

Why do stigmas move in a flexistylous plant?

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Abstract

Flexistylous is a recently documented stilar polymorphism involving both spatial and temporal segregation of sex roles within hermaphroditic flowers. Using the experimental manipulation of stigma movement in self-compatible *Alpinia mutica*, we tested the hypothesis that selection for reducing interference between male and female function drives the evolution and/or maintenance of stigma movement. In experimental arrays, anaflexistylous (protogynous) flowers served as pollen donors competing for mating opportunities on cataflexistylous (protandrous) flowers. The pollen donors were either manipulated so their stigmas could not move or were left intact, and their success was determined using allozymes to assess the paternity of recipient seeds. We found that manipulated flowers sired a significantly smaller proportion of seeds, showing that stigma movement in unmanipulated plants increased male fitness. This result was strongest under conditions in which pollen competition was expected to be highest, specifically when pollinators visited multiple donor plants before visiting recipient flowers.

Introduction

Hermaphroditism, the presence of female and male reproductive organs within one flower, is the predominant sexual condition in flowering plants. The benefits of this sexual system include economizing on the resources allocated to pollinator attraction and saturating gain curves (Charnov, 1982; Lloyd, 1987). The main cost of hermaphroditism arises from the negative effects of self-pollination. Traditionally, many floral traits have been interpreted as adaptations to avoid self-pollination and the harmful effects of inbreeding (Darwin, 1876; Charlesworth & Charlesworth, 1987). However, many self-incompatible hermaphroditic plants still possess floral traits that might be interpreted as anti-selfing mechanisms; for example, most heterostylous plants are also self-incompatible (Barrett *et al.*, 2000). Therefore, it is necessary to find alternative adaptive explanations for the evolution or maintenance of traits that appear to be anti-selfing mechanisms yet do not serve this function.

Sexual interference is a plausible alternative explanation for understanding the origin and maintenance of floral traits (Lloyd & Yates, 1982). In hermaphroditic animal-pollinated plants, sexual interference stems from functional conflicts between pollen dispersal and receipt during pollination and mating. When pollen and receptive stigmas are presented simultaneously within the same self-incompatible flower, there is the potential for gamete wastage and reduction in mating opportunities (Barrett, 2002). Female fitness may be negatively affected by male function in three main ways (Cesaro *et al.*, 2004): (i) the position of the stamens may obstruct pollen deposition on stigmas; (ii) the deposition of the self-pollen may cause pollen clogging; and (iii) self-pollen tubes may usurp ovules and seeds that are then unavailable for cross-fertilization (ovule discounting). Female function can interfere with male function in two nonexclusive ways: styles may physically restrict pollinator access to pollen or styles may remove pollen from a pollinator's body as it leaves the flower. Both types of interference by female function could result in lost opportunities for pollen export (Harder & Wilson, 1998). Sexual interference may act among flowers on the same inflorescence or different inflorescences within an individual plant (Harder *et al.*, 2000). In many plants

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with large floral displays, geitonogamy (between-flower self-pollination) is probably the major source of sexual interference that can cause severe loss of female and male function (Eckert, 2000). However, the importance of sexual interference for influencing floral evolution and mating strategies is still not well understood.

Hermaphroditic plants could suffer significant reproductive costs from sexual interference, resulting in selection for various floral traits that reduce these costs. Segregation of the sex function, either by the spatial separation of stigmas and anthers (herkogamy; Webb & Lloyd, 1986) or by the temporal separation of stigma receptivity and anther dehiscence (dichogamy; Lloyd & Webb, 1986; Bertin & Newman, 1993), has been viewed as an adaptation that reduces interference and enhances reproductive success. For between-flower interference, some floral strategies, such as dichogamy on vertical inflorescences (Harder *et al.*, 2000; Jersakova & Johnson, 2007), enantiostyly (Jesson & Barrett, 2005) and small floral display size (Lau *et al.*, 2008) could act to reduce geitonogamous self-pollination, thus allowing more pollen to be available for export to other plants. Lloyd & Webb (1986) pointed out that sexual interference is one of the most important selective forces shaping floral evolution. However, the sexual interference hypothesis has only been experimentally tested in a limited number of plant species (Kohn & Barrett, 1992; Griffin *et al.*, 2000; Harder *et al.*, 2000; Fetscher, 2001; Cesaro *et al.*, 2004; Routley & Husband, 2006; Jersakova & Johnson, 2007; Vallejo-Marin & Rausher, 2007; Quesada-Aguilar *et al.*, 2008), and most studies of sexual interference have focused on the role of either self-pollen deposition in reducing the access of outcross pollen to ovules or pollen fate during pollination (reviewed by Barrett, 2002). Direct experimental evidence for the decreasing siring success because of the sexual interference is scarce.

Flexistily, a recently documented floral strategy in the Zingiberaceae, combines both herkogamy via stigma movement and heterodichogamy via temporal differences in sexual functions in a single stylar polymorphism (Cui *et al.*, 1995; Li *et al.*, 2001, 2002; Zhang *et al.*, 2003; Takano *et al.*, 2005; Ren *et al.*, 2007). Populations are composed of equal frequencies of two style morphs of hermaphrodites that differ in the direction of movement that stigmas undergo during the 1-d flowering period. In the cataflexistylous (protandrous) morph, stigmas begin in an unreceptive upward position and move downwards into a receptive position where stigmas can contact pollinators (Fig. S1a,b). In contrast, in the anaflexistylous (protogynous) morph, the movement of stigmas is in the opposite direction (Fig. S1c,d). Stigmas of the two morphs reciprocally move in the middle of the 1-d flowering period. Anthers are held in the same position throughout flowering, but shed pollen only when stigmas are in the upward position where they cannot contact pollinators, promoting disassortative mating between floral morphs.

Flexistily has been interpreted as promoting outcrossing by avoiding self-pollination (Li *et al.*, 2001). However, our previous results showed that selection solely for promoting outcrossing is insufficient to explain the evolution of stigma movement (Sun *et al.*, 2007). First, the heterodichogamous component of flexistily is sufficient by itself to limit self-pollination. Second, if stigma movement functions to avoid self-fertilization, then the anaflexistylous styles with their stigmas that were outcrossed by the other morph in the morning would have no need to curve up at noon. Because flexistily cannot be explained as a trait to avoid self-pollination, we investigated an alternative explanation. If both anthers and receptive stigmas in flexistylous plants are housed in approximately the same position within a flower to facilitate pollen deposition and removal, there could be physical interference during pollination, resulting in losses to male fitness. Therefore, synchronous stigma movements on a flexistylous plant resulting in the spatial segregation of the sex roles in inflorescences could increase pollen export during their male phase by reducing levels of within- and between-flower interference.

Here, we ask whether stigma movement in anaflexistylous flowers increases male fitness. To evaluate the extent and mechanisms of pollen-stigma interference in a flexistylous plant, *Alpinia mutica*, we used experimental pollen donor–recipient arrays to compare male fitness of natural and experimentally manipulated flowers during their male phase. Cataflexistylous flowers served as pollen recipients and anaflexistylous flowers served as pollen donors. Donor flowers were either manipulated so that the stigma would not move up or were left unmanipulated (controls). We predicted that if flexistily reduced the interference of female with male function, then the proportion of seeds sired by control donors would be greater than that sired by manipulated donors. We determined the paternity of seeds from recipient plants by using strains for control and manipulated donor plants that differed in allozymes of aspartate amino transferase (AAT). In contrast to previous studies (Fetscher, 2001; Routley & Husband, 2006; Quesada-Aguilar *et al.*, 2008) that assessed male function by measuring pollen transfer, our experiments used the number of seeds sired by successful pollen export to measure directly the effects of stigma movement on male fitness. Finally, by using different plant array designs, we indirectly manipulated the strength of pollen competition, thereby revealing whether the advantages of flexistily increased with increasing pollen competition.

Materials and methods

Study species

Alpinia mutica (Zingiberaceae) is a self-compatible, clonal, flexistylous perennial herb, usually 1–2 m tall. The

inflorescences are terminal on leafy shoots. The floral structure is the same as most gingers, a conspicuous three-lobed labellum produced by the fusion of two staminodes that is flesh-coloured with a red and yellow centre. The labellum forms a tube, the free part of which is expanded and serves as a landing platform for pollinators. During blooming, each inflorescence produces 1–5 open flowers per day; each flower lasts only 1 day. The number of ovules per pistil is variable, but often exceeds 50. *A. mutica* is commonly distributed throughout south-east Asia, but is not a native species in China, so there were no natural pollen sources of *A. mutica* at Xishu-angbanna Tropical Botanical Garden in southern Yunnan Province, China (21°45'N, 101°02'E; 580 m in altitude), where this work was performed. All experimental arrays were carried out in July 2007 and June–July 2008.

Experimental design

To study the effect of stigma movement on pollen export, two types of pollen donor–recipient arrays were established to compare the number of seeds sired by the control and manipulated flowers in 2007 and 2008. For the manipulation to stop stigma movement, the anaflexistylous style was cut off at its base in the morning; this immobilized the stigma that remained in the downward position all day (Fig. 1). In 2007, the experimental array contained 2–6 anaflexistylous plants with 1–2 flowers each as donors in the centre of the array, and 10 or 16 cataflexistylous plants with 1–4 flowers serving as pollen recipients (Fig. 2). Control and manipulated donors had equal numbers of flowers. In 2008, the array contained 16 plants with 1–3 flowers each arranged in a square grid. Donors and recipients were placed in alternating positions within grid, with approximately 1 m between adjacent plants (Fig. 2). On each side of the array, all

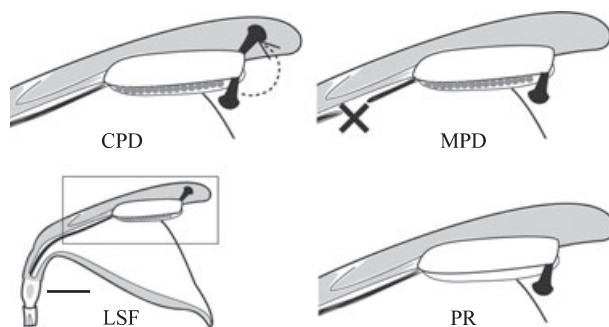


Fig. 1 Diagram for flowers of *Alpinia mutica* used in the experiment. CPD, control pollen donor, an untreated anaflexistylous flower; LSF, longitudinal section of a flexistylous flower; MPD, manipulated pollen donor, an anaflexistylous flower with its style cut off at the base in the morning; PR, pollen recipient, a cataflexistylous flowers with its pollen removed in the morning. The arrow with a dotted line indicates the direction of stigma movement. Scale bar in the bottom-left corner = 1 cm.

plants were selected to have equal numbers of flowers. All arrays were exposed outside to natural pollinators. We replicated the experiment on 14 and 10 days in 2007 and 2008, and a total of 64 and 41 plants were involved in 2007 and 2008, respectively. There were 78 and 154 flowers that served as pollen donors, and 308 and 154 flowers that served as recipients in 2007 and 2008, respectively.

The style treatments of manipulated donors were made during the morning (10:00 to 10:30). To prevent self-fertilization and pollen transfer among recipients, the pollen of recipients was scraped using wet absorbent cotton and the anthers were glued. Plants with similar inflorescence sizes were chosen as pollen donor pairs. The stigmas of pollen donors and recipients finished moving, and pollen of donors was shed, before 15:00. To limit variation in anther dehiscence among donor flowers, we began our experiments only after all anthers had dehisced. Consequently, the experiments were carried out from 15:00 to 19:30 each day. The flowers of pollen recipients were marked, and all plants used were bagged except during the experimental time.

The two array designs were used to indirectly change the magnitude of pollen competition. In the array used in 2007, control and manipulated donor plants were clustered so that pollinators were more likely to accumulate pollen from each before visiting a recipient plant (Fig. 2). In 2008, the intermingling of donor and recipient plants increased the chance that only one donor plant would be visited before a recipient was visited. Of the two designs, the clustering of donor plants in the 2007 design more closely mimics the spatial distribution of the plants in the wild that often occur as clumps (Sun *et al.*, 2010). To characterize the pattern of pollinator movement in the two array designs, we conducted pollinator observations for 30 min every hour between 15:00 and 19:30. For each pollinator entering the array, we recorded its identity, the number and types of flowers visited, the sequence of visits and the duration of each bout until it left the array.

Using observations of pollinator visits, we estimated the potential for pollen competition between control and manipulated donor flowers using two methods. First, we counted only those visits between two sequential visits to recipient flowers, assuming that the recipient stigma efficiently removes most available pollen. The second method summed all visits prior to the visit to any recipient flower, thereby presuming longer pollen carry-over (e.g. Thomson & Plowright, 1980; Thomson, 1986).

To assign paternity of seeds sired, all the plants used for experimental arrays were homozygous at the polymorphic allozyme locus AAT. In the array, manipulated donors and recipients had the same homozygous genotype, and control donors had the other homozygous genotype, such that siring success of each type of donor could be determined by scoring seeds. The genotypes of control donors vs. manipulated donors and recipients were switched after 6 and 8 experimental days in 2007

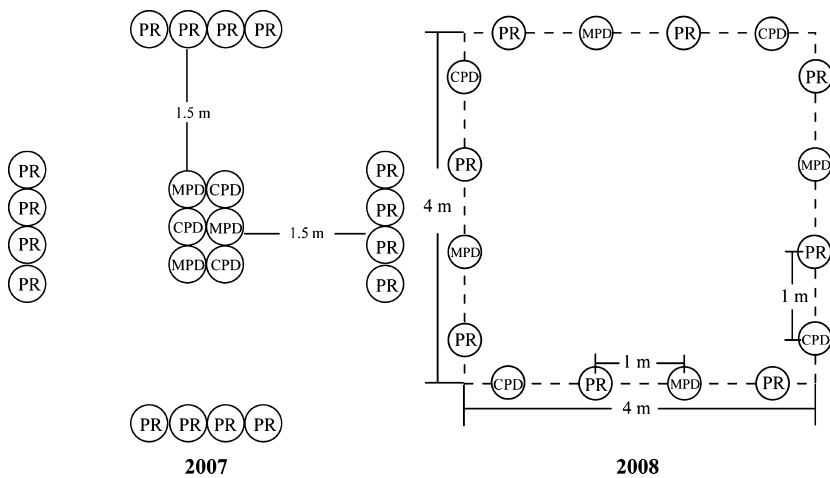


Fig. 2 Diagram for experimental arrays of *Alpinia mutica*. CPD, control pollen donor; MPD, manipulated pollen donor; PR, pollen recipient.

and 2008, respectively. In flexistylous plants, anthers dehiscence and pollen presentation are gradual, leading to residual pollen in anthers of recipients. To exclude the possibility that the seeds were sired by recipients (for example, through incomplete pollen removal), a second locus shikimate dehydrogenase (SKD) was chosen. All pollen donors were heterozygous (FS) and recipients were homozygous (SS) at SKD in both 2007 and 2008. Any seeds expressing the genotype FS at SKD must have resulted from donors, and those with SS at this locus could have resulted from either donors or recipients. We thus only used seeds heterozygous at SKD to assign paternity from the donor types using AAT. The seeds sired from pollen recipients were assayed for the enzyme system AAT (EC 2.6.1.1) and SKD (EC 1.1.1.25) using vertical slab polyacrylamide gel electrophoresis. Alloysymes were extracted by using a Tris-HCL-PVP buffer (pH 7.5) and assayed on 10% polyacrylamide gel. A continuous Tris-glycine buffer system (pH 8.3) was used. All fruits and seeds on the recipient plants were sampled. There were 2885 and 4870 seeds from 76 and 93 fruits scored in 2007 and 2008, respectively.

Statistical analyses

To calculate the relative success of control vs. manipulated donor plants, we performed a generalized linear mixed model (GLMM) analysis in which seeds were scored as either having control or manipulated paternity. Data from the 2 years were analysed separately. Flower and day were treated as random effects, and the data were blocked according to the genotype at the ATT locus of the donor plants (days 1–6 vs. 7–14 in 2007, days 1–8 vs. 9–10 in 2008). This statistical design avoids pseudoreplication by accounting for the possible correlation caused by seeds being scored from the same flowers; an alternative, statistically equivalent procedure would be to analyse the number of seeds per flower under the assumption that paternity is binomially distributed.

Because the experiment was designed to test whether control plants sired more seeds than manipulated plants, we used one-tailed tests of significance.

To account for possible day and block effects in the pollinator visitation data, we used GLMMs in which day was treated as a random effect and block was treated as a fixed effect. For the visitation data, we were interested in explaining the experimental results, rather than the underlying distribution of pollinator visits that would occur if we had performed the experiment on, for example, different days. In principle, it would be possible to know the number of visits without error. However, observations were only made for 30 min every hour; thus, we know precisely the visitation information for half the time, and from this, we must infer the information for the other half of the time. To account for this, we divided the standard errors of the estimates from the GLMMs by the square root of 2 to compute confidence intervals. All analyses were performed in R 2.9.1 (R Development Core Team, 2009), with the GLMMs fit using lmer in the lme4 package (Bates, 2007).

Results

All observed pollinator visits to flowers of *A. mutica* in arrays during 2007 and 2008 were carried out by *Xylocopa* sp. A total of 156 and 126 pollinator bouts were recorded in 26 and 20 h of observations in 2007 and 2008, respectively, during which a total of 1677 and 1982 flower visits were observed. In both years, pollinators visited approximately the same number of control and manipulated donors (49.0% and 47.1% visits to controls in 2007 and 2008, respectively). The GLMM analyses that included day as a random effect and block as a fixed effect revealed no statistically significant block effects; from the models with the block effect removed, the proportions of visits to flowers on control donors in 2007 and 2008 were 0.490 (95% CI = 0.468–0.513) and 0.471 (95% CI = 0.455–0.488).

To assess the potential for pollen competition in the different array designs used in 2007 and 2008, we first compared the proportion of pollinators visiting both control and manipulated donors immediately preceding a visit to a recipient flower vs. those that visited only control or only manipulated donors; for 2007 and 2008, these proportions were 0.617 and 0.180, suggesting greater potential for pollen competition in 2007. This conclusion is made more complex, however, because in 2007, there was a significant block effect in the GLMM; the proportion of pollinators visiting both control and manipulated donors was 0.686 (95% CI = 0.634–0.727) in the first block and 0.500 (95% CI = 0.439–0.561) in the second block. For 2008, there was no block effect, and the estimate for the proportion of pollinators visiting both control and manipulated donors was 0.188 (95% CI = 0.166–0.214).

The second method for assessing pollen competition assumes that pollinators accumulate pollen from all donors visited before visiting a recipient. For 2007 and 2008, the proportion of pollinators visiting both control and manipulated donors at some point prior to visiting a recipient flower vs. those that visited only control or only manipulated donors were 0.838 and 0.813, respectively. The comparable values from the GLMM analyses are 0.854 (95% CI = 0.816–0.881) and 0.808 (95% CI = 0.786–0.827) for 2007 and 2008, respectively, and in neither case was the block fixed effect significant. Thus, measured in terms of the cumulative numbers of control and manipulated donor plants, the potential for pollen competition was similar between 2007 and 2008.

In 2007 and 2008, respectively, 61% and 54% of the recipient seeds were sired by control donors (Fig. 3). In 2007, there was an effect of block, with 81% [= $\text{logit}^{-1}(1.437)$ where 1.437 is the coefficient from the binomial GLMM] and 44% [= $\text{logit}^{-1}(-0.227)$] of the recipient seeds sired by control plants in blocks 1 and 2, respectively. The cause of this block effect is unclear, because the control and manipulated donor plants were matched for genotypes, size and number of flowers. The average of the coefficients from the two blocks is 0.606 ± 0.318 (\pm SE), which is statistically > 0 ($z = 1.91$, $P < 0.028$, one-tailed), implying that the average proportion of seeds sired by control plants is $> 50\%$ [= $\text{logit}^{-1}(0)$]. Furthermore, the proportion of seeds sired by control plants is greater than the proportion of pollinator visits to control plants, 0.490 ($z = 2.031$, $P < 0.021$, one-tailed). The statistical analysis also revealed high variability among fruits in paternity (Fruit random effects); this is to be expected because each flower was visited by few pollinators. Finally, after accounting for the block effect, there was low residual variability among days in the experimental results (Day random effect).

In 2008, there was no overall effect of treatment on siring ability, although control plants sired more seeds than would have been expected from their representa-

tion in the pollen pool. The proportion of seeds sired by control donors was 0.529 [= $\text{logit}^{-1}(0.117)$], which was not statistically different from 0.5 (Table 1). However, the proportion of seeds sired by control donors was marginally statistically significantly greater than the proportion of pollinators visiting control plants, 0.471 ($z = 1.69$, $P < 0.045$, one-tailed). Finally, there was no block effect and no other day-to-day variability (Day random effects), but as expected, high variability among fruits (Fruit random effects).

Discussion

In flexistylous plants, the sexual interference hypothesis explains the movement of stigmas as an adaptation to reduce interference between female and male function, i.e. between stigmas and anthers. This hypothesis therefore predicts that donors with moving stigmas will be more successful at pollen export than are donors with immobile stigmas. In our experiments, in 2007 and 2008 on *A. mutica*, the proportion of seeds sired by control vs. manipulated, stigma-immobilized donors was greater than the null expectation of 50%, although only statistically significantly so in 2007. We also considered the null expectation given by the percentage of control vs. manipulated flowers visited by pollinators, which were 0.490 and 0.471 in 2007 and 2008, respectively. With these null expectations, the siring success of control donors was statistically significant in 2007 and marginally so in 2008. These results give support to the sexual interference hypothesis.

The siring success of control plants was greater in 2007 than in 2008, with the average proportion of seeds sired by control plants equal to 0.647 and 0.529, respectively. In part, this is likely due to differences in the potential for pollen competition between years. In 2007, 0.617 of pollinators visited both control and manipulated flowers immediately before visiting recipient plants, while in 2008 this proportion was only 0.180. We should note, however, that these figures are for only those donor plants visited between recipient plants; if the cumulative visits are used, then these proportions become 0.838 and 0.813 in 2007 and 2008, respectively.

We expected differences in the potential for pollen competition between years owing to differences in array design. In 2007, donor plants (both control and manipulated) were clustered together, with recipient plants surrounding them, whereas in 2008, all plants were more dispersed, with donor and recipient plants intermingled (Fig. 2). Therefore, in 2007, it was more likely that pollinators would visit both control and manipulated donor flowers before visiting recipients. The array design in 2007 was more characteristic of natural conditions, because *A. mutica* generally grows in clumps attributed to vegetative propagation.

Experimental design may be critical in assessments of pollen success in terms of the number of seed sired if

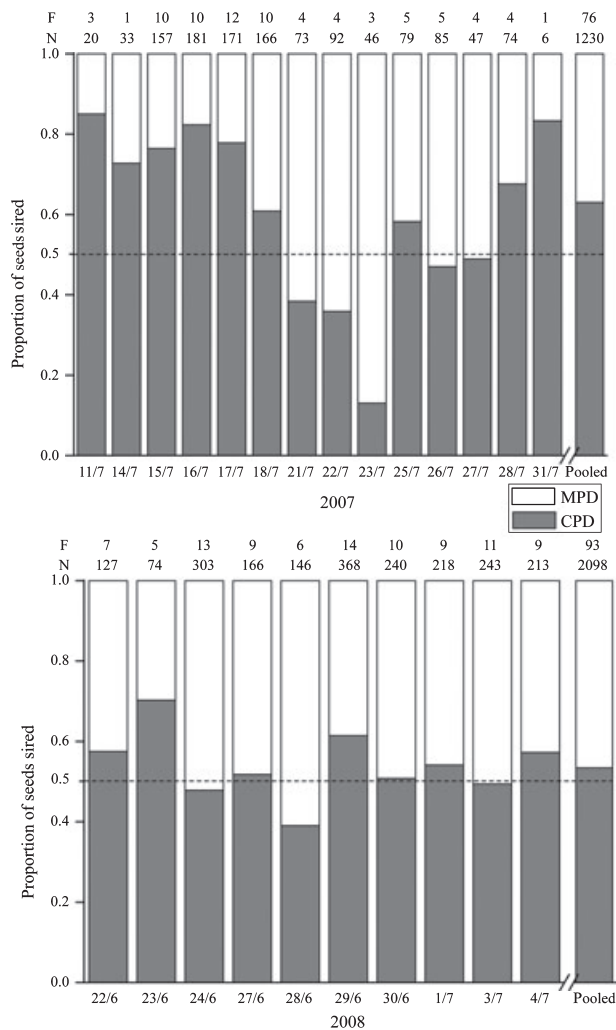


Fig. 3 Proportion of seeds sired by control vs. manipulated pollen donors in arrays of 2007 and 2008. Only heterozygous FS seeds at shikimate dehydrogenase in 2007 and 2008 were assigned paternity using the genotypes at aspartate amino transferase. CPD, control pollen donor; MPD, manipulated pollen donor. F, number of fruits; N, numbers of seeds scored.

experimental arrays affect the strength of pollen competition. For example, Kohn & Barrett (1992) tested the pollen-stigma interference hypothesis in the tristylous *Eichhornia paniculata* (Pontederiaceae) by measuring the ability of the long-styled morph (L) with their styles intact or excised to sire seeds on the mid-styled morph (M). They demonstrated that removal of the style had no effect on the siring success of the L morph on the M morph. However, their 6×8 experimental arrays contained equal numbers of plants of all three morphs, which might limit the strength of pollen competition. In andromonoecious *Solanum carolinense* (Solanaceae), Quesada-Aguilar *et al.* (2008) showed an effect of style length on both pollen deposition and removal when

Table 1 Generalized linear mixed model (GLMM) analysis of the proportion of seeds sired by control vs. manipulated donor plants in 2007 and 2008. The response variable is binary (1 = control, 0 = manipulated) and is assumed to follow a binomial distribution. In the GLMM model, the data are fit through a logit link function, so the coefficients correspond to the logit of the proportion of control donor paternity. Only in 2007 was there a significant effect of block.

	Estimate	SE	z-value	P-value (two-tailed)
Year = 2007; N = 1230				
Fixed effects				
Block 1 (days 1–6)	1.44	0.220	6.54	< 0.00001
Block 2 (days 7–14)	–0.227	0.230	–0.986	0.324
Random effects				
Fruit (n = 76)	0.874			
Day (n = 14)	0.0834			
Year = 2008; N = 2098				
Fixed effects				
Intercept	0.117	0.138	0.849	0.396
Random effects				
Fruit (n = 93)	1.50			
Day (n = 10)	0.000			

flowers were exposed to pollinators foraging among natural donors in the field. However, Vallejo-Marin & Rausher (2007) found that pistil reduction did not increase siring success in *S. carolinense* when using the polygonal grid array consisting of eight pollen recipients, seven pollen donors with perfect flowers and seven pollen donors whose pistils were removed. We suggest that studies on pollen transfer success consider multiple experimental designs that might vary the intensity of pollen competition.

Our results show that stigma movement affects the success of male function. This is consistent with our direct observations; on manipulated plants, the stigma below the anther can remove pollen before pollinators leave the flower. In a previous study (Fetscher, 2001), the bilobed stigma closure of *Mimulus aurantiacus* (Phrymaceae) approximately doubled pollen donation to stigmas of subsequently visited flowers. In contrast to male function, female function appears to be affected weakly by stigma movement (Sun *et al.*, 2007). The upward style curvature in both morphs is unlikely to be an anti-selfing mechanism, because the stigmas when held in an upward position either are unreceptive (cataflexistylous morph) or have already received cross-pollen (anaflexistylous morph). Thus, stigma movement appears to have asymmetrical effects, improving male function whereas having little effect on female function.

As a result of the variability in pollinator visitations and pollen transfer, demonstrating an effect of stigma movement on paternal success is difficult. Our experiment was large, with paternity assessed for 1230 seeds

from 76 flowers in 2007 and for 2098 seeds from 93 flowers in 2008. Even though the results showed large magnitudes in the effects of the manipulation, with 0.647 and 0.529 of seeds sired by control plants in 2007 and 2008, respectively, the statistical results showed significant departures from the null expectation in the $0.01 < P < 0.05$ range. The difficulty of showing a potentially strong force of natural selection in the wild is not surprising, given that traits conferring even relatively small advantages may nonetheless be strongly selected for over many generations. The magnitude of the effects we observed, particularly in 2007, suggest a potentially strong selective advantage of stigma movement. Thus, we have provided the first clear indication that the remarkable combination of herkogamy and style movement in flexistylous species may be a response to selection to avoid the interference by stigmas of pollen dispersal strategies.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Two floral morphs of *Alpinia mutica*.

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