed in Barrett *et al.*, 2000a). The observation that pollinating bees visiting enantiostylous flowers contact the stigma with the sides of their bodies, making any pollen collected unavailable for receipt by flowers of the same stylar deflection, suggests that enantiostyly promotes cross-pollination in a manner functionally analogous to heterostyly (Todd, 1882; Wilson, 1887; Knuth, 1906; Webb & Lloyd, 1986). Bowers (1975) used fluorescent dyes to examine pollen transfer in the annual herb *Solanum rostratum* (Solanaceae), a species with monomorphic enantiostyly. She found that the reciprocally deflected pollinating anther, a common feature of enantiostylous flowers, consistently deposited pollen on either the left or right side of bees' bodies below the wing. This suggests that pollen is segregated into two pools on the bodies of pollinators, promoting pollen transfer between flowers of opposite stylar orientation. However, for monomorphic enantiostylous species, the presence of both left- and right-styled flowers on the same individual may lead to geitonogamous pollination (Bowers, 1975; Dulberger, 1981; Fenster, 1995; Graham & Barrett, 1995). This could have deleterious consequences due to both inbreeding depression and pollen discounting (Char-

lesworth & Charlesworth, 1987; Harder & Barrett, 1996). The function of monomorphic enantiostyly has therefore been considered enigmatic.

To understand the function of enantiostyly, and make inferences about the role of pollination in the evolution of monomorphic enantiostyly and dimorphic enantiostyly, we developed a pollen-transfer model to investigate the relative importance of geitonogamy in enantiostylous and non-enantiostylous populations. We then tested the main assumptions of this model by investigating mating patterns and fertility in experimental garden arrays of *S. rostratum* in which stylar conditions were manipulated to simulate various enantiostylous and non-enantiostylous conditions.

Our study addressed three specific questions: (1) Does enantiostyly promote pollen transfer between flowers of opposite stylar deflection? (2) What are the levels of outcrossing in non-enantiostylous, monomorphically and dimorphically enantiostylous populations? (3) How do the different stylar conditions influence the geitonogamous and intrafloral components of selfing? Here we provide a detailed exposition of our experimental results thus extending our previous preliminary findings (Jesson & Barrett, 2002a), including new data on mating patterns and female fertility.

MODEL OF GEITONOGAMOUS POLLEN TRANSFER

We have argued elsewhere that any consideration of the significance of geitonogamy for the evolution of enantiostyly should be made relative to the ancestral condition (Barrett, Jesson & Baker, 2000b; Jesson, Barrett & Day, 2003a). Based on phylogenetic evidence, the ancestral condition for enantiostyly seems likely to have involved a straight-styled morphology lacking the reciprocal arrangement of sex organs (Graham & Barrett, 1995; Bohs & Olmstead, 1997; Olmstead & Palmer, 1997; Harrison, Möller & Cronk, 1999; Jesson & Barrett, 2003). Moreover, since dimorphic enantiostyly occurs in only seven species in three families each of which contain numerous species with monomorphic enantiostyly (Simpson, 1990; Graham & Barrett, 1995; Jesson & Barrett, 2003), it is also probable that the former is derived from the latter.

We assume that a plant produces a large number of pollen grains, many of which are wasted due to the vagaries of the pollination process (see Harder & Wilson, 1998a). The number of grains eventually deposited on stigmas of other flowers is the product of the number removed from each flower (R) and the proportion deposited on the pollinator's body and available for pollen transfer (π), i.e. πR .

Let us first consider geitonogamy in a straight-styled hermaphroditic plant. Based on earlier models of pollen transfer (Bateman, 1947; Plowright & Hartling,

1981; de Jong, Waser & Klinkhamer, 1993; Klinkhamer & de Jong, 1993; Barrett, Harder & Cole, 1994; Morris *et al.*, 1994; Iwasa, de Jong & Klinkhamer, 1995; Rade-maker, de Jong & Klinkhamer, 1997; Harder & Wilson, 1998a, b), we assume that when a pollinator visits a straight-styled flower, pollen is picked up from the anthers and a proportion (ρ) of pollen on the pollinator's body is deposited on the stigma. We assume that ρ is constant for all pollen grains on the pollinator's body. Also, since geitonogamy will be the same regardless of the visitation sequence of the pollinator, we label flowers 1 to v where 1 is the first flower visited and v is the last. Therefore, the amount of self pollen deposited on flower j from flower i (where $i < j$) is

$$d_{ij} = \pi R \rho (1 - \rho)^{j-i-1} \quad (1)$$

The total geitonogamy experienced by flower j (denoted G_j) is therefore:

$$G_j = \sum_{i=1}^{j-1} d_{ij} \quad (2)$$

The total numbers of geitonogamous pollen grains deposited on a plant (G_T^{NE}) is the sum of geitonogamy experienced by all flowers visited on the plant:

$$G_T^{NE} = \frac{\pi R (\rho v + (1 - \rho)^v - 1)}{\rho} \quad (3)$$

We now examine geitonogamy in a monomorphic enantiostylous plant. We assume that geitonogamous pollen transfer occurs only between left- and right-styled flowers (and vice versa) visited on the same inflorescence, but not between flowers of the same stylar orientation. Let $d_{ij}(s)$ be the amount of pollen transferred from flower i to flower j , given that a bee visits flowers in a particular sequence (s). Unlike the case of straight-styled plants, i and j represent simply some (arbitrary) labelling scheme that we assume is fixed. This means that the number of flowers of each type visited between i and j depends on the visitation sequence (s).

Geitonogamous pollen deposition on flower i (given that flower i is left-styled) is the sum of deposition from all right-styled flowers visited; the converse is true for right-styled flower j :

$$G_i^L(s) = \sum_{j=1}^{nr} d_{ji}(s) \quad (4)$$

$$G_j^R(s) = \sum_{i=1}^{nl} d_{ij}(s)$$

where nl is the number of left-styled flowers, and nr is the number of right-styled flowers. Note that some values of d_{ij} might be zero for a particular sequence, s , if flower j is visited after flower i with that sequence.

Total geitonogamous pollen deposited on a left-styled flower, given a certain sequence [$G_T^L(s)$], is the

sum of pollen deposited on all left-styled flowers visited in the sequence. Total geitonogamy on right-styled flowers can be similarly calculated:

$$G_T^L(s) = \sum_{i=1}^{nl} G_i^L(s) \quad (5)$$

$$G_T^R(s) = \sum_{j=1}^{nr} G_j^R(s)$$

If all possible visitation sequences to a monomorphic enantiostylous plant are labelled as $s = 1$ to $s = v!$, then the total geitonogamy averaged over all possible sequences (\bar{G}_T) is:

$$\begin{aligned} \bar{G}_T &= \sum_{s=1}^{v!} \frac{1}{v!} \{G_T^R(s) + G_T^L(s)\} \\ &= \sum_{j=1}^{nr} \sum_{i=1}^{nl} \frac{1}{v!} \sum_{s=1}^{v!} d_{ij}(s) + \sum_{i=1}^{nl} \sum_{j=1}^{nr} \frac{1}{v!} \sum_{s=1}^{v!} d_{ji}(s) \end{aligned} \quad (6)$$

Let $\frac{1}{v!} \sum_{s=1}^{v!} d_{ij}(s) = E[d_{ij}(s)]$ and $\frac{1}{v!} \sum_{s=1}^{v!} d_{ji}(s) = E[d_{ji}(s)]$.

The amount of pollen transferred from flower i to flower j given sequence s is determined by the number of flowers visited between flower i and j , given sequence s [i.e. $z(s)$]. In particular,

$$d_{ij}(s) = \pi R \rho (1 - \rho)^{z(s)} \quad (7)$$

The number of possible sequences visited where there are z flowers between flowers j and i is $(v - z)!(v - z - 1)$, where v is the total number of flowers visited on a plant. Thus the expected amount of pollen transferred from flower i to j is:

$$E[d_{ij}(s)] = \sum_{z=0}^{v-2} \frac{(v-2)!(v-z-1)}{v!} \pi R \rho (1 - \rho)^z \quad (8)$$

$$E[d_{ji}(s)] = \frac{\pi R}{v(v-1)} \frac{\rho v - 1 + (1 - \rho)^v}{\rho}$$

Substituting this expression in Eqn. 6 gives average total geitonogamy for an monomorphic enantiostylous plant:

$$\begin{aligned} \bar{G}_T &= 2n_r n_l \frac{\pi R}{v(v-1)} \frac{\rho v - 1 + (1 - \rho)^v}{\rho} \\ &= 2l(1-l)v \frac{\pi R}{(v-1)} \frac{\rho v - 1 + (1 - \rho)^v}{\rho} \end{aligned} \quad (9)$$

where l is the proportion of left-styled flowers on a plant.

Using Eqn. 3, the proportional reduction in geitonogamy in a monomorphic enantiostylous plant compared to a straight-styled plant is therefore:

$$\frac{\bar{G}_T}{G_T^{NE}} = 2 \frac{v}{v-1} l(1-l) \quad (10)$$

The model predicts that geitonogamous pollination will be influenced by the number of pollinator visits,

Table 1. Predicted patterns of mating and fertility in arrays of *Solanum rostratum* with contrasting styler conditions. *Abbreviations:* N, non-enantiostyly (straight-styled); ME, monomorphic enantiostyly; DE, dimorphic enantiostyly. L and R, arrays in which all plants are either left- or right-styled, respectively. Predictions for autogamy and geitonogamy will hold for plants of different phenotypes, as well as for entire arrays

Treatment	Precision of pollen transfer	Total seed set	Intrafloral selfing	Geitonogamy	Outcrossing rate
N	high	high	high	high	low
L or R	low	low	low	low	high
M	intermediate	intermediate	low	intermediate	intermediate
D	intermediate	intermediate	low	low	high

and the proportion of left- and right-styled flowers on a plant. In addition, geitonogamy in a monomorphic enantiostylous plant should be less than that of a straight-styled ancestor. This is because every visit between flowers on a straight-styled plant could potentially result in geitonogamous pollen transfer, whereas geitonogamy in a monomorphic enantiostylous plant depends on the number of oppositely deflected styles visited previously. According to this interpretation, monomorphic enantiostyly functions to reduce geitonogamy, not to promote it as had been previously considered by some authors (see above).

This model is based on several assumptions that can be examined using experimental approaches to measure components of pollination and mating (Table 1). Within-plant pollen transfer should be greater among flowers of straight-styled plants and lower in enantiostylous plants, because in the latter case transfers within floral types should be much less frequent compared to those between flower types. Hence, total seed set is expected to be highest in non-enantiostylous straight-styled plants compared to enantiostylous plants. As dimorphic enantiostylous plants only have one flower type, geitonogamous transfer in these plants is predicted to be the lowest. Levels of geitonogamy in monomorphic enantiostyly should be roughly intermediate between dimorphic enantiostyly and non-enantiostyly, depending on the ratio of left- and right-styled flowers on a plant. It is also possible that enantiostyly will influence levels of intrafloral self-pollination. By increasing stigma-anther separation within a flower (herkogamy; Webb & Lloyd, 1986), levels of self-pollination are likely to be reduced in enantiostylous plants, compared to those with straight-styles, although this will depend on the degree of herkogamy in straight-styled plants.

METHODS

STUDY SPECIES

Solanum rostratum Dunal is a self-compatible, summer annual found in open, disturbed habitats from

Mexico to the Great Plains of the USA, and occasionally as a ruderal weed in Ontario, Canada where our experiments were conducted (Bowers, 1975; S. C. H. Barrett, unpubl. observ.). Flowers are borne on branched racemes and usually bear 1–25 1- or 2-day flowers depending on temperature (Bowers, 1975; L. K. Jesson, unpubl. data). Flowers are weakly zygomorphic, yellow, showy, nectarless, and heterantherous, with a large, yellow-brown anther (pollinating anther) and four shorter bright yellow anthers (feeding anthers). Pollen is dispersed from two apical pores in each anther. Plants of *S. rostratum* produce similar numbers of left- and right-styled flowers each day and the direction of deflection is predictable, with left- and right-styled flowers alternating at each node (Jesson *et al.*, 2003b). Field observations of pollinators (Bowers, 1975; L. K. Jesson, pers. observ.) indicate that the most common visitors to *S. rostratum* are species of *Bombus*, although other bee species (e.g. *Xylocopa*, *Anthophora*, *Psaenythia*, *Dialictus* and *Melissodes*) also visit flowers. *Solanum rostratum* is 'buzz-pollinated' with bees grasping the base of the feeding anthers with their mandibles and 'milking' the anthers, while emitting a buzzing sound (see Buchman, 1983).

Plants of *S. rostratum* used in the array experiments were grown from seed from 12 families collected from a natural population on a roadside near Cedar Vale in southern Kansas. To choose plants for experimental arrays designed to estimate outcrossing rates, we screened leaves and selected individuals homozygous at the polymorphic allozyme locus *Pgm-1*. A second locus *Pgi-1* had four alleles, and we chose individuals for the experimental populations that were either homozygous or occasionally heterozygous at this locus.

EXPERIMENTAL ARRAYS

Through the manipulation of flowers, we established five types of arrays in an experimental garden at Toronto in 1999: entirely left-styled (L), entirely right-

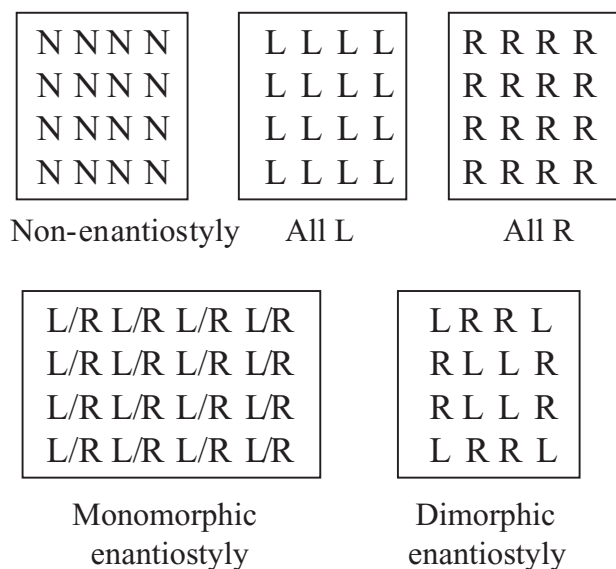


Figure 1. The stylar conditions used in experimental arrays of *Solanum rostratum*. This species naturally exhibits monomorphic enantiostyly. For non-enantiostyly, the stigma was tied to the pollinating anther resulting in a straight-styled condition. In the L, R and dimorphic arrays, flowers were removed from plants to create phenotypes uniform for stylar direction. All treatments involved plants with the same number of flowers.

styled (R), dimorphic enantiostyly (with 1 : 1 ratios of left- and right-styled plants), monomorphic enantiostyly (unmanipulated plants with both left- and right-styled flowers) and straight-styled (Fig. 1). We created the non-enantiostylous straight-styled treatment by tying the base of the stigma to the base of the pollinating anther with clear nylon thread. We tied all stigmas in all treatments to control for the effects of thread on pollinator behaviour or plant fertility.

For L, R and dimorphic enantiostylous treatments, we removed flowers of one type to produce plants that were entirely left- or entirely right-styled. We randomly arranged equal numbers of left- and right-styled plants in each dimorphic enantiostylous array. Experimental arrays contained 16 plants with flowers trimmed from each plant so that daily display size was held constant at six flowers per plant. If sufficient plants with six flowers were not available, we placed two plants of the same genotype together in an array to create a single floral display. In total 110 plants were involved in the experiment. Most plants were used in more than one experimental array (usually two or three arrays). Only two plants were used in five or more arrays.

We conducted array experiments in two locations at the University of Toronto in September 1999, on fine

sunny days. The first location was an experimental garden (hereafter Garden) and the second a rooftop garden (hereafter Roof) approximately 1 km apart. We replicated each treatment on separate days and conducted three replicates of L and R arrays and four replicates each of dimorphic enantiostylous, monomorphic enantiostylous, and non-enantiostylous arrays.

We placed plants in a square grid (approximately 30 cm between adjacent plants), recorded pollinator visitation and at the end of the day, all flowers were marked with a treatment- and day-specific colour combination of acrylic paint. When seeds reached maturity we collected the fruits and recorded fruit and seed set per capsule. We counted all seeds in all fruits produced by a plant. Where possible, we sowed 30 seeds per fruit into pots of sterilized soil and sand mix. We assayed ten seeds per fruit and leaves on young seedlings for *Pgi-1* (seeds and leaves) and *Pgm-1* (leaves only) to quantify outcrossing rates based on seeds vs. seedlings. We used Ritland's (1990) maximum likelihood outcrossing rate program (MLTR) to estimate the female outcrossing rate (\hat{t}) and its standard error (based on the standard deviation of 1000 bootstraps).

To determine levels of geitonogamy, we blocked the pores of the pollinating anther of one randomly selected flower per plant, by applying water-soluble glue to the tip of the anther. Preliminary experiments indicated that no pollen was subsequently released from the anthers (L. K. Jesson, pers. observ.). The feeding anthers were not glued, as this would influence visitation by pollinators. Self pollen grains on the stigma of the glued focal flower must result from either geitonogamy, or transfer from the feeding anthers. Self pollen grains on stigmas of unglued flowers result from both geitonogamy, and intrafloral transfer from the feeding or pollinating anther. When fruit from glued and unglued flowers reached maturity, we counted the number of seeds in the fruit, and all seeds were sown to ensure sufficient numbers to estimate outcrossing rates. Ten seedlings per fruit were assayed electrophoretically as above. We used the difference in the selfing rate between the two flowers as a measure of geitonogamy (see 'Estimation of components of selfing' below).

To determine levels of pollinator activity and to investigate if visitation rates varied between experimental treatments, we made 15-minute observations of pollinators hourly for 3 hours each day. Observations began 30 min after first sighting of a pollinator in an array. We recorded the time that a pollinator entered an array and the number of flowers visited on each plant. To determine whether there was pollinator discrimination between left- and right-styled flowers, we recorded the sequence of visits in arrays that had

both flower types. On 3 days in monomorphic enantiostyly arrays we also noted the position on the pollinator's body that contacted stigmas.

STATISTICAL ANALYSES

To determine whether pollinators discriminated between array type and location, we employed nested mixed-effects ANOVA using *S-plus 4* (Mathsoft, 1997) to examine differences in the two responses: total time a pollinator spent in the array, and the total number of flowers visited in an array. We log-transformed total time spent by a pollinator and square-root transformed total number of flowers visited to account for heteroscedasticity of variances. Because we were specifically interested in whether the two locations had different levels of pollinator visitation, we treated both location and array type as fixed effects, whereas date was considered to be a random effect. We examined differences in the number of flowers visited per inflorescence, as well as differences in pollinator visitation to left- and right-styled flowers using linear mixed effect models (Venables & Ripley, 1994; Mathsoft, 1997) to account for covariance between repeated measures of the same pollinator. We used log-likelihood ratio tests to assess significant effects (Venables & Ripley, 1994). Both responses were square-root transformed for this analysis. Reported means and standard errors are back-transformed data; standard errors are reported as upper standard error (USE) and lower standard error (LSE).

We employed linear mixed effects models to assess differences in female fertility (total seed production per plant) between the different array treatments, due to repeated measures on individual plants (i.e. plants were involved in more than one array), and log-transformed the data. As for the above analyses, we treated location and array type as fixed effects. We examined differences in \hat{t} between the four experimental treatments using ANOVA, with the effect of the outcrossing rate of each array weighted by the inverse of its squared standard error to account for variation in the estimates (Sokal & Rohlf, 1995). To test for differences between means, we separated treatment effects into a priori orthogonal contrasts.

ESTIMATION OF COMPONENTS OF SELFING

We estimated the contributions of autogamy and geitonogamy using a technique developed by Schoen & Lloyd (1992) as described in Eckert (2000). We estimated the fraction of seeds that were selfed (s) for manipulated (s_m) and unmanipulated (s_u) flowers using MLTR (Ritland, 1990). We calculated the fraction of seeds produced by geitonogamy from the pollinating anther as:

$$g_u = \frac{s_m(1-s_u)}{1-s_m}$$

and the contribution from autogamy as:

$$a_u = s_u - g_u$$

This method takes into account the differences in absolute siring success by outcross and geitonogamous pollen in manipulated and unmanipulated flowers, due to competition from autogamous pollen (see Eckert, 2000 for derivation of these formulas). We derived standard errors for the estimates by performing the same calculation for each of the 1000 sets of bootstrap values for the estimates of s and calculating the standard deviations of the resulting distribution. Statistical departures from zero were indicated if < 5% of a distribution fell below zero.

ESTIMATION OF INTRAMORPH VS. INTERMORPH POLLEN TRANSFER

To examine whether pollen transfer between flowers of opposite styler orientation is greater than pollen transfer within flower forms, we estimated the percentage of seeds resulting from either intermorph or intramorph matings. In dimorphic enantiostylous populations, plants of each morph were homozygous at one of the two alleles at *Pgm-1*. We considered any seedlings heterozygous at *Pgm-1* the result of intermorph mating. Seedlings that were homozygous at this locus could have resulted from either intramorph or self matings. We therefore estimated the selfing rates of these homozygous seedlings using MLTR as described above. We calculated the proportion of intermorph, intramorph and selfed seedlings for both left- and right-styled maternal plants.

RESULTS

POLLINATOR VISITATION

All observed visits to flowers of *S. rostratum* in arrays were by bumblebees with greater than 90% of the visits by *Bombus impatiens*. There was no significant effect of array type on the mean number of flowers visited per plant (log-likelihood ratio = 4.11, $P = 0.39$). Bumblebees did not discriminate between left- and right-styled flowers. In dimorphic enantiostylous arrays there was no difference between left- and right-styled individuals in the number of flowers visited (log-likelihood ratio = 2.46, $P = 0.12$).

There was a significant difference in the total number of flowers visited between locations ($F_{1,18} = 7.67$, $P < 0.05$), with an average of 224 flowers (USE = 271, LSE = 193) visited per array at the Garden location, compared to 66 flowers (USE = 142, LSE = 28) at the Roof location. The mean number of flowers visited on a plant also differed between the two locations (log-

likelihood ratio = 11.81, $P = 0.008$). On average, 3.08 flowers per plant (USE = 3.13, LSE = 3.03) were visited at the Garden, whereas 2.49 flowers per plant (USE = 2.56, LSE = 2.43) were visited at the Roof. The behaviour of pollinators also differed between locations. Bees at the Garden visited an average of 3.94 plants (USE = 4.20, LSE = 3.68) in an array, whereas bees at the Roof visited on average 8.44 plants (USE = 10.09, LSE = 6.94).

The majority of bee visits ($N = 187$) to flowers of *S. rostratum* resulted in contact of the stigma on either the left or right side of the pollinator. Most visits resulted in contact of the stigma with the pollen basket (36% of recorded visits) and either the left or right side of the abdomen (13.9%) or the back of the abdomen (17.1% of visits). Only 7% of visits resulted in either no contact, or contact to other parts of the pollinator (usually the front of the abdomen, or the last tergite segment). These patterns of contact are likely to result in segregated pollen deposition on the stigma.

FEMALE FERTILITY

The type and location of an array significantly influenced the total number of seeds produced per plant (Fig. 2). Female fertility was consistently higher at the Garden than at the Roof, with the exception of the non-enantiostylous treatment in which female fertility was equivalent among array types. This resulted in a significant location by treatment interaction (log-likelihood ratio = 10.96, $P = 0.023$). Splitting

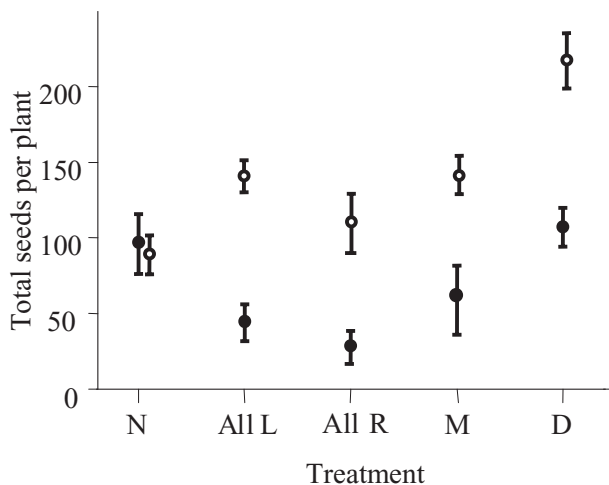


Figure 2. The effect of experimental treatment on the total number of seeds per plant. Garden (○); Rooftop (●). There is a significant interaction of location with treatment. N is non-enantiostylous (straight-styled), L is entirely left-styled, R is entirely right-styled, M is monomorphic enantiostylous, and D is dimorphic enantiostylous.

treatment effects into a priori contrasts revealed that, as predicted, the total seed set of plants in enantiostylous arrays (monomorphic enantiostylous and dimorphic enantiostylous) was significantly higher than arrays of either all left-styled plants (L) or all right-styled plants (R) at the Roof. However, this was not the case at the Garden (Table 2). Also, as predicted, the female fertility of dimorphic enantiostylous arrays was not significantly different from monomorphic enantiostylous arrays in either location (Table 2). Female fertility in the non-enantiostylous array was not significantly different than all other arrays ($P = 0.07$).

OUTCROSSING RATES

We predicted that outcrossing rates would be highest in the L, R and dimorphic enantiostylous arrays, lower in monomorphic enantiostylous arrays and lowest in the straight-styled non-enantiostylous arrays (Table 1). This expectation was partially supported. We found that outcrossing rates were highest in dimorphic enantiostylous arrays, intermediate in L, R and monomorphic enantiostylous arrays, and lowest in non-enantiostylous arrays (Fig. 3). However, this result varied between seed and seedling stages, resulting in a significant treatment \times seed/seedling interaction (Table 3). Outcrossing rates were also influenced by location. In general, arrays at the Roof experienced lower outcrossing than those at the Garden. This was particularly evident in the straight-styled treatment where there was a 30% reduction in the outcrossing

Table 2. Orthogonal contrasts of total seed set per plant in experimental arrays of *Solanum rostratum*. Linear mixed effects models revealed a significant location \times treatment interaction, so treatment contrasts are presented separately for each location. * $P < 0.05$; † $P < 0.1$. M, monomorphic enantiostylous; D, dimorphic enantiostylous; N, non-enantiostylous (straight-styled); L and R, arrays in which all plants are either left- or right-styled, respectively

Effect	Contrast	Value	Approx. SE	z ratio
Garden	Location	-1.63	0.22	-7.28*
	L vs. R	0.23	0.43	0.54
	M vs. D	-1.20	0.49	-2.44
	L and R vs. M and D	-0.88	0.32	-2.72
	N vs. rest	0.48	0.12	4.04†
Roof	L vs. R	0.01	0.61	0.02
	M vs. D	-0.63	0.55	-1.15
	L and R vs. M and D	-2.76	0.38	-5.62*
	N vs. rest	-0.34	0.21	-1.65

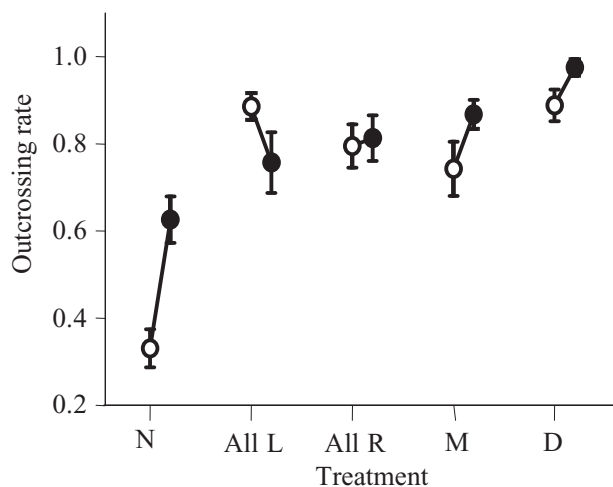


Figure 3. Outcrossing rates of *Solanum rostratum* in the experimental treatments based on analysis of seeds (○) and seedlings (●). Standard errors are based on the standard deviation of 1000 bootstrap estimates. N is non-enantiostylous (straight-styled), L is entirely left-styled, R is entirely right-styled, M is monomorphic enantiostylous, and D is dimorphic enantiostylous.

Table 3. Analysis of variance of outcrossing rates in experimental populations of *Solanum rostratum* with contrasting styler conditions. Outcrossing rates were weighted by the squared inverse of the standard error of the estimate. * $P < 0.05$; ** $P < 0.001$

Effect	d.f.	Sum of Sq	Mean Sq	F
Location	1	82.04	82.04	47.73**
Seeds vs. Seedlings	1	318.97	318.97	185.61**
Treatment	4	514.53	128.63	74.85**
Seeds/seedlings × Treatment	4	7.88	13.54	7.88**
Seeds/seedlings × Location	1	10.10	10.10	5.87**
Location × Treatment	1	43.42	10.85	6.31*
Residuals	20	34.37	1.76	

rate at the Roof. Orthogonal contrasts indicated that outcrossing rates in dimorphic enantiostylous arrays were not significantly different than in the L and R arrays, at both the Garden and Roof, supporting the predictions from Table 1 (Table 4). As predicted, monomorphic enantiostylous arrays had significantly lower outcrossing rates than dimorphic enantiostylous, L and R arrays in the Garden, but not at the Roof. Outcrossing rates in non-enantiostylous arrays were significantly lower than all other arrays at both the seed

Table 4. Orthogonal contrasts of outcrossing rates in experimental arrays of *Solanum rostratum*. Treatment contrasts are presented separately for each location. * $P < 0.05$; ** $P < 0.001$. M, monomorphic enantiostylous; D, dimorphic enantiostylous; N, non-enantiostylous (straight-styled); L and R, arrays in which all plants are either left- or right-styled, respectively

Effect	Contrast	d.f.	F
Garden	L vs. R	1	0.036
	L and R vs. D	1	0.35
	L, R and D vs. M	1	5.65*
	N vs. rest	1	12.74**
Roof	L vs. R	1	0.14
	L and R vs. D	1	0.07
	L, R and D vs. M	1	0.14
	N vs. rest	1	2.19

and seedling stages at the Garden, but not the Roof (Fig. 3, Table 4).

Outcrossing rates were generally lower for seeds (mean $t = 0.711$, SE = 0.085) than seedlings (mean $t = 0.813$, SE = 0.09). This difference was most evident in the non-enantiostylous treatment, with seed outcrossing rates 89% higher than for seedlings (Fig. 3). These differences in outcrossing rate are likely the result of inbreeding depression during germination and seedling establishment.

COMPONENTS OF SELFING

The contribution of geitonogamy to the total selfing rate varied with array type. Dimorphic enantiostylous arrays experienced the lowest levels of geitonogamy (mean = 2.2%, range = 0.7–3.7% of total seeds produced). In contrast, all other array types had significant contributions from geitonogamous selfing. In monomorphic enantiostylous arrays the percentage of seeds resulting from geitonogamy averaged 16.5% (range = 9.3–29.4). Contrary to our predictions, geitonogamy represented a significant contribution to selfing in the L and R arrays, averaging 16.5 and 23.3%, respectively. The geitonogamous contribution to all seeds produced in the straight-styled non-enantiostylous arrays averaged 21.4% (range = 1.6–41.3%).

In all arrays the contribution of autogamous selfing was not significantly different from zero. However, the errors around these estimates were considerably larger than for estimates of geitonogamy. The mean contribution of autogamy in non-enantiostylous arrays was 12.0% (SE = 15.0). In contrast, the mean contribution of autogamy in dimorphic enantiostylous arrays averaged only 0.7% (SE = 3.3).

ESTIMATION OF POLLEN TRANSFER FROM MATING PATTERNS

In dimorphic enantiostylous arrays, the probability of intermorph pollen transfer was substantially greater than for intramorph and self pollen transfers. For left-styled plants, transfer from right-styled plants accounted for 74.3% of all mating events (SE = 8.9, range = 68.7–84.3). In contrast, intramorph mating and self-fertilization accounted for only 15.0% (SE = 3.7, range = 5.7–29.0), and 10.7% of all matings (SE = 4.1, range = 0.02–0.22), respectively. For right-styled maternal plants, intermorph pollen transfer resulted in 65.9% of all mating events (SE = 1.5, range = 60.8–68.6), intramorph matings averaged 25.2% (SE = 3.8, range = 15.7–38.0) and selfing averaged 8.9% (SE = 2.5, range = 1.2–16.0). Thus, approximately 75% of all outcrossed mating resulted from pollen transfer between plants of opposite floral form demonstrating that dimorphic enantiostyly promotes intermorph mating.

DISCUSSION

Our study used experimental manipulations of floral design to investigate the prediction that enantiostyly functions to reduce geitonogamous self-pollination, thus allowing more pollen to be available for export to other plants. We structure our discussion around the three questions that we initially set out to address in our study. First, does enantiostyly promote pollen transfer between flowers of opposite stylar deflection? Second, what is the influence of non-enantiostylous, monomorphic enantiostylous, and dimorphic enantiostylous conditions on outcrossing rate? Third, how do these different stylar conditions affect geitonogamous and intrafloral components of selfing? We conclude by examining the implications of our experimental results for the evolutionary pathways and selective mechanisms involved in the origins of mirror-image flowers.

DOES ENANTIOSTYLY PROMOTE POLLEN TRANSFER BETWEEN FLOWERS OF OPPOSITE STYLAR DEFLECTION?

Many investigators have proposed that enantiostyly functions to promote the transfer of pollen between flowers of opposite stylar orientation (Todd, 1882; Wilson, 1887; Knuth, 1906; Ornduff, 1974; Bowers, 1975; Ornduff & Dulberger, 1978). Our results provide experimental support for this suggestion (see Jesson & Barrett, 2002a). In dimorphic enantiostylous arrays, mating patterns indicate that intermorph pollen transfer was three times more frequent than intramorph pollen transfer. This disassortative mating between the style morphs will maintain polymorphism in natural populations of dimorphic

enantiostylous plants and can result in equilibrium frequencies of 1 : 1 of left- and right-styled plants (Jesson & Barrett, 2002b).

The promotion of disassortative mating likely results from the partitioning of pollen into two pools on the left and right sides of the pollinator's body. In *S. rostratum*, our observations of the position of stigma contact on bumblebees indicated that over 50% of all visits led to contact on either side of the body, involving either the abdomen or pollen baskets. This consistent positioning has been noted in earlier studies of enantiostylous species. For example, Bowers (1975) reported that in *S. rostratum*, dyes placed either on the pollinating anther or on the sides of bees' bodies, were more likely to be deposited on stigmas than when dyes were placed on feeding anthers. In *Monochoria vaginalis*, pollen was observed to be consistently deposited on either the left or right hind leg of the major pollinator, *Apis indica* (Iyengar, 1923). This observational evidence supports the occurrence of pollen segregation on different sides of pollinators' bodies.

Our observations of pollen deposition on the sides of pollinators' bodies supports the idea that pollen from one floral form will be deposited on the stigma of the opposite floral form. However, the promotion of intermorph mating through segregated pollen pools may only result from deposition of pollen from the pollinating anther. Experiments with pollen analogues in *Chamaecrista fasciculata* found no difference in pollen transfer to stigmas from feeding anthers or pollinating anthers (Wolfe & Estes, 1992), and in both *S. rostratum* and *C. fasciculata* pollen from feeding anthers is viable and therefore capable of participating in mating (Bowers, 1975; Wolfe & Estes, 1992). Our finding of significant geitonogamy in arrays of a single stylar orientation was unexpected. This suggests that pollen from the feeding anthers may contribute significantly to geitonogamy. Wolfe & Estes (1992) found considerable transfer of pollen from feeding anthers to stigmas of *C. fasciculata*. It is possible that in arrays composed of a single style form, the absence of pollen dispersal from pollinating anthers of the alternate style form resulted in increased mating opportunities by pollen from the feeding anthers, especially during buzzing by the pollinator.

Plants at the Rooftop had significantly fewer pollinator visits, lower female fertility, and lower outcrossing rates than those at the Garden. This suggests that either pollinators limited seed set and/or increased selfing led to early acting inbreeding depression at the Rooftop. Under these conditions seed set was significantly higher in enantiostylous arrays (both monomorphic and dimorphic) than in arrays with a single stylar orientation. It is possible that when pollinators are infrequent, more pollen is transferred between

flower forms than between plants or flowers of a single stylar orientation (including self-pollen transfer). In contrast, there was no difference in seed set in arrays when pollinator visitation was abundant. If visitation rates are high, differences in the effectiveness of pollen transfer may be outweighed by the sheer number of pollinator visits, and even imprecise pollen dispersal results in seed set.

WHAT LEVELS OF OUTCROSSING OCCUR IN ARRAYS WITH DIFFERENT STYLAR CONDITIONS?

Dimorphic enantiostyly has traditionally been interpreted as a mechanism to promote cross-pollination (Wilson, 1887; Ornduff & Dulberger, 1978; Helme & Linder, 1992). In contrast, the function of monomorphic enantiostyly has been considered problematic because mixed floral forms within an inflorescence have been thought to promote geitonogamy and hence reduce outcrossing (Bowers, 1975; Dulberger, 1981; Helme & Linder, 1992; Fenster, 1995; Graham & Barrett, 1995). Outcrossing rates were significantly higher in both monomorphic enantiostylous and dimorphic enantiostylous arrays, compared to straight-styled non-enantiostylous arrays (Table 3). This finding demonstrates that monomorphic enantiostyly promotes outcrossing when compared to a straight-styled phenotype. Outcrossing rates in seeds produced by arrays with plants fixed for stylar direction (i.e. dimorphic, L and R arrays) were significantly higher than monomorphic enantiostylous arrays at the Garden. Plants that displayed a uniform stylar direction were therefore less susceptible to geitonogamy and mostly experienced cross-pollination.

Two other experimental studies have examined outcrossing rates in enantiostylous species. Fenster (1995) used *Chamaecrista fasciculata*, a species with monomorphic enantiostyly, to test the hypothesis that enantiostyly promoted self-fertilization, by increasing geitonogamy. He placed either an all-left-styled or an all-right-styled individual into arrays of unmanipulated plants. These treatments were compared to arrays in which the focal individuals were unmanipulated (monomorphic enantiostyly). Focal plants with a single stylar orientation had significantly higher outcrossing rates than individuals with monomorphic enantiostyly.

Similarly, Barrett *et al.* (2000a) compared outcrossing rates in experimental arrays of monomorphic enantiostylous *Monochoria korsakowii* and arrays manipulated to be dimorphic enantiostylous with a 1:1 ratio of left- and right-styled plants. These authors found that outcrossing rates were substantially higher in the dimorphic enantiostylous than in the monomorphic enantiostylous treatment. Therefore the results of both experimental studies, in combina-

tion with our own findings, support the proposal that dimorphic enantiostyly has likely evolved to reduce the amount of geitonogamy that can occur with monomorphic enantiostyly.

HOW DO THE DIFFERENT STYLAR CONDITIONS INFLUENCE GEITONOGAMOUS AND INTRAFLORAL COMPONENTS OF SELFING?

Our model predicts that enantiostyly functions to reduce geitonogamy, thus increasing both maternal outcrossing and outcrossed siring success (see Jesson *et al.*, 2003a). The observed differences in outcrossing rates that we detected between treatments may be due to either geitonogamous or intrafloral selfing. Estimates of the components of selfing indicated that both non-enantiostylous and monomorphic enantiostylous arrays experienced significant levels of geitonogamy and intrafloral selfing. The large standard errors associated with these estimates prevented us from clearly differentiating between the magnitude of these components.

However, the level of geitonogamy in dimorphic enantiostylous arrays was not significantly different from zero. This indicates that dimorphic enantiostyly is more effective in reducing geitonogamy than monomorphic enantiostyly, as predicted. Moreover, differences in outcrossing rate between monomorphic enantiostylous and dimorphic enantiostylous arrays must be due to changes in geitonogamy rather than intrafloral selfing, because only the design of the inflorescence, not the flower, was altered between these treatments.

Geitonogamy and intrafloral selfing can influence male fitness though changes in pollen transfer, but they can also reduce female fitness through their effects on seed set and inbreeding depression. Comparison of the outcrossing rates indicated a significant increase in the outcrossing rates between seed and seedling stages, especially in straight-styled arrays. This difference could have arisen from selection against selfed seed during germination and suggests that significant inbreeding depression occurs in *S. rostratum*. Inbreeding depression is also a potential explanation for the reduced seed set that we obtained in the straight-styled treatment in the Garden (see Fig. 2), although controlled pollinations by Bowers (1975) did not reveal differences in fruit or seed set following self- and cross-pollinations.

PATHWAYS IN THE EVOLUTION OF ENANTIOSTYLY

The functional comparisons among floral phenotypes investigated in this study are relevant to the pathways involved in the evolution of enantiostyly. We have assumed that the ancestral state for monomor-

phic enantio styly is a straight style. However, it is possible that the morphology of manipulated flowers in our experiments does not accurately represent ancestral phenotypes. For example, our straight-styled treatment had a single pollinating anther positioned close to the style. The sequence in which stamen dimorphism and stylar deflection become associated during the evolution of enantio styly is unclear (see Jesson & Barrett, 2003). Several sister taxa of enantio stylous species have a stamen dimorphism and are straight-styled (e.g. *Cyanella hyacinthoides* and *Dilatris viscosa*). Selection for enantio styly in these phenotypes would likely reduce geitonogamy while still maintaining the precision of pollination. However, other buzz-pollinated enantio stylous species lack stamen dimorphism. For example, in *Saintpaulia ionantha* and *Monochoria australasica* the style is deflected away from a central cone of morphologically similar anthers. In a comparative study of the evolution of enantio styly in the monocotyledons, the gain of heteranthery and enantio styly often occurred on the same branch of a phylogeny (Jesson & Barrett, 2003). Thus the location(s) of anthers in the ancestral condition to monomorphic enantio styly remains uncertain.

The sequence involved in the evolution of enantio styly may involve transition from a morphology in which the stigma and anthers are close together, followed by the deflection of the style, and only later is the reciprocity of style and anthers selected (Jesson & Barrett, 2003; Jesson *et al.*, 2003a). In our experiments we did not examine the case where the stigma was deflected without the corresponding deflection of a reciprocal stamen. This is because this condition is difficult to create in *S. rostratum* and removing the pollinating stamen would influence the total amount of pollen available for fertilization. However, the L and R treatments can be considered equivalent to this evolutionary step – the style is well away from the feeding anthers, and the role of the pollinating anther in mating is likely to be minimal. Arrays that were fixed for one stylar direction had outcrossing rates significantly higher than straight-styled arrays. This result suggests that the initial selection for stylar deflection could have been to reduce intrafloral selfing and stigma–anther interference. Subsequent evolution of the reciprocal pollinating anther in monomorphic enantio styly could then have occurred to increase the precision of pollination. In the Roof environment, which was characterized by low pollinator visits, the seed set of *S. rostratum* was significantly lower in arrays of a single style orientation than in both enantio stylous arrays. This implies that the reciprocal stigma and anther position that characterize many enantio stylous species has been selected because reciprocity increases the precision of cross-pollination.

There is phylogenetic evidence indicating that dimorphic enantio styly is derived from monomorphic enantio styly (reviewed in Jesson & Barrett, 2003). The transition to dimorphic enantio styly may have occurred via the invasion into a monomorphic enantio stylous population of a mutant fixed for one stylar direction (see Jesson *et al.*, 2003a). If so, L or R populations could represent an intermediate stage in the evolution of dimorphic enantio styly. Fenster's (1995) experiments suggest that if a mutant fixed for direction invaded a monomorphic enantio stylous population, the rare mutant would have slightly higher fitness than resident monomorphic enantio stylous plants. The results from our experimental arrays indicated that the female fitness of entirely left- or right-styled plants was not different from that of plants in monomorphic enantio stylous arrays, although outcrossing rates were slightly higher. Thus, with the existing experimental data it is equivocal whether a mutant fixed for direction that invaded a monomorphic enantio stylous population would spread to fixation, creating an all left- or all right-styled population. Regardless of the potential intermediate step, the evolution of dimorphic enantio styly from monomorphic enantio styly would still require the invasion of two mutants fixed for opposite stylar directions. The rarity of this event may limit the evolution of dimorphic enantio styly and help to explain its infrequent occurrence in angiosperms.

Our experiments with *S. rostratum* demonstrated that the seed fertility and outcrossing rates of dimorphic enantio stylous arrays were higher than monomorphic enantio stylous arrays. This could imply that dimorphic enantio styly confers greater fitness benefits than monomorphic enantio styly, leading to the question of why dimorphic enantio styly has not evolved in *Solanum*. The steps involved in the evolutionary transition to dimorphic enantio styly are not known with any certainty, but the overall rarity of this genetic polymorphism implies that there must be strong constraints on its evolutionary origin (Jesson & Barrett, 2003; Jesson *et al.*, 2003a, b).

In *Solanum* these constraints may be associated with the developmental basis of inflorescence architecture; *S. rostratum* exhibits pendulum symmetry, where flowers at alternating nodes exhibit styles of opposite deflection (Charlton, 1998; Jesson *et al.*, 2003b). Patterns of stylar deflection are rigidly determined by the growth patterns of the inflorescence, rather than through an external or genetic trigger. In *S. rostratum*, the origin of a mutant fixed for stylar direction would therefore require dramatic remodeling of inflorescence architecture. If such a change occurred it seems likely that it would result in a strong fitness penalty. Hence it is perhaps no coincidence that dimorphic enantio styly is absent from groups such as

Solanum and *Chamaecrista* that exhibit pendulum symmetry. Functional interactions between floral design and inflorescence display seem likely to have played an important role in the evolution of mirror-image flowers.

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