

Frequency-Dependent Pollen Discounting Contributes to Maintenance of a Mixed Mating System in the Common Morning Glory *Ipomoea purpurea*

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ABSTRACT: Pollen discounting, a reduction in outcross success associated with increased selfing, was evaluated in the common morning glory *Ipomoea purpurea*. A field experiment was conducted to estimate selfing rates and outcross success using small arrays of plants with large or small anther-stigma distance (ASD). To evaluate the effect of genotypic composition on the mating-system parameters, arrays were composed of five different frequencies of small- and large-ASD genotypes. While the selfing rates of genotypes with small ASD were consistently higher than genotypes with large ASD regardless of the genotypic frequency, outcross success was negatively frequency dependent. The genotype that was at lower frequency in the array had higher outcrossing success in three out of the four array types with unequal frequencies. This advantage-when-rare phenomenon can contribute to preventing the fixation of either extreme ASD-morph and maintaining a mixed mating system in *I. purpurea*.

Keywords: pollen discounting, herkogamy, mating system, frequency-dependent selection, *Ipomoea purpurea*.

The ability of some plants to self-fertilize represents a potential selective advantage compared to individuals that can only cross-fertilize (Fisher 1941). This potential advantage can be understood by realizing that there are three pathways through which a hermaphroditic genotype can transmit its genes to the next generation: (1) through ovules; (2) through pollen used for self-fertilization (self pollen); and (3) through pollen used for outcrossing (outcross pollen). By contrast, an obligatory outcrossing genotype can transmit genes only through

pathways 1 and 3. Consequently, as long as the hermaphroditic genotype uses these two pathways as efficiently as the outcrossing genotype, the additional pathway (2) means that the hermaphrodite will transmit more copies of its mating-system-specifying genes than will the outcrossing genotype. Hence, in the absence of other factors, alleles that promote self-fertilization in plants should, in theory, spread through a population and become fixed (Fisher 1941; Lloyd 1979; Charlesworth 1980; Holsinger et al. 1984).

Other factors are unlikely to be absent, however. In particular, theoretical investigations have suggested two mechanisms that could oppose the transmission advantage associated with increased selfing and either completely prevent the evolution of selfing or, in some cases, maintain a stable mixed mating system (Maynard Smith 1977; Lloyd 1979; Charlesworth 1980; Feldman and Christiansen 1984; Holsinger et al. 1984; Lande and Schemske 1985; Campbell 1986; Charlesworth and Charlesworth 1987; Holsinger 1988; Charlesworth et al. 1990; Holsinger 1991a; Uyenoyama and Waller 1991a, 1991b, 1991c; Damgaard 1996). One of these mechanisms is inbreeding depression, a reduction in the fitness of offspring produced by inbreeding. The phenomenon of inbreeding depression has long been studied in hermaphroditic plants (e.g., Darwin 1876; Schoen 1983; Mitchell-Olds and Waller 1985; Schemske and Lande 1985; Charlesworth and Charlesworth 1987; Dudash 1990; Holtsford and Ellstrand 1990; Ågren and Schemske 1993; Carr et al. 1997; and many more), and generally appears to be common, thus having the potential to explain why many species remain completely outcrossing. By contrast, the second mechanism, pollen discounting, has not received equivalent attention as a mechanism for inhibiting the evolution of selfing.

Pollen discounting occurs when an increase in the selfing rate occurs at the expense of siring success through outcross pollen, that is, when there is a negative correlation between success using pathway 2 and success

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using pathway 3 (Holsinger et al. 1984). Theoretical investigations suggest that if this trade-off is strong enough, pollen discounting can completely offset the transmission advantage of selfing and prevent the evolution of selfing (Nagyaki 1976; Holsinger 1991*b*). Weaker pollen discounting, in conjunction with inbreeding depression, can also act to maintain a mixed mating system, often manifested as genetic variation for tendency to self (Uyenoyama and Waller 1991*a*; Damgaard 1996). Despite this potentially important role of pollen discounting in shaping plant mating systems, only a few empirical studies have attempted to assess even whether pollen discounting occurs in hermaphroditic species (Ritland 1991; Holsinger 1992; Rausher et al. 1993; Holsinger and Thomson 1994; Kohn and Barrett 1994; Harder and Barrett 1995). While about half of these studies have revealed the occurrence of pollen discounting, only three of them (Ritland 1991; Rausher et al. 1993; Kohn and Barrett 1994) examined the specific issue of whether genetic variants affecting selfing exhibited the type of trade-off associated with pollen discounting. In only one case (Kohn and Barrett 1994) could such a trade-off be detected to be context dependent. Current empirical evidence is thus equivocal regarding whether pollen discounting frequently influences the evolution of plant mating systems.

In attempting to explain the maintenance of mixed mating systems, theoretical investigations have largely ignored the possibility that pollen discounting may be frequency dependent. Nevertheless, substantial indirect evidence indicates that this possibility should be considered seriously. Several reports indicate that changes in selfing rates and success through outcross pollen (“outcross success”) may accompany changes in population composition (Thomson and Barrett 1981; Schoen and Clegg 1985; Epperson and Clegg 1987*b*; Holsinger 1992; Holtsford and Ellstrand 1992; Rausher et al. 1993; Kohn and Barrett 1994; Harder and Barrett 1995). For example, even though pollen discounting was not detected, a few studies found that whether white and pigmented flowers differ in selfing rate and outcross pollen success in *Ipomoea purpurea* depends on the relative frequencies of these two morphs (Schoen and Clegg 1985; Epperson and Clegg 1987*b*; Rausher et al. 1993). Other examples demonstrate that the morphology of conspecific individuals in a population can influence the relative attractiveness to the pollinators (Stanton et al. 1986) or the probability that outcross pollen may land on a receptive stigma and germinate (Thomson and Barrett 1981), suggesting that in some cases an individual’s relative outcross success may depend on the relative frequencies of similar and dissimilar genotypes in the population. The frequency dependence of outcross success in di- and tri-stylous species

(Barrett et al. 1987) represents an extreme manifestation of this phenomenon. Even though evidence is lacking that in hermaphroditic species with continuous anther-stigma separation such a frequency-dependent pattern of outcross success occurs, it would not be surprising if the magnitude of their correlation—the magnitude of pollen discounting—were also frequency dependent, since outcrossing success and selfing rate each may be frequency dependent individually. Although negative frequency dependence (an increase in pollen discounting with increasing frequency of selfing genotypes) might be expected to stabilize a mixed mating system, we are aware of only one attempt to determine whether the magnitude of pollen discounting changes with the relative frequencies of selfing and outcrossing genotypes in a population (Kohn and Barrett 1994).

As suggested by Ritland (1991), species that exhibit easily quantifiable genetic variation affecting selfing rates constitute excellent systems in which to examine the role of pollen discounting in mating-system evolution. One such species is the annual morning glory *I. purpurea*. In this species, evidence suggests that genetic variation in the distance separating anthers and stigmas (anther-stigma distance, or ASD) may cause variation in the relative amount of selfing and outcrossing that genotypes exhibit. In particular, greenhouse experiments with this species have revealed that ASD within a flower is negatively genetically correlated with autogamous seedset, that is, seed production without pollinators (Ennos 1981; Epperson and Clegg 1987*a*). This character also commonly exhibits negative phenotypic correlations with selfing rates under field conditions in other species (Holtsford and Ellstrand 1992; Belaousoff and Shore 1995; Carr et al. 1997; Karron et al. 1997). Moreover, some studies on morning glories and other species suggest that this correlation is due to an increased deposition of self pollen on stigmas in flowers with reduced ASD. Such a mechanism could reduce the outcross success of small-ASD genotypes, and thus lead to pollen discounting, because less pollen is available to be transported to other plants by pollinators. The available evidence thus suggests that pollen discounting is likely to occur in *I. purpurea* and may contribute to negating the transmission advantage associated with selfing in small-ASD plants.

The primary objective of this investigation was to determine whether pollen discounting might contribute to maintenance of a mixed mating system in *I. purpurea*. As a prelude, we first confirm that ASD is genetically variable in this species and demonstrate that genotypes differing in ASD also differ in selfing rate under field conditions. We then show that both positive and negative pollen discounting (greater outcross success for geno-

types that self more) occur, depending on the frequency of ASD genotypes; this frequency dependence is of the negative type that would contribute to stabilizing a mixed mating system.

Material and Methods

Natural history of Ipomoea purpurea

Ipomoea purpurea is an annual, weedy species that grows throughout the southeastern United States in disturbed areas, such as gardens, roadsides, and abandoned or poorly weeded crop fields. Plants germinate between late May and early August and flowering typically commences about 6 wk after germination. In our study area, virtually all outcross pollination is accomplished by bumblebees. Capsules mature 4–6 wk after pollination and bear up to six seeds each. Because mature capsules remain unopened for several days, frequent seed collection ensures that all seeds produced can be collected.

The average outcrossing rate for this species has been estimated to be about 65%–70% in natural populations (Ennos 1981; Brown and Clegg 1984). The outcrossing rates for flower-color phenotypes within a population range from 48% for white flowers to 86% for dark pink flowers in a natural population (Brown and Clegg 1984). For experimental populations, the rate typically ranges between 50% and 90%. Other aspects of the natural history of this species are described elsewhere (Simms and Rausher 1989; Rausher et al. 1993).

Experiment 1: Artificial Selection on ASD

The purpose of this experiment was to determine whether ASD is genetically variable in our source population and to produce seeds for plants of extreme ASD for use in subsequent experiments. Two hundred seeds collected from a natural population (the source population) were used as the parental generation for this experiment. This source population grew on a tobacco farm in Durham County, N.C. These seeds (parental generation) were planted in a greenhouse in the spring of 1992 and anther-stigma distance was measured on five flowers of each of the 198 plants that germinated. For these measurements, we defined ASD to be the distance between the tallest anther and the stigma surface (fig. 1). Anther-stigma distance was considered positive if the stigma was above the tallest anther and negative if the stigma was below the tallest anther.

From these plants, one large-ASD selection line and one small-ASD selection line were initiated. For the large-ASD line, the 20 plants with largest ASD were selected and crossed among themselves to form the next generation (F_1 generation) of seeds. Similarly, for the

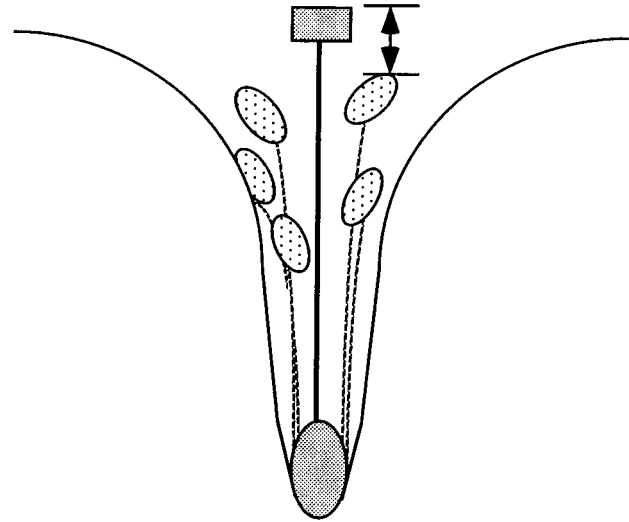


Figure 1: An illustration of anther-stigma distance (ASD). The two-way arrow indicates a positive ASD.

small-ASD line, 20 plants with the lowest ASD were selected and crossed among themselves to generate the next generation of seeds.

A total of 180 crosses were conducted (90 within both the large-ASD and the small-ASD line) to create the F_1 seeds. One seed from each cross was planted in the greenhouse, and ASD was measured on each of at least five flowers and averaged to yield a mean ASD for each plant. The significance of the response to selection was evaluated by performing an ANOVA comparing mean ASD for plants of the two selection lines. Realized heritability of ASD was estimated by the standard equation where h^2 is the realized heritability, R is the response to selection, calculated as the difference between the mean ASD of the two lines in the next generation, and s is the selection differential, the difference in mean ASD in the two groups of selected plants (Falconer 1989).

Experiment 2: Estimate of Mating-System Parameters

Field Experiment. This experiment used plants with extreme ASDs and known marker genotypes to estimate selfing rates and outcross pollen success in the field. Using some of the plants selected for the large- and small-ASD lines described above as parents, we generated seeds for the field experiment by crosses performed in a greenhouse. Selected plants of the parental generation were scored for their genotypes at two allozyme marker loci, Esterase-2 (EST) and phospho-glucomutase (PGM). Hand-pollination was performed within each selected line to produce F_1 experimental plants with desired genotypes at these two loci. As a result of these crosses, all ex-

perimental F_1 plants in the small-ASD group were homozygous for the fast allele (FF) at the EST locus, while all plants in the large-ASD group were homozygous for the slow allele (SS). Within each group, roughly half of the experimental plants were homozygous SS at the PGM locus and the rest were FF. This design allowed us to estimate pollen flow not only between the selected lines but also within lines, which is critical in estimating the mating-system parameters (see below). To increase the number of parental plants used, crosses were performed between any pair of individuals having the potential to produce the desired genotypes. Since not all of the selected parental plants had the appropriate genotypes for producing seeds with desired genotypes, 18 and 11 plants were actually used as parental plants for the F_1 seeds for large- and small-ASD groups, respectively. Early in the summer of 1993, seeds produced by these crosses were scarified and planted in 4-inch pots in the greenhouse. Genotypes at the two marker loci were scored for all of the seedlings and only the ones with the desired genotypes were kept.

Experimental trials were initiated in July 1993, when plants in natural populations were flowering. Individual trials lasted 1 d. The evening before a trial, 20 plants were placed out in a field from which all naturally occurring *I. purpurea* within 500 m had been removed. Plants were arranged in a 5×4 grid system with a 1-m interval between neighboring plants. All of the flowers but one from each experimental plant were removed before the experiment to control for flower number. Because white flowers are often undervisited by pollinators in *I. purpurea* and have increased selfing rates, compared to pigmented flowers (Brown and Clegg 1984; Epperson and Clegg 1987b; Rausher et al. 1993; Fry and Rausher 1997), white-flowered plants were not used in this experiment to avoid confounding effects of flower color and ASD on selfing rates.

To examine whether the mating-system parameters (selfing rates and pollen discounting rates) were frequency dependent, arrays representing five different frequencies of the two ASD types were used. The ratios of small-ASD (S) to large-ASD (L) plants in the five treatments were 1S:19L, 4S:16L, 10S:10L, 16S:4L, or 19S:1L. Each of these frequencies was repeated at least six times between July 27 and September 12, 1993. All of these trials were carried out at the Zoology Department field station at Duke University.

Capsules resulting from the experimental trials were labeled and allowed to develop in the greenhouse. After maturation, F_2 seeds from these capsules were collected and were planted in 72-cell seed-germinating trays. Their allozyme genotypes were scored using standard horizontal starch electrophoresis of seedling leaf tissue.

Estimation of the Mating-System Parameters. For each frequency treatment, we used maximum likelihood analysis to estimate two sets of parameters for each of the two ASD classes produced by the selection experiment. One set of parameters involves the selfing rate—the proportion of seeds that a plant produces through self-fertilization. The parameters s_s and s_L represent the selfing rates of the small- and large-ASD classes, respectively.

The second set of parameters is a set of outcross successes, θ_{i-j} , which correspond to the average proportion of outcrossed seeds produced by a plant of ASD-class j that were pollinated by plants of ASD-class i (Rausher et al. 1993). Since the expected probability of being sired by a small-ASD plant differs slightly for small- and large-ASD maternal plants (see table 1), θ_{s-L} and θ_{s-S} were estimated separately. Because seeds not sired by small-ASD plants must have been sired by large-ASD plants, the expected outcross success for the large-ASD plants is $\theta_{L-S} = 1 - \theta_{s-S}$ for seeds produced by small-ASD mothers and $\theta_{L-L} = 1 - \theta_{s-L}$ for seeds produced by large-ASD mothers; θ_{L-S} and θ_{L-L} were not estimated in the model since they were completely constrained by their relationship with θ_{s-S} and θ_{s-L} .

The conditional probabilities, P_{mo} , that maternal plants of genotype-class m produced offspring of genotype-class o ($m \in \{\text{FFFF}, \text{FFSS}, \text{SSFF}, \text{SSSS}\}$, $o \in \{\text{FFFF}, \text{FFFS}, \text{FFSS}, \text{FSFF}, \text{FSFS}, \text{FSSS}, \text{SSFF}, \text{SSFS}, \text{SSSS}\}$), expressed in terms of the s 's and θ 's, are easily calculated based on the numbers of plants of each genotype within each ASD class in a given array (table 1). For example, in trials with n_1 small-ASD FFFF and n_2 small-ASD FFSS plants, an FFFF seed produced by a small-ASD FFFF maternal plant can arise in two ways: (1) through selfing or (2) through outcrossing by pollen from another small-ASD FFFF plant. The probability of path 1 is just s_s . The probability of path 2 is a compound probability, equal to the probability of not selfing ($1 - s_s$) times the probability that the pollen that sires an outcrossed seed is derived from a small-ASD plant (θ_{s-S}) times the probability that a small-ASD plant is FFFF. This last quantity is in turn the number of other small-ASD FFFF plants ($n_1 - 1$) divided by the number of other small-ASD plants ($n_1 + n_2 - 1$). The sum of the probabilities for paths 1 and 2 yields the entry in the upper-left cell of table 1. Probabilities for other cells are computed in similar fashion.

The overall likelihood (L) of observing the data (the F_2 genotype pattern) given the probability model is

$$L = C \prod_{m,o} (P_{mo})^{N_{mo}},$$

where C is a constant that is independent of the parameters of interest and therefore was not evaluated, and N_{mo}

Table 1: The probability (P_{mo}) of observing a particular offspring genotype o for a seed produced by a particular maternal genotype m

Offspring genotypes	Maternal genotypes			
	Small ASD		Large ASD	
	FFFF ($m = 1$), n_1	FFSS ($m = 2$), n_2	SSFF ($m = 3$), n_3	SSSS ($m = 4$), n_4
FFFF	$S_s + (1 - S_s) \times \theta_{s-s} \times (A)$	0	0	0
FFFS	$(1 - S_s) \times \theta_{s-s} \times (B)$	$(1 - S_s) \times \theta_{s-s} \times (C)$	0	0
FFSS	0	$S_s + (1 - S_s) \times \theta_{s-s} \times (D)$	0	0
FSFF	$(1 - S_s) \times (1 - \theta_{s-s}) \times (E)$	0	$(1 - S_L) \times \theta_{s-L} \times (G)$	0
FSFS	$(1 - S_s) \times (1 - \theta_{s-s}) \times (F)$	$(1 - S_s) \times (1 - \theta_{s-s}) \times (E)$	$(1 - S_L) \times \theta_{s-L} \times (H)$	$(1 - S_L) \times \theta_{s-L} \times (G)$
FSSS	0	$(1 - S_s) \times (1 - \theta_{s-s}) \times (F)$	0	$(1 - S_L) \times \theta_{s-L} \times (H)$
SSFF	0	0	$S_L + (1 - S_L) \times (1 - \theta_{s-L}) \times (I)$	0
SSFS	0	0	$(1 - S_L) \times (1 - \theta_{s-L}) \times (J)$	$(1 - S_L) \times (1 - \theta_{s-L}) \times (K)$
SSSS	0	0	0	$S_L + (1 - S_L) \times (1 - \theta_{s-L}) \times (L)$

Note: Parameter S is the probability of selfing and θ is the probability that an outcrossed seed was sired by pollen from small-ASD plants. The subscripts, L and S, represent the large-ASD and small-ASD groups, respectively; θ_{s-s} represents the proportion of the outcross seeds produced by small-ASD maternal plants that were sired by other small-ASD plants; θ_{s-L} represents the proportion of the outcross seeds produced by large-ASD maternal plants that were sired by small-ASD plants; and n_m is the number of maternal plants of genotype m in the array. Letters in parentheses are as follows: $A = 0$ if $n_1 + n_2 = 1$, otherwise $A = (n_1 - 1)/(n_1 + n_2 - 1)$; $B = 0$ if $n_1 + n_2 = 1$, otherwise $B = n_2/(n_1 + n_2 - 1)$; $C = 0$ if $n_1 + n_2 = 1$, otherwise $C = n_1/(n_1 + n_2 - 1)$; $D = 0$ if $n_1 + n_2 = 1$, otherwise $D = (n_2 - 1)/(n_1 + n_2 - 1)$; $E = n_3/(n_3 + n_4)$; $F = n_4/(n_3 + n_4)$; $G = n_1/(n_1 + n_2)$; $H = n_2/(n_1 + n_2)$; $I = 0$ if $n_3 + n_4 = 1$, otherwise $I = (n_3 - 1)/(n_3 + n_4 - 1)$; $J = 0$ if $n_3 + n_4 = 1$, otherwise $J = n_4/(n_3 + n_4 - 1)$; $K = 0$ if $n_3 + n_4 = 1$, otherwise $K = n_3/(n_3 + n_4 - 1)$; and $L = 0$ if $n_3 + n_4 = 1$, otherwise $L = (n_4 - 1)/(n_3 + n_4 - 1)$.

is the number of seeds of genotype-class o produced by maternal plants of genotype-class m . Estimates for the s 's and θ 's were obtained by maximizing the likelihood of the overall model using Mathematica software (Wolfram 1991). Variances of the estimated parameters were obtained from the inverse of an information matrix whose elements were calculated from the second derivatives of the likelihood function with respect to all possible combinations of the parameters. A detailed description of the calculation can be found in Weir (1996, chap. 2).

One possible limitation of this analysis is that it assumes that $_{-}FF$ and $_{-}SS$ genotypes contribute equally, on a per capita basis, to outcrossing within an ASD type. We tested this assumption by including in the likelihood analysis an additional parameter, ϕ , representing the ratio of outcross successes of $_{-}FF$ and $_{-}SS$ individuals. Results of this analysis were virtually identical to the analysis presented here. Specifically, for four of the five array types, ϕ did not differ from the null hypothesis of $\phi = 1$, and estimates of the selfing rates and the θ 's were identical to two decimal places with corresponding estimates from the model without ϕ . Only in the 16S:4L arrays did including ϕ significantly improve the fit of the model. Nevertheless, in these arrays, the estimated value of ϕ was 0.97, a minor deviation from the expected value of 1.0 under the null hypothesis of equal outcross success. Moreover, including ϕ in the analysis for this array type resulted in slight changes to only two of the other parameters: the selfing rates and siring success of small-

ASD plants increased by 0.07 and 0.02, respectively. These results indicate that our assumption that $_{-}FF$ and $_{-}SS$ genotypes contribute equally to outcrossing within an ASD group (i.e., $\phi = 1$) is reasonable. For simplicity, we present here the results of the analysis omitting ϕ .

Hypothesis Testing. For each array type, two null hypotheses about mating-system parameters were tested: (1) large- and small-ASD plants have equal selfing rates, and (2) large- and small-ASD plants have equal outcross success. In other words, the proportions of outcrossed seeds sired by small-ASD plants and large-ASD plants were equal to their frequencies in the arrays. These hypotheses were tested using log-likelihood ratio tests between the original model and a reduced model with restrictions on the parameters derived from the hypotheses being tested. The restrictions derived from hypothesis 1 will be $s_s = s_L$, and from hypothesis 2 will be θ_{s-L} and θ_{s-s} equal to their expected values, which are the proportion of small-ASD plants among the potential pollen parents (see table 2). A significant result for a test is interpreted as a rejection of the null hypotheses.

To determine whether the deviation of the observed θ 's from their expected values was due to the heterogeneity of data among replicates, to the deviation between the pooled data and their expected values, or both, a replicated goodness-of-fit (G) test (Sokal and Rohlf 1981) was performed (appendix). Results from these tests showed that even though there was a high degree of variation be-

Table 2: Estimates of selfing rates (S) and proportion of outcrossed seed sired by the small-ASD morph (θ) of *Ipomoea purpurea* in the field experiment

Array type ($N_S : N_L$) and parameters	Observed (SE)	Test I	Expected θ 's	Test II
1:19				
S_S	.600 (.070)	* a	...	***a
S_L	.328 (.068)		...	
θ_{S-S}	
θ_{S-L}	.276 (.051)		1/19 = .053	
4:16				
S_S	.366 (.067)	**a	...	***b
S_L	.094 (.057)		...	
θ_{S-S}	.267 (.077)		3/19 = .158	
θ_{S-L}	.385 (.045)		4/19 = .211	
10:10				
S_S	.330 (.063)	**a	...	b
S_L	.057 (.049)		...	
θ_{S-S}	.587 (.063)		9/19 = .474	
θ_{S-L}	.545 (.051)		10/19 = .526	
16:4				
S_S	.366 (.053)	**a	...	b
S_L	.111 (.058)		...	
θ_{S-S}	.771 (.038)		15/19 = .789	
θ_{S-L}	.813 (.067)		16/19 = .842	
19:1				
S_S	.220 (.051)	a	...	**a
S_L	.053 (.051)		...	
θ_{S-S}	.898 (.020)		18/19 = .947	
θ_{S-L}	

Note: Subscripts S and L refer to estimates for the small- and large-ASD morphs, respectively. Note that θ_{S-S} and θ_{S-L} represent proportion of the outcrossed seeds sired by small-ASD morph; therefore, the expected values for them are calculated as the proportion of small-ASD plants among potential outcross pollen donors for the two types of plants. Expected θ_{S-S} equals $[(N_S - 1)/N - 1]$ and θ_{S-L} equals $(N_S/N - 1)$. N is the number of plants; Test I is $S_S = S_L$; and Test II is $\theta_{S-S} = \text{expected } \theta_{S-S}$, and $\theta_{S-L} = \text{expected } \theta_{S-L}$.

^a df = 1.

^b df = 2.

* $P < .05$.

** $P < .005$.

*** $P < .001$.

tween replicates, the overall pattern of the observed data still differed significantly from the expected data calculated using formulas in table 1 and the expected values of θ 's. Based on these results, we determined that it is appropriate to use the data pooled from all six replicates for the parameter estimation and for testing null hypothesis 2.

Results

Experiment 1: Artificial Selection on ASD

The mean ASD for the parental generation before selection was 0.35 ± 1.54 mm. The two selected groups of pa-

rental plants used to produce the F_1 large- and small-ASD groups differed in their means by about 3.5 SDs. Specifically, the stigma of plants in the large-ASD group was 3.1 mm above the tallest anther, while in the small-ASD group, the stigma averaged 2.3 mm below the tallest anther (fig. 2), yielding a selection differential of 5.4 mm. In the F_1 progeny, the mean ASD in the upward selection line was 2.19 ± 0.88 mm, while in the downward selection line it was -1.47 ± 0.98 mm (fig. 3). This response to selection was highly significant, as demonstrated by a simple ANOVA ($F = 723.73$, $df = 1, 196$, $P < .001$). The realized heritability of ASD was calculated to be 0.68, in-

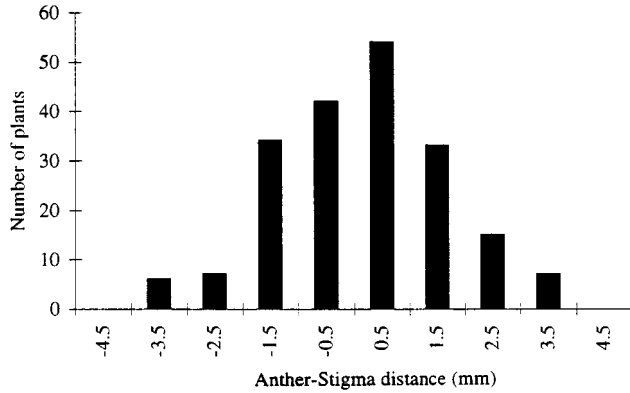


Figure 2: Frequency distribution of anther-stigma distance (ASD) for the parental population. $\bar{X} = 0.35$ mm, $SD = 1.54$ mm, $N = 198$.

dicating substantial genetic variation affecting ASD in the source population. These results also indicate that the two ASD classes in experiment 2 differed genetically for anther-stigma distance.

Experiment 2: Estimation of Mating-System Parameters

Selfing Rates. In each array type, the maximum-likelihood estimate of the selfing rate was larger for the small-ASD plants than for the large-ASD plants. While estimates of selfing rates varied somewhat among the different array types, the difference between selfing rates of the large-ASD and small-ASD groups was independent of the frequency of the two morphs (fig. 4). Differences in selfing rates of the two ASD groups lay within the narrow range of 0.255–0.273, except for arrays in which small-ASD plants were in the highest frequency (19S:1L). In this case the selfing rate difference was slightly lower, about 17%. Although this selfing rate difference was not quite

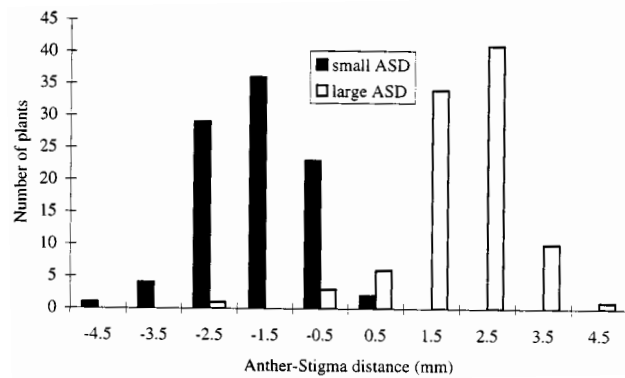


Figure 3: Frequency distribution of anther-stigma distance in the F_1 generation for the two selected lines. Plants in large- and small-ASD groups showed a strong response to one generation of selection on anther-stigma distance.

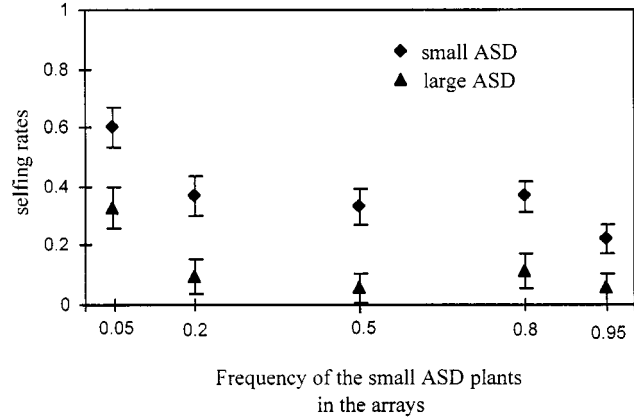


Figure 4: Estimated selfing rates of small- and large-ASD plants in the five array types. Bars around the estimated selfing rates are the standard error associated with the maximum likelihood estimates. The results for the log-likelihood ratio test are listed in table 2.

significant ($\chi^2 = 3.3$, $.05 < P < .1$), the differences were significant in each of the remaining frequency treatments (table 2). Thus, as expected, reduced anther-stigma separation leads to higher selfing rates in the field. There was, however, no evidence that the magnitude of the selfing-rate difference between long- and short-ASD plants depended on the relative frequency of the two types.

Outcross Success. Estimated outcross success exhibited a strong frequency-dependent trend (table 2, fig. 5). Relative outcross success for small-ASD plants was significantly greater than that for large-ASD plants (i.e., θ_{S-L} and θ_{S-S} exceeded their expected values under the null hypothesis that large- and small-ASD plants contribute equally per capita to the outcross pollen pool) when small-ASD plants were in the minority (i.e., arrays with 1S:19L and 4S:16L; table 2). This difference is in the opposite direction of that expected if pollen discounting were occurring and reflects what may be termed “negative” pollen discounting.

This benefit in male reproductive success disappeared when the small-ASD plants were at intermediate frequency in the arrays (10S:10L and 16S:4L). At these frequencies, relative outcross success of small-ASD plants did not differ significantly from expectation. Neither pollen discounting nor negative pollen discounting occurred.

Finally, when small-ASD plants were present at the highest frequency (19S:1L), their relative outcross success dropped significantly below the expected value (table 2). At this frequency, pollen discounting occurred.

This pattern of shifting from negative to positive discounting as the proportion of small-ASD plants in an array increased occurred regardless of whether the mater-

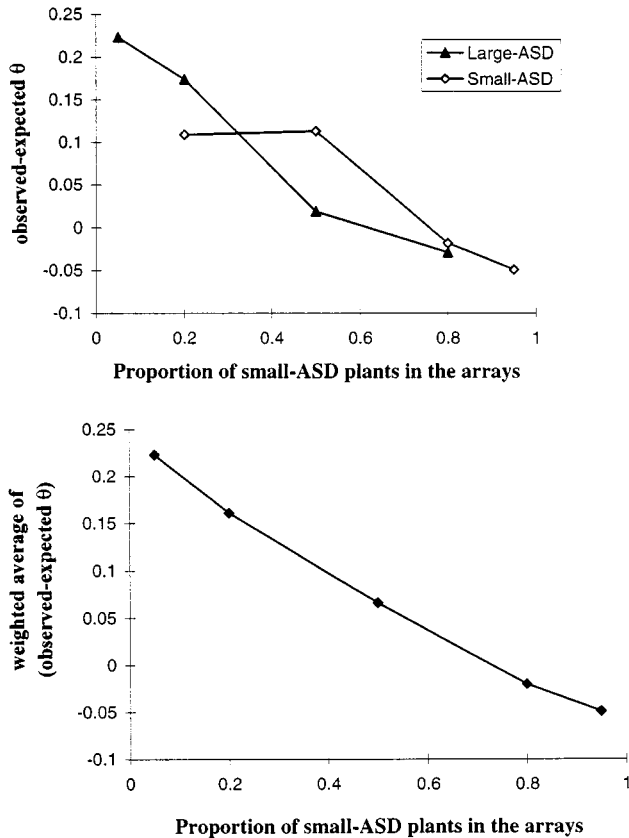


Figure 5: Deviation of siring success of small-ASD plants (θ_{s-}) from expectation. *A*, Lines represent the deviation of θ_{s-} when maternal plants are from large-ASD (θ_{s-L}) or small-ASD (θ_{s-S}) groups. *B*, Weighted-average of the deviation of θ_{s-S} and θ_{s-L} from their expectations, calculated by weighting the values in *A* by the frequency of each ASD group in the array.

nal parent of the outcross seeds was a large-ASD plant or a small-ASD plant (fig. 5A). Moreover, the mean deviation of siring success of small-ASD plants (θ_{s-} , where \bullet = variable ASD) from expectation, calculated as a weighted average of the deviations of θ_{s-L} and θ_{s-S} from their expectations (where the weights are the proportions of large- and small-ASD plants in the array), exhibits an almost linear trend (fig. 5B). The frequency at which relative outcross success of plants in the two ASD groups appears to be equal (i.e., at which θ_s equals its expectation) corresponds to about 70% small-ASD plants in an array. Above this frequency, positive pollen discounting occurs, while below it, negative discounting occurs.

Discussion

ASD and Transmission Bias

Two results from this study are important in establishing that variation in anther-stigma distance is likely to result

in a transmission bias that has the potential to cause evolutionary change in mean ASD, as envisioned generally for selfing traits by Fisher (1941). First, results of the selection experiment revealed that there was substantial genetic variation underlying phenotypic variability for ASD in *Ipomoea purpurea*. This observation is consistent with the earlier findings of Ennos (1981), who estimated a similar heritability (0.52 vs. 0.67 reported here) of ASD in a different *I. purpurea* population using parent-offspring regression and suggests that genetic variation for ASD is widely distributed in *I. purpurea*. These results are complemented by another study that demonstrated that the genetic variation of ASD could still be detected when measured in field conditions (Chang 1997). It thus appears that ASD is capable of evolving if subject to transmission bias or other forms of selection.

The second result, that selfing rate is greater for small- than for large-ASD genotypes, indicates that genes affecting ASD are actually mating-system modifiers. Genotypes producing small ASD exhibited increased levels of selfing at all relative frequencies of the two ASD groups examined. This result confirms the suggestion, based on observations of greenhouse-grown plants in which anther-stigma distance was negatively correlated with autogamous seed production, that ASD variation is likely to affect selfing rates under field conditions where pollinators are present (Ennos 1981; Epperson and Clegg 1987a). Our results are also consistent with investigations of other species (Holtsford and Ellstrand 1992; Motten and Antonovics 1992; Astholm and Nyman 1994; Carr and Fenster 1994; Belaoussoff and Shore 1995; Karron et al. 1997) that suggest that anther-stigma distance can generally be expected to influence selfing rates.

While there is some question as to whether the effect of ASD on selfing rate was significant for the 19S:1L arrays, we note that the trend was similar and almost as great in magnitude as for the other array types, for which selfing-rate differences were highly significant. We thus believe that the marginal significance of this effect in the 19S:1L arrays reflects insufficient statistical power rather than a true lack of affect of ASD on selfing rate in this treatment and conclude that genes that modify ASD in *I. purpurea* influence selfing rates regardless of relative frequency.

Pollen Discounting versus Biased Transmission

As noted previously, theoretical investigations have shown that alleles that promote selfing, such as alleles for small ASD in *I. purpurea*, are expected to become fixed in the absence of forces that counteract the transmission advantage associated with selfing (Fisher 1941). However, this fixation has not occurred in populations of *I. pur-*

purea that have been examined since there still exist alleles producing large ASD. This observation suggests that some mechanism, such as inbreeding depression or pollen discounting, must be operating to prevent small-ASD alleles from going to fixation. In a separate investigation (Chang 1997), we found that inbreeding depression was relatively weak for the source population examined here (the fitness of selfed progeny was approximately 20% lower than that of outcrossed progeny under field conditions). Similarly, Pear (1983) failed to detect evidence of inbreeding depression in a population in Georgia. These results indicate that inbreeding depression is unlikely to be the sole mechanism preventing the fixation of small-ASD plants (Chang 1997) and lead us to consider whether pollen discounting could explain the persistence of the long-ASD alleles.

Despite the consistent effect of the ASD on selfing rates across all frequency treatments, evidence for pollen discounting was found only for arrays in which small-ASD plants were at high frequencies. At lower frequencies, negative discounting was manifested, which would tend to favor an increase in frequency of the small-ASD alleles. Nevertheless, this frequency-dependent pattern of pollen discounting could contribute to maintenance of large-ASD alleles, since it indicates that, as small-ASD alleles approach fixation, their selective advantage due to the transmission bias associated with increased selfing becomes opposed by reduced outcross success.

Simple calculations of the average per capita success at transmitting genes to offspring for the two ASD groups suggest that the magnitude of pollen discounting acting in the 19S:1L arrays is sufficient to counteract the observed selfing advantage in those arrays. For these arrays, the ratio of the fitness of large-ASD genotypes (W_L) to the fitness of the small-ASD genotypes (W_S) is

$$\frac{W_L}{W_S} = \frac{f_s}{(1-f_s)} \times \frac{(1-f_s)(1+S_L) + [f_s(1-S_S) + (1-f_s)(1-S_L)](1-\theta_{SS})}{f_s(1+S_S) + [f_s(1-S_S) + (1-f_s)(1-S_L)]\theta_{SS}},$$

where $f_s = 0.95$ = frequency of small-ASD plants in the array and $f_L = 0.05$ = frequency of large-ASD plants in the array. Inserting estimated values of the parameters (see table 2) yields $W_L/W_S = 1.35$. It thus seems that the magnitude of pollen discounting at these extreme frequencies is more than sufficient to counterbalance the transmission advantage the small-ASD plants enjoy because of greater selfing. Pollen discounting at these frequencies thus seems a plausible explanation for the failure of alleles causing a reduced anther-stigma distance to spread to fixation.

In most models of pollen discounting, a given level of

pollen discounting introduces a critical selfing rate, above which alleles favoring increased selfing will go to fixation, and below which alleles favoring reduced selfing will become fixed (see the review in Uyenoyama et al. 1993; see also Charlesworth 1980; Holsinger et al. 1984; but see Gregorius et al. 1987; Holsinger 1991b). Thus, according to these models, mating systems tend to evolve to the extremes of either complete outcrossing or complete selfing. This all-or-nothing behavior arises because the magnitude of pollen discounting is considered fixed, independent of frequency. By contrast, in our experiment, the magnitude of pollen discounting not only varies with the relative frequency of different ASD genotypes, but, in addition, discounting switches from positive to negative at low frequencies of small-ASD plants. At these frequencies, small-ASD genotypes have a greater outcross success than large-ASD genotypes, reinforcing the transmission advantage associated with increased selfing. Consequently, at low frequencies of small ASD, both pollen discounting and a transmission “dis-advantage” apparently act to prevent large-ASD alleles from spreading to fixation. The frequency dependence of whether positive or negative pollen discounting occurs thus likely contributes to maintenance of a mixed mating system in *I. purpurea*.

These conclusions may depend to some extent on the genetic model used to calculate total transmission success of different ASD genotypes. In the above calculations, we have treated ASD as if it were essentially dichotomous, using extreme genotypes produced by artificial selection. However, ASD is a typical continuous, normally distributed character (fig. 2) and is likely under polygenic control. We conjecture that, if the magnitudes of selfing and pollen discounting are approximately linearly related to ASD, our basic conclusion that an intermediate selfing rate can be maintained by frequency-dependent pollen discounting will remain valid even for polygenic control of ASD. The validity of this conjecture must await more detailed modeling of the interaction between selfing and pollen discounting with polygenic controlled mating-system modifiers, as well as empirical determination of the degree of selfing and magnitude of pollen discounting in genotypes with intermediate ASD.

The Mechanism of Pollen Discounting

Pollen discounting is traditionally viewed as arising because pollen used for self-fertilization is not available for outcrossing. In this experiment, plants with small ASD may have higher probabilities of depositing self pollen simply because of the small, or sometimes nonexistent, distance between anthers and stigma. The increased selfing diverts more pollen away from that available for

outcrossing, thus reducing outcross success (e.g., cleistogamous flowers; see Waller 1979). However, it is difficult to explain the pollen discounting observed in our experiment in this way. In particular, if small-ASD plants are less successful outcrossers because they deposit more of their pollen on their own stigmas, we see no reason to believe this would not occur at all relative frequencies of small- and large-ASD plants. Yet discounting did not occur at intermediate or low frequencies of small-ASD plants, despite the fact that the magnitude of the difference in selfing rates between ASD classes was constant across frequency treatments.

An alternative explanation of the difference in selfing rates observed could be due to the difference in the order of presentation for anther and stigma in the two groups of plants (Webb and Lloyd 1986). Webb and Lloyd (1986) suggested that anthers and stigma that are located closely to each other would also be presented to the pollinators at once. This simultaneous presentation of the pollen-receiving and pollen-donating surfaces may result in interference between the processes of receiving foreign pollen from and donating pollen to the pollinators. In contrast, plants with large ASD may have the benefit of avoiding such self-interference (Webb and Lloyd 1986) and may thus be able to both receive more foreign pollen and donate more pollen to pollinators for outcrossing. Since the order of the presentation of anthers and stigma cannot be separated from the absolute ASD in this experiment, it is not clear which of the two factors influenced the selfing rates of *I. purpurea*. Nevertheless, the consideration of possible interaction between the location of anthers and stigma in the flower and their pollinators suggests several plausible explanations for the observed pattern of pollen discounting.

One mechanism that may have produced this pattern involves the effects of anther position on outcross success. The role of position effects in creating frequency-dependent outcrossing success has been well documented for heterostylous plants (Barrett et al. 1987; Kohn and Barrett 1994). However, because this mechanism depends on stigmas, as well as anthers, being at one of two or three discretely different heights in different genotypes, it is unlikely to apply to *I. purpurea*. Nevertheless, we can imagine interactions between floral morphology and pollinator behavior that could produce a similar effect. For example, we can imagine that the relative height of the tallest anthers might influence the path a bee takes when entering a flower to collect nectar and, thus, what parts of its body are dusted with pollen from anthers of different heights. If, under these circumstances, when large-ASD plants are in the majority and are thus the most common type encountered by a bumblebee, bees become conditioned to enter a flower in a way that causes pollen

placed by short anthers to be preferentially deposited on subsequent stigmas, small-ASD plants will enjoy an outcross advantage. If, by contrast, when small-ASD plants are in the majority, bees become conditioned to entering via a different path that causes pollen placed by long anthers to be preferentially deposited on subsequent stigmas, large-ASD plants will then enjoy an outcross advantage and relative outcross success will be frequency dependent.

An even simpler possible mechanism for producing the observed frequency dependence in relative outcross success would be saturation of the pollen-carrying capacity of pollinators. This mechanism could operate if pollen were placed on different parts of a bumblebee by anthers of different height. When long-anthered (small-ASD) plants are rare and short-anthered (large-ASD) plants are common, the position of pollen deposition on bees' bodies would be saturated for short-anthered plants, and visits to these plants would result in very little pollen pickup by the pollinator. By contrast, the position of deposition would not be saturated for the long-anthered plants, and visits to these plants would pick up most pollen. The long-anthered plants would thus effectively get more of their pollen onto the pollinator than the short-anthered plants, resulting in a greater per capita outcross success by long-anthered plants. The opposite would occur, for the same reason, when short-anthered plants were rare.

While these kinds of scenarios are admittedly speculative, we describe them to demonstrate that apparent pollen discounting—a negative correlation between selfing rate and outcross success among genotypes—may arise via mechanisms that do not involve inherent trade-offs in availability of pollen for selfing versus availability for outcrossing. In the above example, such an inherent trade-off undoubtedly occurs: small-ASD plants self more because more self pollen is deposited on self stigmas and thus less is available for outcrossing. However, the difference between ASD classes in number of pollen grains available for outcrossing is probably a very small fraction of the total number exported and would by itself probably yield pollen discounting of undetectable small magnitude. Instead, this inherent discounting is, we suggest, either overridden or greatly accentuated by characteristics of the pollinator that determine whether tall or short anthers will deposit pollen at locations on the bee's body most conducive to subsequent pollination.

These considerations suggest several generalizations about the evolution of mating systems that bear further investigation. First, in many cases, whether genetic variants that differ in degree of selfing also exhibit pollen discounting will depend on the behavior and morphology of the particular pollinators that visit a plant. Discounting may thus be found in some populations having a certain

suite of pollinators but not in other populations having a different suite. Second, between-population variation in average selfing rate, and in characters responsible for selfing rate (e.g., ASD), may often be produced by variation in pollinator fauna causing spatial variation in whether discounting opposes fixation of alleles that increase selfing. Finally, pollinator behavioral responses to changes in the relative frequencies of different floral morphologies may often generate frequency-dependent pollen discounting that contributes to maintenance of mixed mating systems.

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APPENDIX

Replicated Goodness-of-Fit Tests for Null Hypothesis 2: Large- and Small-ASD Plants Have Equal Outcross Success

Material and Methods

To determine whether the deviation of the observed θ 's from their expected values was due to the heterogeneity of data among replicates, to the deviation between the

pooled data and their expected values, or both, a replicated goodness-of-fit (G) test (Sokal and Rohlf 1981) was performed. Expected values of each offspring genotypes used in these tests were calculated based on the formula in table 1. Expected θ 's based on null hypothesis 2 (see table 1) were used in the calculation. Since there was no a priori expected selfing rates for the plants, the maximum likelihood estimates of selfing rates were used for calculating the expected values. Because values for each possible maternal-offspring genotype combinations were calculated separately, the number of categories for these G tests were nine for 1S:19L and 19S:1L arrays and 16 for the others.

Results

The highly significant G_{total} values (table A1) confirm the deviation of observed θ 's from their expected values. Partitioning the total deviation into a component representing heterogeneity among replicates and one representing deviation of the pooled data from the null expectation revealed that, for all arrays, there was significant among-replicate heterogeneity. Nevertheless, for four of the five array types, the pooled estimates of θ deviated significantly from expectation even when the effects of heterogeneity among replicates were taken into account. Moreover, the one exception (16S:4L array) is the same array that showed no evidence of pollen discounting in the likelihood analysis. This analysis indicates that despite high heterogeneity among replicates, the observed deviations of the θ 's were real, supporting the conclusion that the magnitude of pollen discounting was negatively frequency dependent.

Table A1: Replicated goodness-of-fit tests for the replicates within each frequency array

	1S:19L	4S:16L	10S:10L	16S:4L	19S:1L
G_h	54.84 ⁺ (40)	148.49** (75)	168.98** (75)	139.86** (75)	102.23** (40)
G_p	53.14* (8)	49.30* (15)	26.41* (15)	6.52 (15)	16.38* (8)
G_{total}	107.98** (48)	197.79** (90)	195.39** (90)	146.38** (90)	118.61** (48)

Note: G_h represents the G value for the goodness-of-fit test for the heterogeneity among replicates within a frequency type; G_p represents the G value for the goodness-of-fit test between the pooled data and the expected data (see text for the calculation); $G_{total} = G_h + G_p$. Numbers in parentheses are the df for the G tests.

⁺ .1 < P < .05.
 * P < .05.
 ** P < .001.

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