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Reviewed work(s):

Source: *International Journal of Plant Sciences*, Vol. 174, No. 2 (February 2013), pp. 179-188

Published by: [The University of Chicago Press](#)

Stable URL: <http://www.jstor.org/stable/10.1086/668786>

Accessed: 08/02/2013 19:19

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BREEDING SYSTEM EVOLUTION IN *OXALIS ALPINA*: ASYMMETRICAL EXPRESSION OF TRISTYLOUS INCOMPATIBILITY

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Premise of research. Tristyloous populations of *Oxalis alpina* have three floral morphs (short-, mid-, and long-styled), and populations appear to be at different stages in the evolution of distyly via loss of the mid-styled morph. In tristylous populations transitioning to distyly, asymmetrical incompatibility reactions could affect style-morph frequencies and slow the evolution of distyly. We hypothesized that greater success of pollen from long-styled relative to short-styled morphs leads to an excess of mid-styled progeny from mid-styled morphs and favors retention of tristyly despite other forms of selection acting against the mid-styled morph.

Methodology. We quantified morph ratios of progeny from naturally pollinated plants. We also compared pollen tube and seed production of mid-styled plants following single-source pollinations using pollen from long- versus short-styled pollen parents, and we investigated the proportion of short-styled progeny following pollinations of mid-styled plants with mixtures of pollen from short- and long-styled plants.

Pivotal results. Three lines of evidence generally support the hypothesis that reduced performance of short-styled pollen donors may favor retention of mid-styled plants. (1) Naturally pollinated mid-styled plants exhibited reduced numbers of short-styled progeny for two years in one of two tristylous populations with modified tristylous incompatibility. (2) In populations transitioning toward distyly, mid-styled plants pollinated by short-styled plants had fewer pollen tubes and lower seed production than crosses using long-styled plants as pollen parents. (3) Hand-pollinations of mid-styled plants using mixtures of pollen from short- and long-styled plants resulted in reductions in the proportion of short-styled progeny, consistent with the differences in pollen tube numbers produced by the paternal morphs.

Conclusions. Asymmetry in paternity among different pollen donors via pollen tube performance influences the evolution of the breeding system of *O. alpina*, by affecting the relative proportions of different floral morphs and potentially counteracting selection through inbreeding depression and differential fitness of alleles controlling floral morphs.

Introduction

The majority of flowering-plant species are hermaphroditic, but breeding systems that lead to outcrossing are very common. Symmetric frequency-dependent selection underlies many of these breeding systems and is expected to lead to equal representation of mating types within populations (Charlesworth 1979; Heuch 1979; Barrett et al. 2000). Disruption of frequency-dependent selection may result in breeding-system transitions with profound effects on genetic variation in populations, and it may alter the extent of diversification of lineages (Barrett et al. 1989; Ferrer and Good 2012). Up to half of flowering-plant species exhibit incompatibility systems that prevent self-fertilization but also prevent fertilization between individuals with the same incompatibility reaction (Igić et al. 2008). In many species, incompatibility varies within and among populations (Good-Avila and Stephenson 2003; Goodwillie et al. 2004; Colautti et al. 2010). We studied populations of a heterostylous species to examine the potential for variation in incompatibility among

different floral morphs to influence the evolution of the breeding system.

Heterostylous species are either distylous, where two self-incompatible floral morphs occur in populations, or tristylous, where three morphs are present in a population. In tristylous populations, the three genetically controlled floral morphs are either short-, mid-, or long-styled (fig. 1A). In most heterostylous species, incompatibility reactions prevent seed production following pollinations between anthers and stigmas of different levels from the same or different flowers (illegitimate pollinations; Darwin 1877), whereas pollinations between anthers and stigmas of the same level are capable of producing seeds (legitimate pollinations; fig. 1A). The complementary arrangement of male and female reproductive organs of different floral morphs, termed “reciprocal herkogamy,” promotes outcrossing and may reduce pollen wastage on incompatible stigmas (Darwin 1877) and increase male fitness (Kohn and Barrett 1992; Lloyd and Webb 1992; Baena-Díaz et al. 2012). Self-incompatibility reduces the expression of inbreeding depression and affects the maternal component of fitness (Barrett and Shore 2008). In populations with typical distylous or tristylous incompatibility, symmetric frequency-dependent fitness of style morphs leads to their equal representation in populations (Charlesworth 1979; Heuch 1979).

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Manuscript received July 2012; revised manuscript received September 2012.

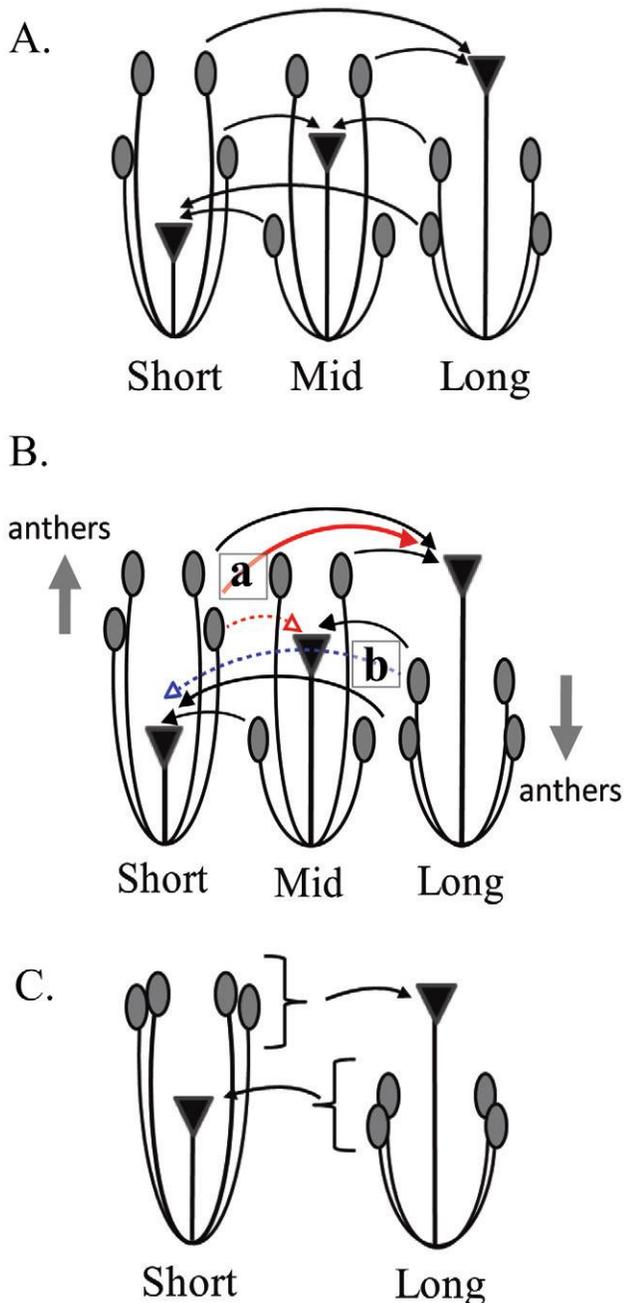


Fig. 1 Three potential stages in the evolution of distyly from tristily in *Oxalis alpina*. Some populations of *O. alpina* may maintain the mid-styled morph at stage B, while others may lose the mid-styled morph (stage C). Ovals represent anthers, triangles represent stigmas, thin arrows represent direction of pollination, and vertical arrows represent changes in anther position (Sosenski et al. 2010). For each description, S, M, and L refer to the short-, mid-, and long-styled morphs, respectively, and s, m, and l refer to the anther whorl position. A, Typical tristylous incompatibility. Only legitimate crosses between stigmas and anthers at the same level produce seeds (arrows); e.g., pollen from mid-level anthers of long-styled plants is normally compatible only with stigmas of mid-styled individuals ($M \times mL$; where M refers to the maternal plant, m refers to the anther whorl, and L refers to the style morph of the paternal plant). The MAR population exhibits typical tristylous incompatibility. B, Modified

tristylous populations of *Oxalis alpina* (Oxalidaceae) appear to be at different stages in the transition to distyly (Weller et al. 2007; Sosenski et al. 2010; Kutaka et al. 2011). In some populations, modifications of tristylous incompatibility characterized by greater success of typically illegitimate pollinations between short- and long-styled plants (e.g., $S \times mL$, $L \times mS$; fig. 1B, 1C; see fig. 1A for notation) may lead to selection against mid-alleles because of lower transmission of alleles controlling the mid-styled morph (genic selection; Weller et al. 2007; Kutaka et al. 2011). Alternatively, Charlesworth (1979), using a modeling approach, suggested that greater expression of inbreeding depression in mid-styled morphs could result in loss of this morph from populations (genotypic selection). In addition to genic selection against mid-alleles and genotypic selection against mid-genotypes, other mechanisms also could influence morph dynamics in tristylous populations. We examine how differences in seed production of crosses between morphs (specifically, seed production of mid-styled plants pollinated by long-styled paternal parents [$M \times mL$] vs. that of those pollinated by short-styled paternal parents [$M \times mS$]) may influence the frequency of the mid-styled morph in populations of *O. alpina*.

The expression of style morphs is controlled by two linked diallelic loci in *O. alpina* and other species of section *Ionoxalis* (Weller 1976). The S allele at the short/nonshort locus (S or s) is dominant and epistatic to the M allele at the mid-/long locus (M or m). Short-styled plants normally have one copy of the dominant S allele (S) at the short/nonshort locus (Weller 1976; S. G. Weller, A. K. Sakai, C. A. Lucas, J. J. Weber, C. A. Domínguez, and F. E. Molina-Freaner, unpublished data); mid-styled plants are homozygous recessive (ss) at the short/nonshort locus and have at least one copy of the M allele at the mid-/long locus; long-styled plants are homozygous recessive (ssmm) at both loci (table 1; Weller 1986). An excess of the mid-styled morph in the progeny of mid-styled maternal parents described by Weller (1986) was largely due to a deficiency of short-styled progeny, presumably because of reduced seed production from short-styled pollen donors. Maternal mid-styled plants will produce short-styled progeny only if they inherit the dominant S allele at the short/nonshort locus from the pollen of a short-styled plant ($M \times mS$ cross). If maternal mid-styled plants receive pollen from a long-styled plant ($M \times mL$ cross)

tristylous incompatibility. The two illegitimate crosses between short- and long-styled plants ($L \times mS$ and $S \times mL$) can produce seeds, and mid-anther whorls have shifted in position. Both CHIR and PA have modified incompatibility, where the $L \times mS$ cross exhibits full fecundity (red arrow a) and the $S \times mL$ cross (blue arrow b) exhibits partial fertility for CHIR and full fecundity for PA. The legitimate $M \times mS$ cross exhibits reduced fecundity (dashed red arrow), while there is no reduction in fecundity for the $M \times mL$ cross (Kutaka et al. 2011). C, Distylous incompatibility. Seed production of the two illegitimate crosses between short- and long-styled plants ($L \times mS$; $S \times mL$) is similar to that of legitimate crosses ($L \times lS$; $S \times sL$). Distyly may be less likely to evolve in populations with decreased seed production of the $M \times mS$ cross, because mid-styled morphs will produce more mid-styled and fewer short-styled offspring. Figure adapted from Kutaka et al. (2011).

Table 1
Genotypes of Tristyloous Floral Morphs in a Tetraploid Population of *Oxalis alpina* (Oxalidaceae)

Style morph	Genotype
Short-styled	$S_ _ _ ; _ _ _ _ _$
Mid-styled	$ssss; M_ _ _$
Long-styled	$ssss; mmmmm$

Note. Expression of style morphs is controlled by two linked loci. The *S* locus controls expression of the short versus non-short-styled phenotype; the *M* locus controls expression of the mid-styled versus long-styled phenotype. An underscore indicates that either form of the allele (*S* or *s*; *M* or *m*) is possible. Short-styled individuals with more than a single *S* allele have not been detected, probably because of strong self-incompatibility (Weller 1976; S. G. Weller, A. K. Sakai, C. A. Lucas, J. J. Weber, C. A. Domínguez, and F. E. Molina-Freaner, unpublished data).

and if the dominant *M* allele at the mid-/long locus is inherited from the maternal parent, the progeny will be mid-styled (table 2; Weller 1986). Pollinations occur with equal frequency among all style morphs in natural populations of *O. alpina* (Weller 1981; Baena-Díaz et al. 2012), indicating that a reduced frequency of short-styled plants in the progeny of mid-styled maternal plants might result from postpollination events, potentially including differential pollen tube growth and fertilization, depending on the paternal style morph. Such differences could affect the evolutionary transition from tristylous to distylous in this species.

We investigated the potential for greater success, via greater numbers of pollen tubes and fertilization, of the $M \times mL$ cross relative to the $M \times mS$ cross in populations of *O. alpina* (Oxalidaceae) that varied in the extent of modification of heterostylous incompatibility. We addressed the following questions: (1) Is there a deficiency of short-styled progeny from naturally pollinated mid-styled maternal plants? If long-styled paternal parents produce more offspring than short-styled paternal parents, we predicted that naturally pollinated mid-styled plants would produce fewer short-styled progeny than expected. (2) Are differences in the success of short- and long-styled paternal parents driven by differences in pollen tube numbers, seed production, or germination? (3) When both short- and long-styled plants pollinate mid-styled plants simultaneously, is there still a deficiency of short-styled progeny? We predicted that when $M \times mS$ and $M \times mL$ pollinations occur simultaneously, differences in pollen tube numbers between these two crosses would result in a deficiency of short-styled progeny from mid-styled maternal plants.

Material and Methods

Study Species and Populations

Oxalis alpina (Rose) Knuth (section *Ionoxalis*: Oxalidaceae) is a perennial species ranging from Guatemala to the southwestern United States (Denton 1973). *Oxalis alpina* is not monophyletic (Gardner et al. 2012), but distylous and tristylous populations are uniformly autotetraploid through-

out the study area in the Sky Islands, a series of isolated mountain ranges in the Sonoran Desert region of Arizona, New Mexico, and northern Mexico (Weller and Denton 1976), and on the basis of molecular markers, these populations are likely to share a common ancestor (Pérez-Alquicira et al. 2010). Our study populations differed in several breeding-system characteristics, including the extent of modification of heterostylous-incompatibility reactions (Weller et al. 2007). The primary pollinators of *O. alpina* are two bee species (*Heterosarus*; Andrenidae); pollinators of *O. alpina* are similar throughout the Sky Islands, and they show no preferences for the floral morphs (Weller 1981; Baena-Díaz et al. 2012).

We chose populations of *O. alpina* that exhibit a range in modification of tristylous incompatibility (tristylous self-incompatibility index [triSI]), variation apparently associated with selection against the mid-styled morph. In highly modified populations, all pollinations between short- and long-styled plants are capable of producing seeds, potentially presenting a selective disadvantage to mid-styled plants. The triSI is a measure of the amount of change in incompatibility, calculated as the ratio of the seed production of illegitimate to that of legitimate pollinations between short- and long-styled plants, where low values indicate typical tristylous incompatibility, i.e., the mean of $[(S \times mL)/(S \times sL)]$ and $[(L \times mS)/(L \times lS)]$ seed production (data from Weller et al. 2007). As illegitimate pollinations between long- and short-styled morphs ($S \times mL$ and $L \times mS$) become more compatible, the triSI approaches 1 (Weller et al. 2007; table 3). Increased modification of tristylous incompatibility may present a disadvantage to the transmission of *M* alleles, which are inherited largely from mid-styled plants. Genic selection against the *M* alleles is consistent with the decrease in relative frequency of mid-styled plants in populations with increased modification of tristylous incompatibility (Weller et al. 2007; table 3; fig. 1B).

Among the three populations used for this study, the Sierra La Mariquita (MAR) population is unmodified, i.e., the population has typical tristylous-incompatibility relationships; seed production from legitimate pollinations is greater than seed production from any other type of pollination (table 3: triSI = 0.12). Populations from the Pinos Altos Mountains (PA) and Chiricahua Mountains (CHIR) are fully modified; seed production from illegitimate pollinations between short- and long-styled morphs (i.e., $S \times mL$

Table 2
Expected Proportions of Short- and Nonshort-Styled Progeny Resulting from Legitimate Crosses to Mid-Styled Plants from Short-Styled or Long-Styled Pollen Donors

Cross	Expected proportion of progeny (%)	
	Short-styled	Nonshort-styled
$M \times mS$	50	50
$M \times mL$	0	100

Note. Greater success of crosses to mid-styled plants from long-styled pollen donors ($M \times mL$) may influence style-morph frequencies because only mid- and long-styled plants (nonshort) will occur in the progeny from the $M \times mL$ cross.

Table 3

Morph Ratios, Tristylosus SI Indexes, Reciprocity Indexes, Outcrossing Levels, and Pollen Production of Three Populations of *Oxalis alpina*

Population	L : M : S (<i>n</i>)	triSI	R_1	Multilocus t_m outcrossing \pm SE (<i>n</i>)		Pollen grains/anther \pm SE (<i>n</i>)	
				2006	2007	mS	mL
MAR (960)	30:36:34 (308)	.12	.72	.84 \pm .04 (11)	.88 \pm .32 (14)	690.4 \pm 10.8 (8)	848.9 \pm 87.4 (10)
PA (971)	41:21:38 (309)	.95	.77	.53 \pm .06 (16)	.68 \pm .08 (26)	285.0 \pm 55.8 (9)	304.4 \pm 64.2 (10)
CHIR (727)	48:25:27 (508)	.92	.79	.80 \pm .10 (13)	.62 \pm .10 (29)	575.0 \pm 48.0 (10)	791.6 \pm 19.99 (10)

Note. Population name (MAR = Sierra La Mariquita; PA = Pinos Altos; CHIR = Chiricahua), population voucher numbers (960 and 971, US, Weller and Sakai; 727, UC), and morph ratios (long-:mid-:short-styled plants) in field populations (*n* = number of plants surveyed) are given. The triSI (tristylosus self-incompatibility index) is a measure of the amount of change in incompatibility, where low values indicate typical tristylosus incompatibility, i.e., the mean of $[(S \times mL)/(S \times sL)]$ and $[(L \times mS)/(L \times lS)]$ seed production (data from Weller et al. 2007). The reciprocity index (R_1) measures changes in morphology of short- and long-styled plants as the mid-styled morph decreases in frequency; as the index approaches 1, the two anther whorls of the short- and long-styled morphs converge with each other and approach the level of the stigma of the opposite morph (Sosenski et al. 2010). Multilocus (t_m) outcrossing levels (*n* = number of maternal families) were determined with progeny grown from seeds of mid-styled maternal parents collected in 2006 and 2007 (J. J. Weber, S. G. Weller, A. K. Sakai, O. V. Tsyusko, T. C. Glenn, C. A. Domínguez, F. Molina-Freaner, et al., unpublished data). Pollen production/anther is the mean number of pollen grains (*n* = number of individuals measured) from the mid-level anther whorl of short-styled (mS) and long-styled (mL) plants. Pollen counts for CHIR were obtained from Weller (1986).

and $L \times mS$ crosses) is equivalent to seed production from legitimate pollinations and is greater than that from any other illegitimate pollination (table 3: triSI = 0.95 for PA, 0.92 for CHIR).

Morph Ratios of Progeny from Naturally Pollinated Mid-Styled Plants

For each population and year, we determined floral morph segregations of progeny from naturally pollinated mid-styled plants (*N* mid-styled plants, *N* capsules collected, *N* flowering progeny, proportion of total progeny that flowered by 2011, respectively: MAR in 2006: 48, 70, 503, 90.3%; PA in 2006: 39, 45, 121, 69.1%; CHIR in 2006: 55, 86, 285, 94.2%; MAR in 2007: 43, 48, 202, 93.6%; PA in 2007: 51, 62, 320, 93.6%; CHIR in 2007: 55, 75, 256, 91.8%). We were usually able to collect only one capsule per mid-styled plant in the field; most maternal families had fewer than 10 flowering progeny. Two populations had several large (>10 progeny) families (MAR in 2006: 20 large families; PA in 2007: 9 large families). Large and small families of mid-styled plants did not differ in the proportion of short-styled progeny (MAR in 2006: $t = 10.15$, $df = 68$, $P = 0.090$; PA in 2007: $t = 6.09$, $df = 60$, $P = 0.142$; data arcsine square-root transformed), and thus we pooled all the progeny by morph within each population each year without respect to family to calculate morph ratios.

We compared the observed morph segregation patterns to the segregation patterns expected on the basis of the genotypes of different morphs (tables 1, 2), adjusting the expected numbers for differences in selfing level and pollen production among morphs. The expected morph ratios of the progeny were adjusted by first accounting for the mean level of selfing of mid-styled plants in 2006 and in 2007 in each population. Selfing levels for the mid-styled morph were obtained using the same parents and progeny examined here (J. J. Weber, S. G. Weller, A. K. Sakai, O. V. Tsyusko, T. C. Glenn, C. A. Domínguez, F. Molina-Freaner, et al., unpublished data; table 3). For each population and year, we subtracted from the to-

tal flowering progeny the number of nonshort progeny that would be result of selfing of the mid-styled morph (selfing in the mid-styled morph will not result in short-styled progeny; table 1). We then adjusted for differences in pollen production in the short- and long-styled morphs, where the expected number of short-styled progeny of mid-styled plants (ExpS) is

$$\text{ExpS} = \frac{(P_S \times \text{Pollen}_{mS}) / [(P_S \times \text{Pollen}_{mS}) + (P_L \times \text{Pollen}_{mL})]}{2},$$

where P_S is the proportion of short-styled plants in the field population, P_L is the proportion of long-styled plants in the field population, and Pollen_{mS} and Pollen_{mL} are pollen production values for the mid-stamen whorls of the short- and long-styled morphs, respectively (per-whorl calculations based on pollen production/anther; table 3). The expected number of short-styled progeny was divided by 2 because only half of the pollen produced by short-styled plants carries the *S* allele. We compared the observed and expected numbers of short and nonshort progeny of naturally pollinated mid-styled plants for each of two years in each of three populations, using χ^2 tests.

Pollen Tube Number, Seed Production, and Seedling Emergence Following Single-Source $M \times mL$ or $M \times mS$ Pollinations

Number of pollen tubes. In a pollinator-free greenhouse, we applied single-source pollen to mid-styled plants to determine the number of pollen tubes, seed production, and percent seedling emergence for $M \times mL$ and $M \times mS$ pollinations in each population. Because preliminary observations indicated that pollen tubes were capable of reaching the base of the style in 24 h, we assessed the mean number of pollen tubes at the base of floral styles from hand-pollinations at 24 h (*N* maternal plants/population; table 4). For each mid-styled maternal plant, pairs of $M \times mL$ and $M \times mS$ pollinations were performed on separate flowers each morning (1–3 long-styled paternal parents and 1–3 short-styled paternal parents for each

Table 4

Population, life stage	Treatment category (<i>N</i>)		Fixed effects				Random effects		
	M × mL	M × mS	Source of variation	df	<i>F</i>	<i>P</i>	Source of variation	χ^2	<i>P</i>
Sierra La Mariquita:									
<i>N</i> pollen tubes ^a	18	18	Treatment	1, 81	.78	.380	Family	16.5	<.0001
							Trmt × fam	.0	1.000
Seeds/pollination	20	19	Treatment	1, 191	.03	.854	Family	24.1	<.0001
							Trmt × fam	.0	1.000
Percent emergence ^b	19	19	Treatment	1, 13	2.49	.139	Family	11.6	<.001
							Trmt × fam	.0	1.000
Pinos Altos Mountains:									
<i>N</i> pollen tubes ^a	19	20	Treatment	1, 35	11.40	.002	Family	.0	1.000
							Trmt × fam	.1	.752
Seeds/pollination	16	16	Treatment	1, 15	24.89	<.001	Family	4.5	.034
							Trmt × fam	12.0	<.001
Percent emergence ^b	10	8	Treatment	1, 9	8.20	.019	Family	5.3	.021
							Trmt × fam	1.4	.237
Chiricahua Mountains:									
<i>N</i> pollen tubes ^a	19	19	Treatment	1, 17	84.92	<.0001	Family	.6	.439
							Trmt × fam	.1	.752
Seeds/pollination	16	16	Treatment	1, 15	28.84	<.0001	Family	3.1	.078
							Trmt × fam	24.8	<.0001
Percent emergence ^b	14	10	Treatment	1, 9	3.07	.113	Family	2.5	.114
							Trmt × fam	.4	.527

Note. *N* = number of maternal families in each pollination (treatment) category; family = maternal family; trmt × fam = treatment category × maternal family interaction. *F*, *P*, and χ^2 are reported from restricted maximum likelihood analyses in PROC MIXED. For all χ^2 comparisons, *df* = 1. We used residual likelihood values comparing a full model (with both random effects) to reduced models and tested against a χ^2 distribution with 1 *df*. Underlining indicates that result is significant at *P* < 0.05.

^a Square-root transformed.

^b Arcsine square-root transformed.

maternal plant). Stigmas from each pollinated flower were collected at 24 h, fixed in 3:1 ethanol–acetic acid, cleared in 10N sodium hydroxide, and stained in 0.1% aniline blue in 0.1N potassium phosphate (Martin 1959). Pollen tubes were counted at the base of styles using a Zeiss Imager A.1 fluorescence microscope.

Seed production. We assessed the seed production of mid-styled maternal plants (*N* maternal families/population; table 4) following single-source crosses (pollinations/population for M × mL: in MAR: 106, in PA: 75, in CHIR: 100; for M × mS: in MAR: 103, in PA: 85, in CHIR: 105). We used fine forceps to transfer pollen; forceps were washed with ethanol between pollinations to prevent pollen contamination. Ripe capsules were collected 14–15 d after pollination, before explosive dehiscence.

Seedling emergence. Percent seedling emergence was compared for progeny from single-source pollinations of M × mL and M × mS seeds (*N* seeds planted/population for M × mL: in MAR: 1806, in PA: 684, in CHIR: 1033; for M × mS: in MAR: 1764, in PA: 208, in CHIR: 319; see table 4 for *N* maternal plants/population). From each maternal plant × paternal plant combination, we planted 10 seeds per pot (in 2-inch pots), and pots were placed haphazardly with respect to each other on benches in the University of California Irvine greenhouses. The proportion of seeds that emerged per pot was calculated after cotyledons appeared.

Statistics. For each population, we compared the number of pollen tubes (square-root transformed), seed production/pollination, and percent emergence (arcsine square-root transformed) between M × mL and M × mS pollinations, using restricted maximum likelihood (PROC MIXED; Littell et al. 1996; SAS 2002–2005). The degrees of freedom for the fixed effects were estimated using the Kenward-Roger correction, which reduces the probability of making a Type I error for small sample sizes and unbalanced designs (Kenward and Roger 1997; SAS 2002–2005). Treatment category (M × mL or M × mS pollination) was considered a fixed effect. Maternal family and interactions between treatment category and maternal family were considered random effects. We used residual likelihood values comparing the full model (with both random effects) to reduced models and tested against a χ^2 distribution with 1 *df* (Littell et al. 1996).

Morph Ratios of Progeny from Mixed Hand-Pollinations of Mid-Styled Plants

For each of our three study populations, mid-styled maternal plants were hand-pollinated with a 50:50 mixture of pollen from long- and short-styled plants (termed “mixed pollinations”; *N* mid-styled plants, *N* mixed pollinations, *N* flowering progeny, respectively: MAR: 23, 488, 3829; PA: 15, 489, 1455; CHIR: 18, 466, 1445). To prepare the 50:50 mixture, we determined the number of pollen grains pro-

duced by anthers from mid-level anther whorls of long- and short-styled plants of the MAR and PA populations ($N = 8-10$ long- and short-styled plants/population; table 3). For each individual, two anthers from the mid-level anther whorl were collected from newly opened flowers, each anther was suspended in 2 mL of lactophenol containing 0.1% aniline blue, and pollen was counted with a hemacytometer. Plant means were calculated from the two anther counts, and population means and standard error were based on the individual plant means. Pollen counts for CHIR were obtained from Weller (1986). On the basis of the mean number of pollen grains per anther, we calculated the number of anthers that would approximate a 50:50 ratio of pollen grains from the midlevel whorls of the long- and short-styled plants for each population. If half of all progeny from the mixed pollinations was sired by short-styled plants, then 25% of the progeny from each maternal plant was expected to be short-styled (i.e., to carry the dominant *S* allele, based on Mendelian segregation patterns; tables 1, 2). For each population, we tested whether the mean percent of short-styled progeny from each maternal mid-styled plant with mixed hand-pollination was significantly different from 25%, using a two-tailed, one-sample *t*-test, where n is the number of maternal families. For this test, we calculated our expected percentage of short-styled progeny (ExpS) from our mixed hand-pollinations, taking into account the differences in pollen tube growth from single-source pollinations, using

$$\text{ExpS} = \frac{[\text{Ptubes}_{\text{mS}} / (\text{Ptubes}_{\text{mS}} + \text{Ptubes}_{\text{mL}})] \times 100}{2},$$

where $\text{Ptubes}_{\text{mS}}$ and $\text{Ptubes}_{\text{mL}}$ represent the number of pollen tubes produced by single-source $M \times mS$ and $M \times mL$ pollinations, respectively. As above, the expected number of short-styled progeny was divided by 2 because only half of the pollen produced by short-styled plants carries the short allele. For each population, we used a two-tailed, one-sample *t*-test (where n is the number of maternal families) to determine whether the mean percentage of short-styled progeny from mixed pollinations was different from the expected percentage of short-styled progeny. Percentage data were arcsine square-root transformed. All figures and standard errors are based on nontransformed data.

Results

Morph Ratios of Progeny from Naturally Pollinated Mid-Styled Plants

Naturally pollinated mid-styled plants produced significantly fewer than expected short-styled progeny in the unmodified MAR population in 2006 ($\chi^2 = 4.39$, $df = 2$, $P = 0.036$ in 2006; fig. 2) and in the modified CHIR population in 2006 and 2007 ($\chi^2 = 7.28$, $df = 2$, $P = 0.007$ in 2006; $\chi^2 = 15.29$, $df = 2$, $P < 0.0001$ in 2007). In contrast, naturally pollinated mid-styled plants produced significantly more short-styled progeny than expected in the modified PA population in 2006 and 2007 ($\chi^2 = 3.92$, $df = 2$, $P = 0.047$ in 2006; $\chi^2 = 10.21$, $df = 2$, $P = 0.001$ in 2007).

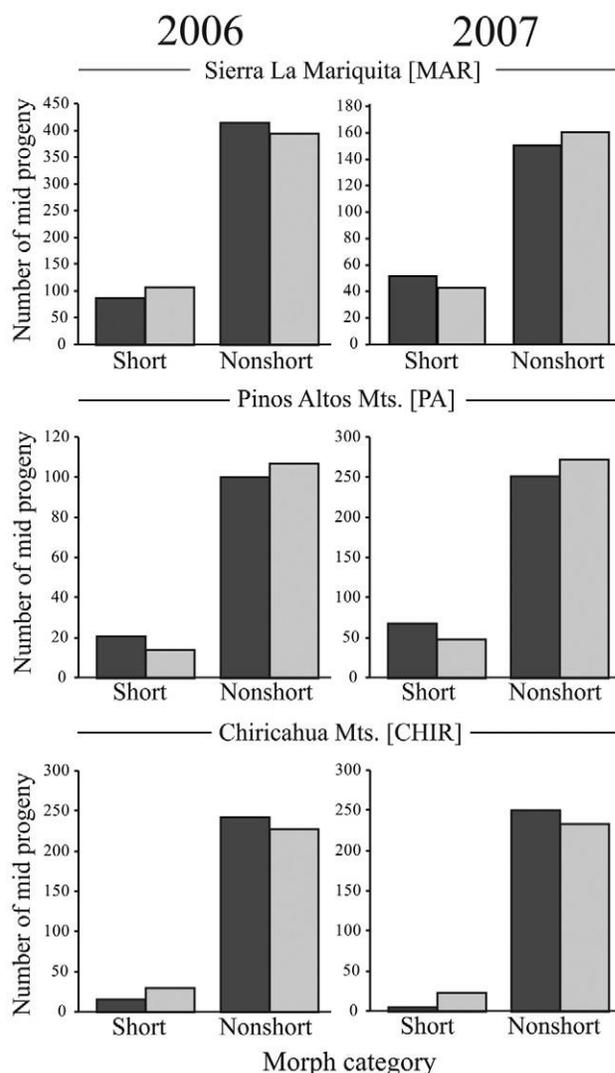


Fig. 2 Observed (dark gray) and expected (light gray) numbers of short and nonshort (mid- and long-styled) progeny from naturally pollinated mid-styled plants in two years in three populations of *Oxalis alpina*. Asterisks indicate significant differences between observed and expected number of short-styled progeny: fewer short-styled progeny were observed than expected in one year for MAR (2006; $\chi^2 = 4.39$, $df = 2$, $P = 0.036$) and in both years for CHIR (2006; $\chi^2 = 7.28$, $df = 2$, $P = 0.007$; 2007: $\chi^2 = 15.29$, $df = 2$, $P < 0.0001$). More short-styled progeny were observed than expected in both years for PA (2006: $\chi^2 = 3.92$, $df = 2$, $P = 0.047$; 2007: $\chi^2 = 10.21$, $df = 2$, $P = 0.001$).

Pollen Tube Numbers, Seed Production, and Seedling Emergence Following Single-Source $M \times mL$ and $M \times mS$ Pollinations

Long-styled pollen donors ($M \times mL$ pollinations) produced more pollen tubes than short-styled pollen donors ($M \times mS$) in the two populations with modified tristylous-incompatibility reactions (PA: $F_{1,35} = 11.40$, $P = 0.002$; CHIR: $F_{1,17} = 84.92$, $P < 0.0001$; fig. 3). CHIR produced 259% and PA produced 71% more pollen tubes from $M \times mL$ pollinations than from $M \times mS$ pollinations. In the unmodified MAR

population, pollen tube numbers did not differ significantly between $M \times mL$ and $M \times mS$ crosses, but pollen tube numbers differed among maternal families ($\chi^2 = 16.50$, $df = 1$, $P < 0.0001$; table 4).

Seed production per pollination was greater for long-styled pollen donors ($M \times mL$) than for short-styled pollen donors ($M \times mS$) in the two modified populations (PA: $F_{1,15} = 24.89$, $P < 0.001$; CHIR: $F_{1,15} = 28.84$, $P < 0.0001$; fig. 3). Seed production following $M \times mL$ and $M \times mS$ pollinations differed among families in PA and CHIR (significant interaction between treatment category and maternal family; PA: $\chi^2 = 12.00$, $df = 1$, $P < 0.001$; CHIR: $\chi^2 = 24.80$, $df = 1$, $P < 0.0001$). Maternal families also differed in seed production in MAR and PA (MAR: $\chi^2 = 24.10$, $df = 1$, $P < 0.0001$; PA: $\chi^2 = 4.50$, $df = 1$, $P = 0.034$). In a few cases, the mean number of seeds per pollination was greater than the mean number of pollen tubes at 24 h for the same cross, although the difference was small and may have occurred because pollen tube and seed production data were based on separate sets of pollinations (for both the $M \times mL$ and $M \times mS$ crosses in MAR and for $M \times mL$ for PA; fig. 3).

In contrast to the results for pollen tubes and seed production, in PA percent emergence was 41.7% greater for the short-styled pollen donor than for the long-styled pollen donor ($M \times mS$ vs. $M \times mL$; $F_{1,9} = 8.20$, $P = 0.019$; fig. 3). In MAR and CHIR, percent emergence did not differ between short- and long-styled pollen (table 4; fig. 3). Percent emergence differed significantly among maternal families in MAR and PA (MAR: $\chi^2 = 11.60$, $df = 1$, $P < 0.001$; PA: $\chi^2 = 5.30$, $df = 1$, $P = 0.021$).

Morph Ratios of Progeny from Mixed Hand-Pollinations of Mid-Styled Plants

Almost all progeny raised from mixed hand-pollinations of mid-styled maternal plants flowered in the greenhouse (95.2%–96.9% of potted plants flowered in each population). In the unmodified MAR population, the observed proportion of short-styled progeny from mixed pollinations (27.7%) was not significantly different from the expectation of 25% ($t = 1.70$, $df = 22$, $P = 0.104$). In the two modified populations, significantly fewer than 25% short-styled progeny were produced from mixed pollinations of mid-styled plants (PA: $t = 3.81$, $df = 14$, $P = 0.002$; CHIR: $t = 7.34$, $df = 17$, $P = 0.0001$; fig. 4). The observed and expected numbers of short-styled progeny differed when the expected proportions were adjusted for the differences in pollen tube numbers between $M \times mS$ and $M \times mL$ hand-pollinations in modified populations (for PA, 18.5% ExpS: $t = 3.82$, $df = 14$, $P = 0.002$; for CHIR, 10.8% ExpS: $t = 2.86$, $df = 17$, $P = 0.011$; and for MAR, 25.4% ExpS: $t = 1.41$, $df = 22$, $P = 0.174$).

Discussion

Nonrandom paternity via differences in the number of pollen tubes among pollen donors not only can affect the siring ability of individuals (Snow and Spira 1991, 1996; Skogmyr and Lankinen 1999; Lankinen et al. 2009) but may also dis-

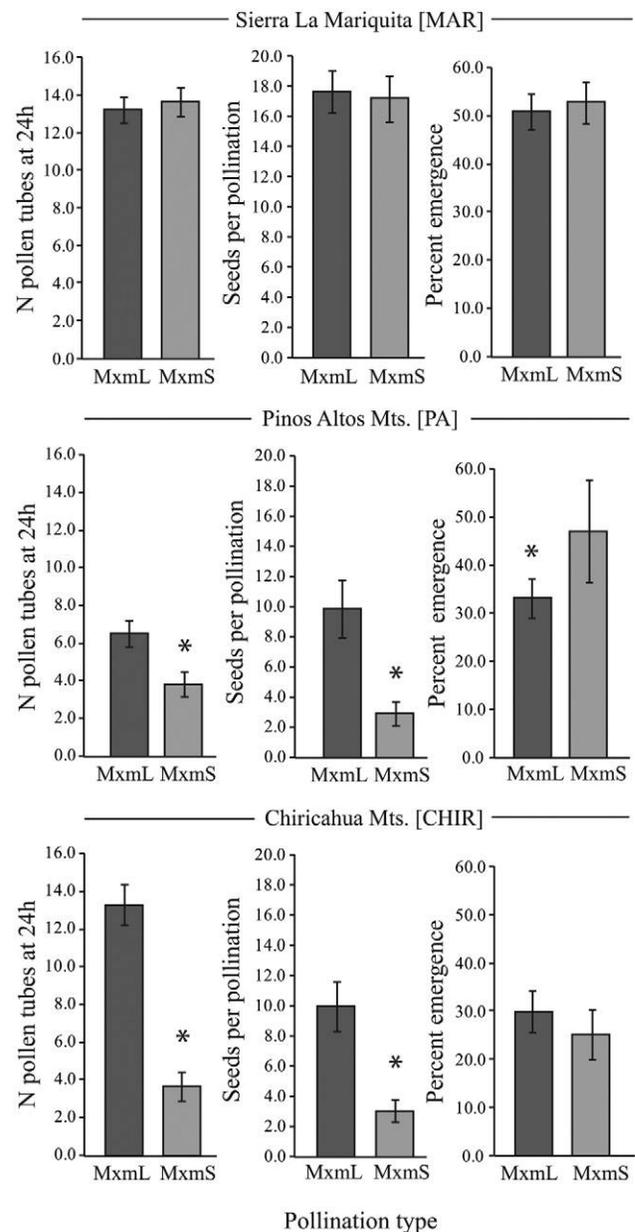


Fig. 3 Mean number of pollen tubes at the base of floral styles at 24 h, number of seeds produced per pollination, and percent emergence from $M \times mL$ (dark gray) and $M \times mS$ (light gray) crosses in three populations of *Oxalis alpina*. A significant treatment effect ($M \times mL$ vs. $M \times mS$) is indicated with an asterisk. In the MAR population, which exhibits typical tristylous incompatibility, treatment effects were not significant. In both PA and CHIR, the long-styled pollen donor ($M \times mL$ crosses) had significantly higher pollen tube and seed production than short-styled pollen donors ($M \times mS$ crosses; $P < 0.002$ for both cases). In PA, percent emergence was greater for progeny from short-styled pollen donors ($M \times mS$) than for that from long-styled pollen donors ($M \times mL$ crosses; $P = 0.019$). Error bars = 1 SE.

rupt frequency-dependent selection and potentially influence morph representation in heterostylous populations, as shown here in *Oxalis alpina*. Differences in pollen tube performance by floral morphs in heterostylous populations may slow the

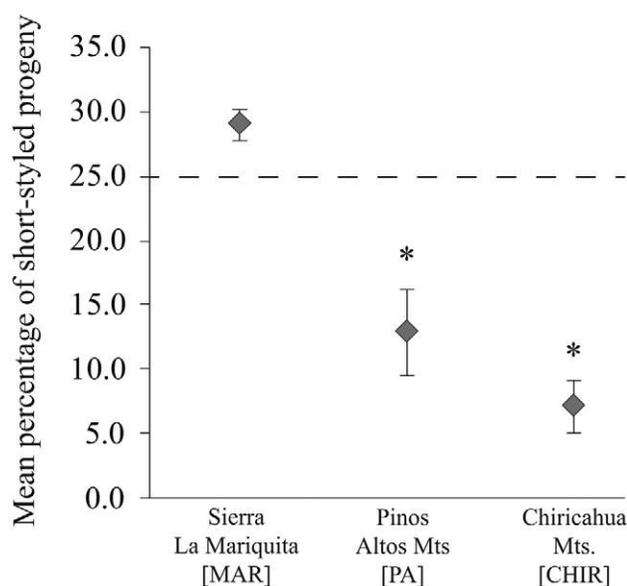


Fig. 4 Mean percentage of total progeny that were short-styled from mid-styled plants hand-pollinated with a 50:50 mixture of short- and long-styled pollen. For MAR, the observed and expected percentages of short-styled progeny were not significantly different (the dashed line represents the expected value of 25%; $t = 1.70$, $df = 22$, $P = 0.104$). For both PA and CHIR, mid-styled families had significantly fewer than expected short-styled progeny, indicated by asterisks (PA: $t = 3.81$, $df = 14$, $P = 0.002$; CHIR: $t = 7.34$, $df = 17$, $P = 0.0001$). Error bars = 1 SE.

evolution of distyly from tristily. In two populations of *O. alpina* that appear to be in transition to distyly, short-styled pollen parents leave fewer offspring than expected relative to long-styled pollen parents when crossed to mid-styled plants. This deficiency was evident in comparisons of single-morph pollinations as well as in mixed pollinations of mid-styled plants and was consistent with the reduced numbers of pollen tubes from the $M \times mS$ cross in mid-styles. Mixtures of pollen on stigmas from multiple pollen donors may also occur in nature, providing the opportunity for competition among pollen grains and nonrandom paternal success (Marshall 1991; Mitchell and Marshall 1998; Kruszewski and Galloway 2006). We did not, however, find evidence of interactions between pollen grains from short- and long-styled parents when they were combined on mid-styled stigmas. Differences in the number of pollen tubes for single-source $M \times mS$ and $M \times mL$ crosses accounted for the differences in siring ability of short- and long-styled parents in both single-source and mixed pollinations.

In populations of *O. alpina*, both genic selection against *M* alleles (Weller et al. 2007) and genotypic selection against mid-styled progeny (Charlesworth 1979; J. J. Weber, S. G. Weller, A. K. Sakai, O. V. Tsyusko, T. C. Glenn, C. A. Domínguez, F. Molina-Freaner, et al., unpublished data) may facilitate the transition to distyly. In populations with modified incompatibility, seed production from legitimate pollinations is similar to that from the two illegitimate pollinations between short- and long-styled morphs (i.e., $S \times mL$ and $L \times mS$; fig. 1B). With a loss in incompatibility differentiation in

the anthers of the mid-whorl of short- and long-styled morphs, two whorls of pollen from a long-styled morph can fertilize short-styled stigmas ($S \times mL$ and $S \times sL$), while only a single anther whorl of the mid-styled morph can fertilize short-styled stigmas ($S \times mS$; fig. 1B). Short-styled morphs have a similar advantage over mid-styled morphs as pollen parents in crosses to long-styled morphs (fig. 1B). Pollen grains of short- and long-styled plants carry fewer *M* alleles than pollen grains of mid-styled plants, and thus the increased transmission advantage for alleles of short- and long-styled plants should select against *M* alleles (Weller et al. 2007). Reduced frequency of the mid-styled morph in populations of *O. alpina* with greater loss of incompatibility differentiation is consistent with this genic model (Weller et al. 2007).

Selection against mid-styled genotypes may also occur because of relatively greater expression of inbreeding depression in the progeny of selfing mid-styled plants (Charlesworth 1979; J. J. Weber, S. G. Weller, A. K. Sakai, O. V. Tsyusko, T. C. Glenn, C. A. Domínguez, F. Molina-Freaner, et al., unpublished data). Charlesworth (1979) predicted that the close proximity of both anther whorls to stigmas within mid-styled flowers should result in higher levels of self-compatibility for mid-styled plants, compared to that for short- and long-styled plants, in populations of *O. alpina*, a prediction realized in highly modified populations but not in populations with typical tristylous-incompatibility reactions (J. J. Weber, S. G. Weller, A. K. Sakai, O. V. Tsyusko, T. C. Glenn, C. A. Domínguez, F. Molina-Freaner, et al., unpublished data). Selfing levels and expression of inbreeding depression at the population level suggest a fitness cost to selfed progeny of mid-styled plants in modified populations of *O. alpina* (J. J. Weber, S. G. Weller, A. K. Sakai, O. V. Tsyusko, T. C. Glenn, C. A. Domínguez, F. Molina-Freaner, et al., unpublished data).

Asymmetries in seed production following $M \times mS$ and $M \times mL$ crosses, as demonstrated here, may result in the retention of the mid-styled morph and therefore tristily in some populations of *O. alpina*, despite selection against the mid-styled morph (Charlesworth 1979; Weller et al. 2007). As predicted, in populations with increased modification of tristylous incompatibility, mid-styled plants are at a selective disadvantage (Weller et al. 2007; J. J. Weber, S. G. Weller, A. K. Sakai, O. V. Tsyusko, T. C. Glenn, C. A. Domínguez, F. Molina-Freaner, et al., unpublished data; table 3; fig. 1B). The outcome of genic and genotypic selection against mid-styled morphs is complicated, however, by the observation that in populations with modified incompatibility, pollen tubes produced by pollen from short-styled plants do not perform as well in mid-styles as pollen tubes from long-styled plants. This advantage of pollen from long-styled plants may lead to retention of the mid-styled morph, because the $M \times mL$ pollination will result in many mid-styled progeny (table 2). In the highly modified CHIR population, for example, the deficit of short-styled progeny from naturally pollinated mid-styled plants is likely driven by the greater production of offspring from legitimate crosses with long-styled ($M \times mL$) parents than from those with short-styled ($M \times mS$) parents. In nature, mid-styled plants may receive mixtures of legitimate short- and long-styled pollen on stigmas, and differ-

ences in pollen tube numbers between short- and long-styled pollen will result in a deficiency of short-styled progeny in modified populations, as shown in the mixed hand-pollinations (CHIR, PA; fig. 4). The pattern of reduced seed production following $M \times mS$ crosses with modification of tristylous incompatibility in the CHIR population is consistent with patterns found in two other similarly modified populations (the White and Animas populations of Weller et al. 2007). Analysis of the potential role of pollen tube growth of $M \times mS$ compared to $M \times mL$ pollinations in the White and Animas populations would be particularly useful, providing replication to the population results presented here. On the basis of results from CHIR and PA, selection against short-styled pollen donors in mid-styles would increase mid-styled morph representation and could counter loss of the mid-morph due to both genic and genotypic selection. Our result is novel among studies of tristylous species and indicates that a complex interaction of factors influences frequency-dependent selection and may affect style-morph frequencies.

In the unmodified MAR population, a history of self-fertilization for some mid-styled families may have contributed to the significant deficit of short-styled progeny raised from field-collected capsules of mid-styled plants for 2006. Legitimate pollinations of mid-styled plants are unlikely to have influenced morph ratios in MAR, because the $M \times mS$ and $M \times mL$ crosses did not differ in number of pollen tubes, seed production, or percent emergence. In this population, however, some mid-styled families that are self-compatible also show evidence of reduced inbreeding depression (Weber et al. 2012). Families with different histories of inbreeding may evolve within a population when selfing has a genetic basis (Uyenoyama et al. 1993; Stone and Motten 2002), and associations between inbreeding depression and inbreeding history may develop, resulting in purging of recessive deleterious alleles in highly selfing families (Uyenoyama and Waller 1991a, 1991b, 1991c; Takebayashi and Delph 2000). Greater levels of self-fertilization in some mid-styled families, leading to greater numbers of mid-styled progeny, may have contributed to the deficit of short-styled progeny in MAR in 2006 (fig. 2).

For the modified PA population, the excess of short-styled progeny from naturally pollinated mid-styled plants contra-

dicted our expectation of deficits of short-styled progeny of mid-styled maternal plants. The excess of short-styled progeny from naturally pollinated mid-styled plants suggests that in some populations, other factors may override these differences in pollen tube and seed production. In the modified PA population, the $M \times mS$ cross had significantly fewer pollen tubes than the $M \times mL$ cross, while in the modified CHIR population, this difference in pollen tube production was much greater. In the PA population, the lower number of pollen tubes from $M \times mS$ relative to $M \times mL$ crosses may also be compensated for in part by a greater percentage of emergence for plants from the $M \times mS$ cross. In the field, asymmetries between short- and long-styled plants as paternal parents of mid-styled offspring may have to be quite large to be reflected in asymmetries in the morph ratios of offspring.

Asymmetry in paternity among different pollen donors via pollen tube performance may influence the evolution of the breeding system of *O. alpina*, counteracting selection related to both inbreeding depression and differential fitness of alleles associated with expression of reproductive morphs. In populations of *O. alpina* with modified tristylous incompatibility, both genic and genotypic selection may select against the mid-styled morph (Weller et al. 2007; J. J. Weber, S. G. Weller, A. K. Sakai, O. V. Tsyusko, T. C. Glenn, C. A. Domínguez, F. Molina-Freaner, et al., unpublished data). In these modified populations, however, selection against the legitimate $M \times mS$ cross may reduce representation of short-styled morphs and therefore stabilize or increase the frequency of the mid-styled morph in the population. Morph dynamics in *O. alpina* appear to result from a complex interaction of selective forces working at several different levels.

Acknowledgments

We thank W. Yang and R. Basile for plant care; A. Nguyen, M. Nguyen, and M. Ku for greenhouse help; and two anonymous reviewers for helpful comments. Support was provided by NSF grant DEB-0614164 (S.G.W. and A.K.S., co-PIs), a GAANN fellowship and funds to J.J.W., and UC-Irvine Undergraduate Research Opportunity Awards to L.N. and D.K.

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