Mirror Image Flowers and their Effect on Outcrossing Rate in Chamaecrista fasciculata (Leguminosae)

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MIRROR IMAGE FLOWERS AND THEIR EFFECT ON OUTCROSSING RATE IN *CHAMAECRISTA FASCICULATA* (LEGUMINOSAE)[1]

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The influence of enantiostyly (reciprocal segregation of anthers and stigmas to different sides of the flower) on outcrossing rate was examined in *Chamaecrista fasciculata* (Leguminosae). I hypothesized that enantiostyly has not evolved to increase the female component of outcrossing and actually acts to increase the selfing rate through geitonogamy. To quantify the role of enantiostyly to outcrossing, plants of known isozyme genotype were manipulated to be either completely left- or right-styled (nonenantiostylous) or to have equal numbers of left- and right-styled flowers (enantiostylous). Flower number was varied to quantify any interaction between floral display size and enantiostyly on outcrossing rate. These "target" plants were surrounded by unmanipulated plants homozygous for the alternative allele. Outcrossing rates of the target plants were determined by scoring the presence or absence of heterozygotes. The contribution of enantiostyly to geitonogamy may be reduced if pollinators discriminate among the floral types. Thus, observations of pollinator movement between flowers on the same plant were made to determine if pollinators discriminate between the floral types. Although pollinators moved randomly between flower types, outcrossing rate was only marginally effected by the presence of enantiostyly. Enantiostylous plants outcrossed at a slightly lower rate than nonenantiostylous plants only when the opportunity for geitonogamy was great. These results suggest that the contribution of enantiostyly to selfing is minimal.

Although enantiostyly, or mirror image flowers, has been recognized for well over a century (Todd, 1882), its adaptive significance has never been confirmed. Hypotheses have been influenced by the parallel in reciprocal position of anthers and stigmas of enantiomorphic flowers with heterostylous species (Darwin, 1877), and generally considered enantiostyly to be a cross pollination (Knuth, 1906) or outcrossing mechanism (Ornduff and Dulberger, 1978; Dulberger and Ornduff, 1980; Dulberger, 1981). In addition to heterostyly, enantiostyly is considered to be a second type of reciprocal herkogamy (Webb and Lloyd, 1986) that promotes pollinations between floral forms and consequently outcrossing (Wilson, 1887). There are two components to cross pollination and outcrossing: the donation of pollen to and the reception of pollen from other individuals. Reciprocal herkogamy will not affect the level of autogamous self-pollination more than regular herkogamy (Ganders, 1979). Therefore, recent models of the evolution of heterostyly specifically and reciprocal herkogamy in general focus on the role of these breeding systems in promoting the male component of outcrossing by increasing the efficiency of pollen donation through intermorph pollination (Webb and Lloyd, 1986).

Enantiostyly is similar to heterostyly in the reciprocal placement of anthers and stigmas in different flowers but differs in two major ways. First, with the exception of *Wachendorfia* and *Barberetta,* Haemodoraceae (Ornduff and Dulberger, 1978), both floral forms (left- and right-styled flowers) occur on all individuals (*Dilatris,* Hae-

modoraceae, Ornduff and Dulberger, 1978; Cyanastraceae, Dulberger and Ornduff, 1980; Solanaceae, Bowers, 1975; Leguminosae, Todd, 1882 and Dulberger, 1981; Nyctaginaceae, Webb and Lloyd, 1986). In addition, somatic enantiostyly is associated with self-compatibility except for two species of *Cyanella* that exhibit weak self-incompatibility (Cyanastraceae, Dulberger and Ornduff, 1980), whereas heterostyly is most often associated with self- or cryptic self-incompatibility (Barrett, 1988). The consequence of these differences is that somatic enantiostyly might actually promote geitonogamous pollinations over nonreciprocal herkogamy with the subsequent increase of selfing rate. If pollinators indiscriminately visit different floral types on a plant, then pollen dusted on one side of the pollinator will likely be deposited on the next flower with its stigma in the reciprocal position. This may impose a cost to somatic enantiostyly if geitonogamy makes a significant contribution to self-fertilization, and if inbreeding depression exists. The adaptive significance of somatic enantiostyly in promoting cross-pollination is obscure if geitonogamy makes a significant contribution to self-fertilization.

The objective of the present study was to investigate whether enantiostyly influences the selfing rate in *Chamaecrista fasciculata* Michx. (Leguminosae: Caesalpinioidaea). The cost of enantiostyly in terms of increasing selfing via geitonogamy was examined by creating nonenantiostylous (pure left- or pure right-styled plants) and enantiostylous phenotypes. I hypothesize that enantiostyly is associated with increased selfing and that the difference between outcrossing rates among enantiostylous and nonenantiostylous plants should increase as the opportunities for geitonogamy increase. Somatic enantiostyly may not promote geitonogamy if pollinators visit only one floral type on a plant. Thus pollinators were observed to determine if they discriminate among floral types or, alternatively, randomly move between them.

MATERIALS AND METHODS

Study organism—*Chamaecrista fasciculata* Michx., partridge pea (formerly known as *Cassia fasciculata* or *Cassia chamaecrista,* Irwin and Barneby, 1982), is a self-compatible, annual legume of old field, prairie, and savanna habitats. Flowers open for 1 day, and plants are somatically enantiostylous (i.e., the style emerges from opposite sides of the flower in alternating flowers in an inflorescence). The androecium contains two sets of anthers: nine upright anthers curved away from the pistil and a tenth anther which parallels the style. The minute stigma is at the end of a gently incurved filliform style.

Chamaecrista fasciculata falls into the "buzz" pollination syndrome (Faegri and van der Pijl, 1979): the large, yellow caesalpinoid flowers provide no nectar rewards, and the pollen is released through terminal pores in the anthers following vibration or "milking" by large bees (Thorp and Estes, 1975; Wolfe and Estes, 1992). During this manipulation by the bee, a cloud of pollen often emerges from the flower (Buchmann, 1983; personal observation). *Chamaecrista fasciculata* has a rigid upper petal (cucullus) that curves over the nine anthers that are deflected away from the pistil. Wolfe and Estes (1992) have demonstrated the cucullus acts as a flight guide, directing the pollinator to first contact the stigma before it sets down on the anthers. *Chamaecrista fasciculata* is exclusively bee pollinated, usually by *Bombus* spp. or Anthophorids (Thorp and Estes, 1975; Lee and Bazzaz, 1982; Fenster, 1991b). Left- and right-styled flowers are equally frequent at the population level (Todd, 1882; Fenster, unpublished data). The distribution of left- and right-styled flowers on a given plant fluctuates daily, but most plants have both floral types present on a given day (Todd, 1882; Thorp and Estes, 1975; Fenster, unpublished data).

Outcrossing rate—Bulk seed was collected from two populations, approximately 3 km apart, in Chester Co. Virginia in Fall 1990, germinated in Spring 1991, and scored for isozyme genotypes in Summer 1991. Polymorphic loci that were found to segregate in Mendelian fashion in controlled crosses were found for PGM (three alleles), PGI (two alleles) and LAP (three alleles) (methods described in Fenster, 1991b).

Three experimental arrays were constructed consisting of 13 or 14 plants with 12 plants on the perimeter homozygous for the common allele (four plants per side of a square array and one or two "target" plants in the center of the array, with each target plant homozygous for different alternative alleles). If two target plants were used in the same array then each had the same floral display and one was assigned to be enantiostylous and the other nonenantiostylous. All plants in an array were derived from seed collected from the same population. Plants were spaced 20–30 cm apart, which approximates densities in natural populations. The perimeter plants were kept in pots in an experimental garden adjacent to the University of Maryland glasshouse in College Park, Maryland. The target plants, homozygous for the alternative alleles, were kept in a pollinator-free glasshouse under sodium-vapor lights. The lights were set to turn on 1–2 hours before sunrise allowing flowers of target plants to open earlier

than the outdoor perimeter plants. Target plants were then manipulated by removing flowers, leaving a 50:50 mixture of left- and right-styled flowers (approximating an alternating sequence of left- and right-styled flowers) or all left- or all right-styled flowers. Care was taken to minimize the creation of gaps in the floral display, and no obvious differences in floral display, other than enantiostyly vs. nonenantiostyly, were apparent. The treatments were conducted on plants with two to 30 flowers to determine if enantiostyly affects outcrossing rate only when the opportunities for geitonogamy increase. Surrounding plants had a similar range of floral display sizes. To avoid any association between marker allele and outcrossing rate, a given target plant was rotated between enantiostylous and nonenantiostylous treatments. Target plants were positioned in the arrays just before the arrival of *Bombus* spp., when both array and target plants were in anthesis (approximately 0630 hours), and were returned to the glasshouse at the end of the day, when flowers were labeled. The pollinators behaved identically to those in the field by buzzing the plants for pollen. Ten target plants were used throughout the experiment. The experiments were conducted from early August to mid-September 1991, after which all target plants were kept in the glasshouse until fruit collection.

Outcrossing rates were determined by the frequency of heterozygote progeny from the target plants. Flower abortion rates are 50%, and pods contain only ten to 12 seeds on average (Fenster, 1991a); thus, in order to have equivalent sample sizes, the smaller floral display size classes were replicated across more days. From one to four fruit from the smallest floral display size class to ten to 20 fruit in the largest size class were sampled per plant per day. For any given day up to 40 seed were genotyped directly (not germinated first) per target plant totaling 42–123 seeds per treatment and floral display size (mean = 89 seed ± 24 *SD* seeds).

Pollinator flight movement—Within-plant pollinator flight movements were observed to determine if bees discriminated between left- and right-styled flowers. Approximately 30 minutes after the first arrival of bees to the arrays (0700 hours), the movement of bees between left- or right-styled flowers on the unmanipulated perimeter plants was observed for 1 hour. Observations were conducted for eight mornings, and all arrays were sampled at least once.

Statistical analyses—Two methods were used to determine the effect of enantiostyly on outcrossing rate. First, the data were analyzed using a two-factor categorical model with enantiostyly/nonenantiostyly and display size as main effects (PROC CATMOD, SAS, Inc., 1985). Chi-square analyses were then conducted to contrast the effect of enantiostyly for the different floral display sizes on outcrossing rate. Sidak's procedure (Rohlf and Sokal, 1981) was used to hold the experiment-wise type I error rate at 5% while conducting simultaneous contrasts between enantiostylous and nonenantiostylous plants for the five floral display size classes. In this analysis each seed is considered an independent data point. Although pollen carryover is extensive in this species and flowers are vis-

ited repeatedly, it is still likely that mating events within a fruit are not independent of one another. To circumscribe this problem, outcrossing rates were also compared between enantiostylous and nonenantiostylous treatments for each plant and contrasted using a paired t-test.

Chi-square analysis was used to determine whether bees moved randomly among the left- and right-styled flowers.

RESULTS

Outcrossing rate—There was a significant effect of both treatments, enantiostylous/nonenantiostylous, and floral display size on outcrossing rate ($\chi^2 = 4.35$, 1 df, $P = 0.037$, $\chi^2 = 17.54$, 4 df, $P = 0.002$, respectively). The interaction between the two treatments was not significant ($\chi^2 = 6.26$, 4 df, $P = 0.180$). All floral display size classes were combined, and the mean outcrossing rate was 0.64 for enantiostylous plants (477 seeds) and 0.69 (417 seeds) for nonenantiostylous plants. Since the overall differences were small, most of the individual contrasts were nonsignificant. There were no significant differences for outcrossing rate between enantiostylous and nonenantiostylous plants for each of the floral display size-classes (Fig. 1). The opportunity for geitonogamy is least for the smallest floral display size class. Therefore, outcrossing rates were contrasted between enantiostylous plants in the smallest floral display size class vs. all other size classes combined and were found to differ significantly (outcrossing rate = 0.73 and 0.61, respectively, $\chi^2 = 7.407$, 1 df, $P < 0.05$). However, no other effect of flower number on outcrossing rate was observed (Fig. 1). Since the level of selfing was higher in the larger floral display size class, the data were pooled for floral displays with more than six flowers and outcrossing rates were contrasted between enantiostylous and nonenantiostylous plants. When the comparison of outcrossing rates was restricted to larger floral displays, the differences between the enantiostylous and nonenantiostylous plants increased slightly but the specific contrast was nonsignificant (outcrossing rate = 0.61 and 0.68, respectively, $\chi^2 = 3.564$, 1 df, ns). Very similar results occurred when the analysis was conducted using each plant, rather than each seed, as a replicate when comparing the effect of enantiostyly on outcrossing rates for floral displays greater than six flowers. Five of the ten plants had less than ten seed in either one of the two treatments and were not used in the analysis while the remaining five had from 44 to 119 seeds for each of the two treatments. Outcrossing rates for these five plants were contrasted, and the mean outcrossing rate for the enantiostylous treatment was 0.61 (2 $SE = 0.05$) and 0.70 for the nonenantiostylous treatment (2 $SE = 0.09$) averaged across the five plants ($t = 3.569$, 4 df, $P = 0.023$).

Pollinator flight movement—Pollinators did not discriminate among the two types of flowers based on within-plant movements. Within plants, bees made 48.4% of 304 movements between flowers of the same orientation and 51.6% of 304 movements between flowers of different orientation, which does not differ from random expectation, assuming a 1:1 ratio of left- and right-styled flowers ($\chi^2 = 0.329$, 1 df, $P > 0.5$).
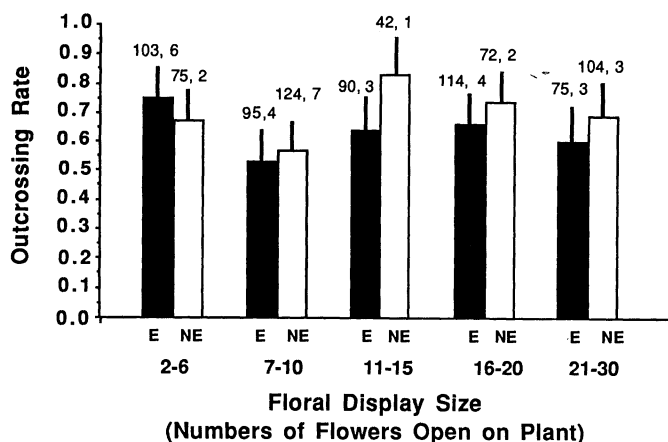

Fig. 1. The effect of enantiostyly and floral display size on outcrossing rate. Filled bars indicate plants with a 1:1 ratio of left- and right-styled flowers (E = enantiostylous). Open bars are plants that are either all left or all right styled (NE = nonenantiostylous). Bars indicate two SEs. Number of seeds sampled to determine outcrossing rates and number of plants used in treatment are provided above each bar.

DISCUSSION

Cost of enantiostyly—In *C. fasciculata*, nine of ten anthers are deflected away from the style, and there is no difference in function and dispersal between the two sets of anthers (Wolfe and Estes, 1992, and see their fig. 1). Thus, it is likely that more pollen will be placed on the side of the bee opposite the style. Consequently, the indiscriminate visitation of pollinators on left- and right-styled flowers suggests that geitonogamy would be lower in an all left- or all right-styled plant. However, the lack of association between flower number and outcrossing rate of plants with greater than six flowers suggests that beyond a threshold number of flowers, floral display size does not continue to contribute to an increase in geitonogamy in *C. fasciculata*. Consequently, the contribution of geitonogamy to selfing rapidly plateaus and in turn limits the contribution of enantiostyly to selfing.

Increasing floral attractiveness associated with increasing numbers of flowers on a plant may result in prolonged pollinator visitation and increased frequency of geitonogamy and hence selfing (Charlesworth and Charlesworth, 1987). Several field studies have observed plant size to be correlated to geitonogamous transfer of pollen (Hessing, 1988; Peakall, 1989; Dudash, 1991; de Jong et al., 1992) and to selfing rate (Crawford, 1984; Sun and Ganders, 1988; M. R. Dudash and S. C. H. Barrett, University of Toronto, Toronto, Ontario, unpublished data). Therefore, the disadvantage of having both left- and right-styled flowers on the same plant in terms of promoting geitonogamy should be reduced for plants with smaller floral displays. Studies with other species, *Lithospermum caroliniense* (Weller, 1980), *Malaviscus arboreus* (Webb and Bawa, 1983), and *Mertensia ciliata* (Geber, 1985), have, as in the work described here, also observed little relation between floral display size and selfing. Potential mechanisms suggested by these studies that may limit the degree of selfing on large plants of *C. fasciculata* include limited foraging of pollinators on individual plants (citations in Lloyd and Schoen, 1992). The outcrossing rate

for enantiostylous plants in the smallest floral display size class is similar to previous values reported from natural populations (0.73 vs. 0.80, respectively; Fenster, 1991b). Therefore, the small differences in outcrossing rate between enantiostylous and nonenantiostylous plants were probably not artifacts of special conditions associated with the artificial array. Pollen tubes from self-pollen are just as likely to fertilize ovules as outcross pollen (Fenster and Sork, 1988), and self-pollinations have no reduction in fruit-set and only a 10% reduction in seed number compared to outcross pollinations (Fenster, 1991a). Thus it is unlikely that incompatibility or inbreeding depression could have 'dampened' differences in selfing rates between treatments.

The increased outcrossing rate of nonenantiostylous plants compared to enantiostylous plants was small but nevertheless confirms that enantiostyly has not evolved as a mechanism to promote maternal outcrossing. This is in agreement with previous hypotheses (Ganders, 1979; Webb and Lloyd, 1986) that the reciprocal placement of stamens and pistils in heterostyly and enantiostyly have likely evolved in response to selection to increase the pollen dispersal phase of cross-pollination. Lloyd (1992) concluded that geitonogamy is a nonadaptive feature of outcrossing because it offers no reproductive assurance and likely results in decreased male contribution to outcrossing. Thus, it is unlikely that enantiostyly could have evolved to increase geitonogamy. *Chamaechrista fasciculata* suffers severe inbreeding depression following selfing (Fenster, 1991c). Therefore, any advantage that enantiostyly confers must outweigh its small contribution to geitonogamy and the consequent expression of inbreeding depression. In many natural populations of *C. fasciculata* the number of flowers open on a given day is often less than six (Fenster, 1991b). Thus enantiostyly probably makes a negligible contribution to selfing and progeny of enantiostylous plants likely do not differ from progeny of nonenantiostylous plants in terms of the expression of inbreeding depression.

Adaptive significance of enantiostyly—Although the above discussion suggests that the maladaptive consequences of enantiostyly are small if present, what might be the selective agent(s) driving the evolution and maintenance of enantiostyly? In particular, what advantages might enantiostyly confer to plant reproduction over other forms of herkogamy, and other forms of reverse herkogamy, e.g., reciprocal placement of the stigma above or below the stamens as in heterostyly? Like heterostyly, enantiostyly may facilitate pollen collection and dispersal by reducing interference between the androecial and gynoecial function (Webb and Lloyd, 1986). Having a pistil protrude among the anthers may decrease the active collection efficiency or number of pollen grains actively gathered by a flower visitor (Inouye et al., 1994). However, reciprocal placement of the pistil above and below the anthers as in heterostyly might interfere with the buzz pollination process. Deflection of the pistil thus removes the pistil from the area of the flower manipulated by the bee. In addition, Dulberger (1981) proposed that enantiostyly may also reduce the probability of damage to a fragile gynoecium during buzz pollination which requires forceful manipulation of the stamens by the bee. Fur-

thermore, the bee's position between the stigma and anthers may reduce the chances of self pollination via a pollen cloud which invariably forms during the bee's manipulation of the anthers. Consequently, enantiostyly reduces the likelihood of within-flower self pollination. This is in contrast to other forms of herkogamy where the anthers and stigmas are on the same side of the pollinator.

Although the above hypotheses may explain the evolution of enantiostyly, they do not explain why both floral types are present on the same plant. Having both floral types on a plant may be an adaptation associated with increasing precision of pollen transfer from pollinator to stigma. The loss of herkogamy is sometimes associated with increased pollen dispersal because pollen is placed on the same part of the pollinator that comes in contact with conspecific stigmas (Campbell, 1989; Barrett, Kohn and Cruzan, 1992). Enantiostylous plants with both floral forms may effectively be placing the stigma and stamens in juxtaposition, yet not suffer from increased selfing from within-flower pollen transfer (Webb and Lloyd, 1986). Therefore, enantiostylous species may be able to place pollen on a restricted portion of the pollinator, which then transfers it to a reciprocal flower type, analogous to species exhibiting heterostyly (Price and Barrett, 1984; Barrett and Glover, 1985). The role of enantiostyly in promoting pollen transfer efficiency clearly needs to be further examined. Although developmental enantiostyly does not promote the cross fertilization of ovules, it may represent an evolutionary response to the convergence of selective forces favoring maximum pollen transfer efficiency and pollination precision (Bowers, 1975; Dulberger and Ornduff, 1980), minimum interference (Webb and Lloyd, 1986), and minimum within-flower transfer of self pollen, while removing the fragile gynoecium from the axis of the flower that experiences the most manipulation by the pollinator during buzz pollination (Dulberger, 1981).

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