THE ROLE OF INBREEDING DEPRESSION AND MATING SYSTEM IN THE EVOLUTION OF HETEROSTYLY

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We investigated the role of morph-based differences in the expression of inbreeding depression in loss of the mid-styled morph from populations of tristylous *Oxalis alpina*. The extent of self-compatibility (SC) of reproductive morphs, the degree of self-fertilization, and the magnitude of inbreeding depression were investigated in three populations of *O. alpina* differing in their tristylous incompatibility relationships. All three populations exhibited significant inbreeding depression. In two populations with highly modified tristylous incompatibility, manifested as increased reciprocal compatibility between short- and long-styled morphs, substantial SC and self-fertilization of mid-styled morphs were detected, and expected to result in expression of inbreeding depression in the progeny of mid-styled morphs in the natural populations. In contrast, significant self-fertility of the mid-styled morph was absent from the population with typical tristylous incompatibility, and no self-fertilization could be detected. Although self-fertilization and expression of inbreeding depression should result in selection against the mid-styled morph in the later stages of the transition from tristyly to distyly, in *O. alpina* selection against the mid-styled morph in the early phases of the evolution of distyly is likely due to genic selection against mid-alleles associated with modified tristylous incompatibility, rather than expression of inbreeding depression.

KEY WORDS: Breeding systems, distyly, Oxalidaceae, self-incompatibility, tristyly.

The majority of flowering plants are hermaphroditic (Yampolsky and Yampolsky 1922; Charlesworth 2002), but few hermaphroditic plants exhibit complete self-fertilization (Igic and Kohn 2006). Mechanisms that promote cross-fertilization and reduce the expression of inbreeding depression include spatial separation of male and female reproductive organs (e.g., herkogamy)

and self-incompatibility (SI). Heterostylous species are both self-incompatible and herkogamous. Populations include either two (distyly) or three (tristyly) genetically controlled floral morphs, and are usually characterized by reciprocal herkogamy (Lloyd and Webb 1986) and cross compatibility between anthers and stigmas occurring at the same level in different floral morphs
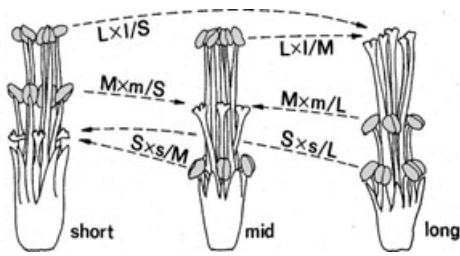
Figure 1. Unmodified heterostylous incompatibility relationships in *Oxalis alpina*. In a typical tristylous species, pollinations leading to fertilization and seed production are those between stigmas and anthers at the same level (e.g., LxlM, where L is the long-styled maternal parent, l is the long-anther whorl of the male parent, and M is the mid-styled morph used as a male parent). These pollinations were termed legitimate by Darwin (1877). Because of tristylous incompatibility, self-pollinations and pollinations between stigmas and anthers at different levels (e.g., MxsM; SxmL; MxlS, termed illegitimate by Darwin 1877), are incompatible and do not normally produce seeds. The arrangement of similar heights of male and female sex organs in the different floral morphs has been termed reciprocal herkogamy (Lloyd and Webb 1986). Diagram from Weller (1976a).

(Fig. 1). Heterostylous breeding systems presumably promote cross-fertilization by reducing self-fertilization via SI (Barrett and Shore 2008) and by increasing transfer of compatible pollen to stigmas (Darwin 1877; Kohn and Barrett 1992; Lloyd and Webb

1992). In heterostylous plants, pollinations between anthers and stigmas of the same level are usually capable of producing seeds and are termed legitimate (Darwin 1877; Fig. 2, panel A), whereas pollinations between anthers and stigmas at different levels often fail to produce seeds and are termed illegitimate (Darwin 1877). Modifications of heterostylous reproductive systems may lead to the evolution of self-compatibility (SC; Kohn et al. 1996), one of the most common breeding system transitions in flowering plants (Ferrer and Good 2012), but transitions that lead to new outcrossing systems are also possible (Weller et al. 2007).

We examined the roles of tristylous incompatibility, mating system, and inbreeding depression in the evolutionary transition from tristyly to distyly in heterostylous *Oxalis alpina* (Oxalidaceae), a species with tristylous populations as well as distylous populations in which the mid-styled morph has been lost (Weller 1976a; Weller et al. 2007). Tristyly co-occurs with distyly in four plant families (Amaryllidaceae, Linaceae, Lythraceae, Oxalidaceae), and distyly is considered to be derived from tristyly in Lythraceae and Oxalidaceae (Weller 1992; Weller et al. 2007; Gardner et al. 2012). In *O. alpina*, phylogeographic studies suggest that transitions to distyly have occurred multiple times (Pérez-Alquicira et al. 2010), and tristylous populations of this species appear to be at different stages in the transition to distyly (Weller et al. 2007; Sosenski et al. 2010; Kutaka et al. 2011).
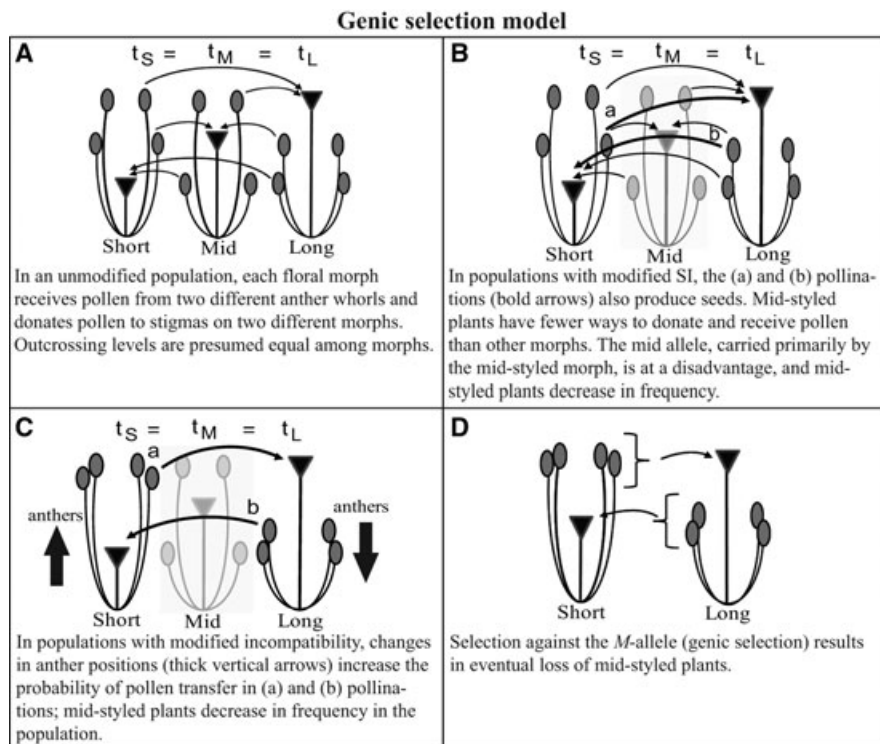
Genic selection model

Figure 2. The genic model for the evolution of distyly from tristyly. Ovals represent anthers, triangles represent stigmas, thin arrows represent directions of pollinations, and vertical arrows represent changes in anther position. Relative outcrossing levels for floral morphs are denoted by: t_S, t_M, and t_L. (a) and (b) refer to the illegitimate LxmS and SxmL pollinations, respectively.

Table 1. Genotypes of tristylous floral morphs in a tetraploid population of *Oxalis alpina* (Oxalidaceae). Expression of style morphs is controlled by two linked loci. The *S*-locus controls expression of the short versus nonshort-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.

Style morph	Genotype
Short-styled	S _ _ _; _ _ _ _
Mid-styled	ssss; M _ _ _
Long-styled	ssss; mmmm

Modifications of the incompatibility system appear to be important initially in the evolutionary transition from tristyly to distyly (Fig. 2, panels A and B; Genic Selection Model; Weller et al. 2007; Kutaka et al. 2011). Although some populations of *O. alpina* have typical tristylous incompatibility relationships where seed production from legitimate pollinations is greater than seed production from all other types of pollinations and mid-styled morphs are common, many populations possess modified tristylous incompatibility, where illegitimate pollinations between short- and long-styled morphs (i.e., SxmL and LxmS crosses) are partially or fully compatible and produce seeds, and mid-styled morphs are usually reduced in frequency (Weller et al. 2007). In modified populations of *O. alpina*, increased reciprocal compatibility between short- and long-styled plants selects against mid-alleles because short- and long-styled morphs carry fewer mid-alleles than mid-styled plants (Table 1). Selection against mid-alleles should reduce the frequency of the mid-styled morph. The reduced mid-styled frequencies in populations of *O. alpina* possessing greater reciprocal compatibility between short- and long-styled morphs are consistent with selection against mid-alleles (Weller et al. 2007). Populations with greater reduction in frequencies of the mid-styled morph also have greater convergence in the position of mid-anther whorls toward the height of the long- or short-styled stigmas (Fig. 2, panel C; Sosenski et al. 2010). These morphological modifications of mid-level stamen positions in short- and long-styled morphs further increase pollen transfer for the illegitimate SxmL and LxmS crosses (Baena-Díaz et al. 2012).

The first changes associated with the evolution of distyly from tristyly appear in the incompatibility system rather than in floral morphology (Fig. 2, panel B). This evolutionary sequence is supported by reciprocal factorial crosses in two populations of *O. alpina* (Kutaka et al. 2011). In a population with apparently typical tristylous incompatibility where average seed production following illegitimate SxmL and LxmS crosses was low and identical to other illegitimate crosses (Weller et al. 2007), a few short- and long-styled plants showed partial compatibility of the SxmL

and LxmS crosses. These initial modifications of incompatibility were not associated with changes in stamen lengths (Kutaka et al. 2011). Although the cause of partial compatibility is unknown, once mutations causing greater compatibility between the short- and long-styled morphs appear in a population, the disadvantage experienced by the mid-allele appears to reduce the frequency of the mid-styled morph (Kutaka et al. 2011). Comparisons with a second population possessing highly modified tristylous incompatibility (SxsL and SxmL crosses have equivalent seed production; LxlS and LxmS crosses have equivalent seed production) indicate that morphological changes evolve after modifications of incompatibility are established in populations. In this population, the two stamen whorls of short- and long-styled individuals have converged toward the height of compatible stigmas (Fig. 2, panels B and C; Kutaka et al. 2011), and individuals with greater modification of incompatibility also had more highly modified stamen positions.

The evolutionary transition from tristyly to distyly may be influenced not only by selection against the mid-allele, but also by differential expression of inbreeding depression among the floral morphs (genotypic selection model; Fig. 3, panels A–D). Charlesworth (1979) suggested that greater expression of inbreeding depression in the progeny of the mid-styled morph, relative to the progeny of short- and long-styled morphs, would lead to the evolution of distyly from tristyly. Proximity of reproductive whorls was expected to be positively related to increased compatibility and illegitimate pollen transfer, either within or between morphs (Charlesworth 1979). Because the stigmas of the mid-styled morph are located next to two stamen whorls rather than only one whorl, this morph was expected to have higher levels of SC, greater self-pollination, and higher selfing levels (lower outcrossing) than either the short- or long-styled morphs (Charlesworth 1979), leading to greater expression of inbreeding depression in the progeny of mid-styled morphs relative to either of the other two morphs (Fig. 3, panel A). The frequency of the mid-styled morph was predicted to decline because of this fitness disadvantage, followed by morphological adjustments of the positions of mid-level stamens of the short- and long-styled morphs that increased the likelihood of transfer of pollen from mid-level anthers to the stigmas of long- and short-styled morphs (Fig. 3, panels B and C). Charlesworth (1979) suggested that these adjustments would decrease pollen flow to mid-stigmas from other morphs, and therefore lead to even greater selfing of mid-styled morphs in populations with modified incompatibility. Increased selfing of the mid-styled morph would lead to an unstable positive feedback resulting in more pronounced expression of inbreeding depression (Fig. 3, panel C). Eventually, loss of the mid-styled morph and evolution of distyly was predicted (Fig. 3, panel D; Charlesworth 1979). In Charlesworth's model, selection against mid-alleles also occurs, although Charlesworth focused on the
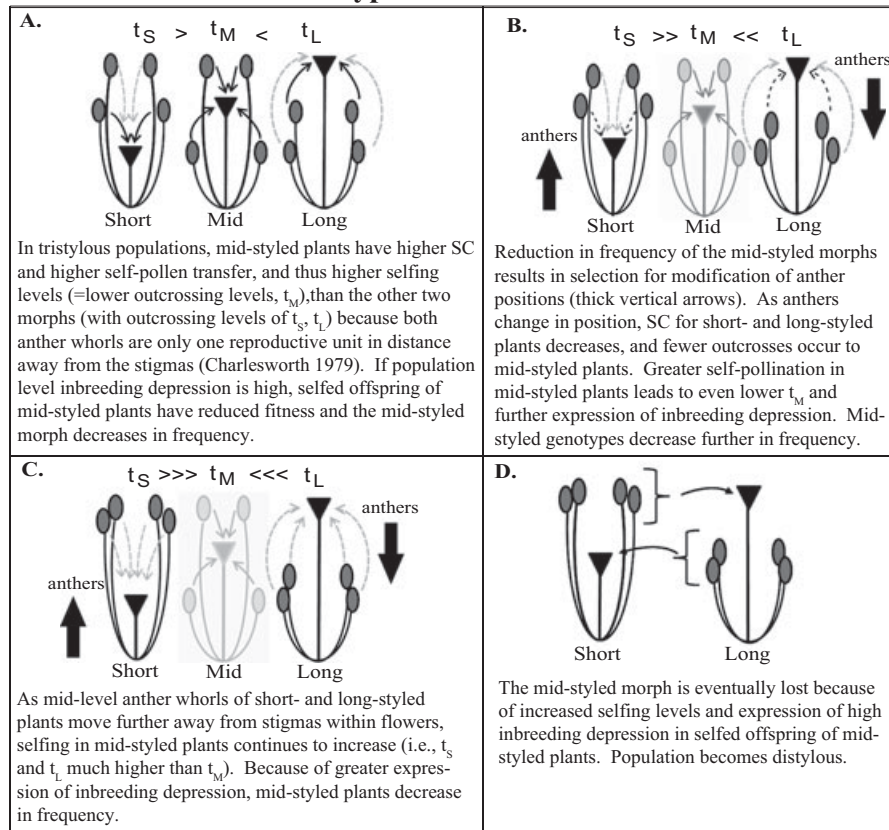
Genotypic selection model

A. $t_S > t_M < t_L$

Short Mid Long

In tristylous populations, mid-styled plants have higher SC and higher self-pollen transfer, and thus higher selfing levels (=lower outcrossing levels, t_M),than the other two morphs (with outcrossing levels of t_S, t_L) because both anther whorls are only one reproductive unit in distance away from the stigmas (Charlesworth 1979). If population level inbreeding depression is high, selfed offspring of mid-styled plants have reduced fitness and the mid-styled morph decreases in frequency.

B. $t_S \gg t_M \ll t_L$

anthers

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Short Mid Long

Reduction in frequency of the mid-styled morphs results in selection for modification of anther positions (thick vertical arrows). As anthers change in position, SC for short- and long-styled plants decreases, and fewer outcrosses occur to mid-styled plants. Greater self-pollination in mid-styled plants leads to even lower t_M and further expression of inbreeding depression. Mid-styled genotypes decrease further in frequency.

C. $t_S \ggg t_M \lll t_L$

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Short Mid Long

As mid-level anther whorls of short- and long-styled plants move further away from stigmas within flowers, selfing in mid-styled plants continues to increase (i.e., t_S and t_L much higher than t_M). Because of greater expression of inbreeding depression, mid-styled plants decrease in frequency.

D.

Short Long

The mid-styled morph is eventually lost because of increased selfing levels and expression of high inbreeding depression in selfed offspring of mid-styled plants. Population becomes distylous.

Figure 3. The genotypic model for the evolution of distyly from tristyly. Notations for diagrams are the same as Figure 2. In the genotypic selection model, dark arrows have the highest probability for pollen transfer and compatibility, followed by dashed arrows and light gray arrows, respectively. Note that except for Panel D, legitimate crosses are not shown.

expectation of greater selfing of the mid-styled morph and differential expression of inbreeding depression as the cause for the transition from tristyly to distyly. The genic and genotypic models are not mutually exclusive, and ultimately elements of both models could account for the transition from tristyly to distyly. Our goal in this study was to determine whether the pattern of variation among populations in the extent of incompatibility modification, selfing, and inbreeding depression matches expectations of theoretical analyses (Charlesworth 1979).

We examined the role of inbreeding depression in the evolution of distyly using three populations of *O. alpina* (Oxalidaceae) that varied in the extent of modification of tristylous incompatibility. We addressed three primary questions to understand the role of selfing and thus the potential for expression of inbreeding depression in the transition from tristyly to distyly: (1) Do mid-styled morphs exhibit greater SC than short- and long-styled morphs within the same population? (2) In populations where the mid-styled morph shows significant SC relative to short- and long-styled morphs, is selfing more common in the mid-styled morph than in other morphs, and is greater selfing in the mid-

styled morph positively associated with increased modification of tristylous incompatibility between the short- and long-styled morphs in different populations? (3) Could expression of inbreeding depression in the progeny of self-fertilizing mid-styled plants result in a selective disadvantage for mid-styled morphs, and contribute to the evolution of distyly? We interpreted the significance of inbreeding depression in mid-styled morphs relative to selection against mid-alleles caused by modifications of trimorphic incompatibility as factors in the evolution of distyly.

Materials 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). Recent phylogenetic investigations indicate that *O. alpina* is not monophyletic (Gardner et al. 2012). Within our geographically more restricted study area in the Sky Islands, a series of isolated mountain ranges in the Sonoran Desert region of AZ, NM, and northern Mexico (Weller et al.

Table 2. Characteristics of three populations of *Oxalis alpina* with a range in breeding system characteristics. Morph ratios are the relative ratios of long-styled:mid-styled:short-styled plants in field populations (sample size of plants surveyed in parentheses), and the tristylous SI index (Tri SI index) indicates the ability of the illegitimate SxmL and LxmS pollinations to produce seeds compared, to the SxsL and LxlS legitimate pollinations, respectively (data from Weller et al. 2007). The degree of seed production of SxmL and LxmS crosses is very low in unmodified populations, intermediate in partially modified populations, and equivalent to SxsL and LxlS crosses in completely modified populations (Weller et al. 2007). The reciprocity index (R_I) accounts for changes in reciprocity between mid-level anther and stigmas of short- and long-styled plants; as the index approaches one, short- and long-styled morphs are more reciprocally herkogamous (see text; Sosenski et al. 2010).

Population (population number, UC)	Geographic coordinates (latitude; longitude)	Morph ratios L:M:S	(Tri) SI index	Classification of incompatibility modification	(R_I)
Sierra La Mariquita (960) [MAR]	31.0537°N; 110.3834°W	30:36:34 (308)	0.12	Unmodified	0.719
Chiricahua Mts. (727) [CHIR]	31.8326°N; 109.3285°W	48:25:27 (508)	0.92	Completely modified	0.793
Pinos Altos Mts. (971) [PA]	32.9223°N; 108.2126°W	41:21:38 (309)	0.95	Completely modified	0.773

2007), tristylous and distylous populations of *O. alpina* are likely to share a common ancestor, based on similarity of molecular markers (Pérez-Alquicira et al. 2010) and uniform tetraploidy for those populations that have been investigated (Weller and Denton 1976). We examined inbreeding depression levels and levels of selfing in three populations that varied in expression of tristylous incompatibility (Sierra La Mariquita, Sonora [MAR], Chiricahua Mts., Arizona [CHIR], and Pinos Altos Mts., NM [PA]; Table 2).

GENETIC BASIS OF HETEROSTYLY IN *O. ALPINA*

Inheritance of style morphs in *O. alpina* is controlled by two diallelic loci, with the *S* allele at the *S*-locus dominant and epistatic to the *M*-allele at the *M*-locus. Short-styled plants normally have one copy of the dominant *S*-allele (*S*) because of SI, mid-styled plants are homozygous recessive (*ss*) at the *S*-locus and have at least one copy of the *M*-allele (*M*) at the *M*-locus, and long-styled plants are homozygous recessive (*ssmm*) at both loci. Tetraploidy does not fundamentally alter inheritance of style morphs (Table 1). The two loci controlling style morph expression are linked in *O. alpina* and related species (Weller 1976b; S. G. Weller et al., unpubl. data). In populations with typical tristylous incompatibility, symmetric frequency-dependent fitness of style morphs, in conjunction with the genetic system underlying style morph expression, leads to equal representation of style morphs (Charlesworth 1979; Heuch 1979).

SI MODIFICATION IN STUDY POPULATIONS

The level of modification of incompatibility in tristylous populations (SI index; Weller et al. 2007) was quantified in each population as the mean number of seeds per pollination formed by two illegitimate hand pollinations (SxmL and LxmS) divided by the mean number of seeds per pollination formed by corresponding legitimate hand pollinations (e.g., SxmL divided by SxsL value). As

the SI index approaches one, the level of modification increases, that is, seed production of the SxmL and LxmS crosses increases, and the short- and long-styled morphs become more fully intercompatible; note that the SI index refers to cross-compatibility between short- and long-styled morphs rather than changes in SC (Weller et al. 2007; Table 2: tristylous SI index). Populations exhibit a range in reciprocal herkogamy between short- and long-styled plants (i.e., as the mid-styled morph declines in frequency, reciprocal herkogamy increases because the position of mid-level anthers of the short-styled morph shifts toward the level of the long stigmas of the long-styled morph, and mid-level anthers of the long-styled morph shift in position toward the level of the short stigmas of the short-styled morph; Sosenski et al. 2010; Table 2).

ASSESSMENT OF SI

We assessed the level of SI by measuring seed production following self-pollinations compared to cross-pollinations in all three floral morphs of the three study populations (Appendix: sample sizes). Individuals were collected from throughout populations to ensure that genets were represented only once (Weller et al. 2007). Using a pollinator-free greenhouse, self-pollinations were made by applying self-pollen to the stigmas of the same flower. Both anther whorls within flowers were used for self-pollinations; for analyses, the seed production for pollinations from the two self-anther whorls was averaged. All cross-pollinations were legitimate crosses, with three to five different paternal parents for each category (32–39 maternal plants were self- and cross-pollinated per population). In statistical analyses, progeny from the different paternal parents were not distinguished. We used fine forceps to transfer pollen; forceps were washed with ethanol after pollination to prevent pollen contamination. Ripe capsules were collected 14–15 days after pollination when seeds were ripe but before explosive dehiscence of the capsules. Additional flowers were tagged

haphazardly on individuals of all three style morphs and were not pollinated by hand to confirm the absence of pollinator activity in the greenhouse.

For each population, we compared differences in seed production per pollination of maternal plants from hand self-fertilization versus hand cross-fertilization for each style morph. We used restricted maximum likelihood to test for significant differences (PROC MIXED; SAS Inc. 2002–2005; Littell et al. 2006). The degrees of freedom for the fixed effects were estimated using the Kenward–Roger correction (Kenward and Roger 1997; SAS Inc. 2002–2005). Treatment category (seeds produced via self-fertilization, cross-fertilization), maternal style morph category (short-, mid-, long-styled), and interactions between treatment and maternal style morph were treated as fixed effects. Maternal family (nested within maternal morph) and interactions between treatment category and maternal family (nested within maternal morph) were treated as random effects. We used residual likelihood values comparing a full model (with both random effects) to reduced models and tested against a χ^2 distribution with one degree of freedom (Littell et al. 2006).

The level of SC among style morphs within each population was characterized for each style morph by an index of SC that compares the seeds per pollination following selfing and cross-pollination (index of SC [ISC]: [average seed production after self-pollination]/[average seed production after cross-pollination]; in fully SC floral morphs, ISC = 1.00 [Bawa 1974; Zapata and Arroyo 1978; Bullock 1985; Igic and Kohn 2006]). Our index of SC undoubtedly includes a component of reduced seed production because of expression of early acting inbreeding depression either as seeds mature or during germination, and thus the ISC results in an underestimate of the extent of SC (Bawa 1974; Zapata and Arroyo 1978; Bullock 1985; Igic and Kohn 2006). To examine the prediction that the relative SC of mid-styled plants across populations will increase with an increase in modification of tristylous incompatibility between short- and long-styled plants, the relative index of SC for mid-styled morphs (relative index of SC [rISC]) compared to short- and long-styled morphs was calculated as (mid-styled ISC/[mean of short- and long-styled ISC]).

We also used the SI index measuring reciprocal compatibility between short- and long-styled morphs and data from 13 tristylous Sky Island populations (including the three populations studied here; Weller et al. 2007) to determine whether populations with significant SC of mid-morphs had greater modification of tristylous incompatibility in short- and long-styled plants. Populations were categorized as those with significant SC of mid-styled morphs ($N = 8$ populations), or those showing no evidence of SC for mid-styled morphs ($N = 5$ populations). The SI index for these two groups was compared using a t test.

MATING SYSTEM

DNA extraction, amplification, and genotyping to estimate outcrossing

Ripe seeds and leaves of *O. alpina* were collected from approximately 30 maternal individuals (when available) of each morph (long-, mid-, and short-styled) in populations from the Sierra La Mariquita (MAR), the Pinos Altos Mts. (PA), and Morse Canyon in the CHIR in late August and early September in 2006 and 2007. More fruiting plants were present in 2007 (Table 3). After a required period of dormancy, seeds were planted in May 2007 and May 2008 in the greenhouse. Leaf samples from each of the progeny were collected and placed in bags with silica gel. Dry leaves from maternal plants and their respective progeny were crushed into powder using a FastPrep instrument (MP Bio). The DNA was extracted using the Qiagen DNeasy Kit (Qiagen, Venlo, Netherlands) from 1047 and 1639 individuals (maternal parents and progeny) from 2006 and 2007, respectively. Maternal families per style morph ranged from 11 to 36 families (Table 3) and progeny arrays per family ranged from 4 to 21 individuals.

For microsatellite analyses, we selected the seven most reliable polymorphic microsatellite loci (Oxa17, 25, 41, 43, 62, 81, and 84). Loci and conditions for amplifications followed previous methods (Tsyusko et al. 2007) except that the primers (one in each pair) were directly labeled with Fam or Ned fluorescent dyes. The polymerase chain reaction (PCR) amplifications were performed using an Applied Biosystems thermal cycler (GeneAmp PCR System 9700, Foster City, CA) in a total of 12.5 μl volume (11.5 μl + 1 μl of DNA): 1$\times$ PCR buffer, 25.0 μg/mL bovine serum albumin (BSA), 0.24μm of each (forward and reverse) primer (10 μm), 2mM (for Oxa17, 62, 81, and 84) or 3mM (for Oxa25, 41, and 43) MgCl$_2$, 0.15 mM deoxyribonucleotide triphosphates (dNTPs) 0.5 U JumpStart Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO), and 5–50 ng DNA template. PCR products were run in panels of two loci on an ABI PRISM 3130xl sequencer (Applied Biosystems, Foster City, CA) and sized using an internal size standard (DeWoody et al. 2004). The genotyping was performed using Genemapper version 4.0 (Applied Biosystems, Foster City, CA). To assign genotypic configurations (for each of the four allele copies for tetraploids), we used the microsatellite DNA allele counting-peak ratios (MAC-PR) method (Esselink et al. 2004).

Estimation of outcrossing

Estimates of multilocus (t_m) outcrossing levels and biparental inbreeding ($t_m - t_s$, due to matings between closely related individuals, where t_s is the single-locus outcrossing level) were calculated using Ritland's program MLTET (Murawski et al., 1994), which estimates t_m and t_s for autotetraploids. The statistics (t_m and t_s) were calculated based on 1000 bootstraps with resampling performed among families. To determine whether the t_m values were significantly different from one (complete outcrossing),

Table 3. Outcrossing levels (standard errors) from 2006 and 2007 for floral morphs in three populations of *Oxalis alpina*. Multilocus (t_m) and single-locus (t_s) outcrossing estimates, biparental inbreeding ($t_m - t_s$), and the inbreeding coefficient (*F*) were calculated using Ritland's program MLTET (Murawski et al. 1994). The inbreeding coefficient (*F*) reported is the two-gene coefficient (the covariance of identity by descent of a pair of randomly sampled alleles with the remaining pair of alleles). *N* is the number of maternal families. Multilocus outcrossing levels in bold are significantly different from 1.00 (complete outcrossing) and indicate the occurrence of selfing.

	First year (2006)					Second year (2007)				
Long-styled	t_m	t_s	$(t_m - t_s)$	F	N	t_m	t_s	$(t_m - t_s)$	F	N
MAR	0.83	0.55	0.27	−0.08	12	0.92	0.69	0.23	0.21	19
	(0.10)	(0.07)	(0.09)	(0.05)		(0.36)	(0.06)	(0.36)	(0.08)	
CHIR	0.92	0.97	−0.05	0.27	11	0.98	0.80	0.18	−0.04	14
	(0.38)	(0.08)	(0.36)	(0.44)		(0.48)	(0.05)	(0.46)	(0.06)	
PA	0.84	0.73	0.12	0.07	11	0.92	0.72	0.20	0.04	36
	(0.24)	(0.07)	(0.21)	(0.13)		(0.22)	(0.05)	(0.21)	(0.04)	
Mid-styled										
MAR	0.84	0.69	0.20	−0.30	11	0.88	0.64	0.23	0.20	14
	(0.04)	(0.09)	(0.21)	(0.01)		(0.32)	(0.06)	(0.30)	(0.15)	
CHIR	**0.80**	0.68	0.12	0.30	13	**0.62**	0.47	0.15	0.33	29
	(0.10)	(0.10)	(0.07)	(0.21)		**(0.10)**	(0.06)	(0.05)	(0.41)	
PA	**0.53**	0.40	0.12	−0.24	16	**0.68**	0.59	0.08	0.33	26
	(0.06)	(0.04)	(0.06)	(0.01)		**(0.08)**	(0.06)	(0.04)	(0.11)	
Short-styled										
MAR	**0.82**	0.57	0.24	0.25	11	0.92	0.60	0.32	0.21	21
	(0.08)	(0.10)	(0.07)	(0.15)		(0.11)	(0.04)	(0.11)	(0.10)	
CHIR	0.88	0.71	0.18	−0.18	11	0.84	0.71	0.14	0.21	21
	(0.17)	(0.10)	(0.15)	(0.07)		(0.14)	(0.06)	(0.12)	(0.15)	
PA	0.97	0.67	0.29	0.25	11	0.94	0.71	0.23	0.05	25
	(0.49)	(0.08)	(0.45)	(0.20)		(0.48)	(0.05)	(0.21)	(0.09)	

bootstrapping values were used to calculate the mean and 95% confidence intervals (CI) for each t_m (Efron and Gong, 1983) for each morph within a population. The value of t_m was considered significantly different from one (i.e., complete outcrossing) if the upper 95% CI did not overlap with one. To test whether morphs differed significantly in estimates of t_m, we calculated the differences between randomly paired bootstrap outcrossing estimates for each pair of morphs. If 97.5% of these differences (as described in Eckert et al. 2009) fell either above or below zero, the two estimates were considered significantly different.

ASSESSMENT OF INBREEDING DEPRESSION

Because *O. alpina* exhibits strong SI, our measurements of inbreeding depression began at percent emergence. Progeny from self- and cross-fertilized pollinations were used to assess the level of inbreeding depression within each maternal family from each study population. From each maternal by paternal plant combination, we planted up to 10 seeds per 2 cm pot and pots were placed haphazardly with respect to each other on benches in the UC Irvine greenhouses. The proportion of seeds that emerged per pot was calculated after cotyledons appeared.

We transplanted progeny from each maternal individual to individual 5 cm pots to establish maternal families (the number of maternal families per population ranged from 19 to 35; Appendix). For each maternal family, transplanted seedlings in the cross-fertilized treatment were evenly distributed among the different paternal plants. The number of flowers per inflorescence, total number of inflorescences, and total number of flowers per plant were recorded at least weekly for every transplanted individual throughout the six-week flowering season. Survival was not used as a measure of fitness because all mortality appeared to result from gnat infestations, and depended on the date seedlings were transplanted rather than intrinsic differences between individuals within populations or between populations. Total biomass (all standing aboveground mass, all bulbs, and roots) was harvested after plants ceased growth and entered dormancy at the end of the growing season. Each plant was dried to constant mass for 12–14 days at 60°C and weighed.

For each population, we compared differences in fitness measures of progeny produced from hand self-fertilization versus hand cross-fertilization of all three style morphs, where sample size was determined by the number of maternal families. Cumulative

relative fitness for each family was calculated as the product of the relative fitness (w_s/w_o; where w_s and w_o are the fitness values for selfed and outcrossed progeny) for percent emergence, total number of flowers, and total biomass. Inbreeding depression (δ) was defined as $1 - (w_s/w_o)$. The relative fitness values for the three life-history stages were not significantly correlated with each other in any pairwise comparison in any population ($P > 0.0167$; adjusted by Bonferroni correction for all three comparisons in all three populations). The cumulative relative fitness of each style morph was calculated as the mean of the cumulative relative fitness values of the maternal families of each style morph. Population-level inbreeding depression was calculated as the relative fitness of each style morph weighted by the proportion of each style morph in the field. Statistical methods (including fixed and random effects) were similar to those used to compare differences in seed production per pollination from hand self-fertilization versus hand cross-fertilization.

Results

SELF-COMPATIBILITY

The mid-styled morph, relative to the other two morphs, produced more seeds following self-compared to cross-pollination in the two populations with modified tristylous incompatibility; the interaction between treatment category and style morph (without family nested within style morph) was significant in two of the three populations (CHIR and PA; Table 4, Fig. 4). Mid-styled plants in the unmodified MAR population had the lowest self-compatibility (ISC) and rISC (Table 5).

Greater SC of the mid-styled morph was associated with greater modification of tristylous incompatibility (measured by the SI index) throughout the Sky Island populations of *O. alpina*. Populations with significant SC in the mid-styled morph had a significantly higher average SI index (SI = 0.62, SE = 0.10, $N = 8$) than populations where the mid-styled morph showed no evidence of SC (average SI index = 0.23, SE = 0.06, $N = 5$; $t = 7.67$; $P = 0.018$, df = 11).

SELFING LEVELS

Mid-styled plants had significant levels of selfing (t_m significantly <1) in CHIR and PA (2006, 2007; Table 3; Fig. 5, t_m ranging from 0.53 to 0.80), and short-styled plants had significant levels of selfing in MAR (2006, Table 3; Fig. 5; $t_m = 0.82$). Mid-styled families had higher selfing levels (lower t_m) than short- and long-styled families in both CHIR (2007) and PA (2006 and 2007; Fig. 5).

When the same floral morph was compared across populations in 2006, selfing levels for mid-styled plants were higher in PA than in CHIR, and short-styled plants had higher selfing levels in MAR than in PA. Selfing levels for mid-styled plants in the completely modified CHIR (in 2007) and PA (in 2006 and 2007) populations were higher than in the unmodified MAR population.

INBREEDING DEPRESSION—*PERCENT EMERGENCE*

Percent emergence showed significant inbreeding depression only in PA, with selfed progeny exhibiting lower percent emergence than outcrossed progeny (Table 4; Fig. 6).

NUMBER OF FLOWERS

In MAR, levels of inbreeding depression were lower for total flower number in progeny of long-styled maternal parents ($\delta = -0.25$ [0.34]) than either mid-styled ($\delta = 0.58$ [0.15]) or short-styled maternal parents ($\delta = 0.53$ [0.14]; Fig. 6), based on the significant interaction between treatment category and style morphs (without family nested within style morph). The total number of flowers showed significant inbreeding depression at the population level in completely modified CHIR ($\delta = 0.60$ [0.04]).

BIOMASS

Biomass showed significant inbreeding depression at the population level in unmodified MAR ($\delta = 0.29$ [0.01]), and completely modified CHIR ($\delta = 0.28$ [0.10]) and PA ($\delta = 0.07$ [0.11]; Fig. 6). Biomass differed by family for all three style morphs following self- and cross-pollination in CHIR (significant interaction between treatment category [self- or cross-pollinated] and maternal family [nested within style morph]; Table 4; Fig. 6).

POPULATION-LEVEL INBREEDING DEPRESSION

Cumulative levels of inbreeding depression (SE) for the two fully modified populations were moderate, 0.61 (0.07) in CHIR and 0.50 (0.04) in PA, Table 5). In contrast, population-level inbreeding depression from unmodified MAR was much lower (0.32 [0.10]).

Discussion

Our results suggest that modifications of tristylous incompatibility favoring evolution of distyly also lead to changes in morph-specific selfing levels and expression of inbreeding depression. In the absence of modifications of tristylous incompatibility, however, inbreeding depression does not appear to be a significant factor in the evolution of distyly.

MID-STYLED MORPHS HAVE GREATER SC THAN SHORT- AND LONG-STYLED MORPHS IN POPULATIONS WITH MODIFIED INCOMPATIBILITY

In Charlesworth's (1979) model, compatibility between anthers and stigmas is related to floral morphology and the difference in height between the levels of the reproductive organs (Fig. 3). In this model, self-pollen deposition and SC are assumed to be

Table 4. Fitness of progeny produced from hand self- and cross-fertilized pollinations (treatment), for maternal plants (nested within style morph) from three populations (Sierra La Mariquita [MAR], Chiricahua Mts. [CHIR], and the Pinos Altos Mts. [PA]); Sds/poll = seeds per pollination, Pct emerge = percent emergence, and *N* = number of maternal families. Numerator, denominator degrees of freedom (df), F, P, and χ^2 are reported from restricted maximum-likelihood analyses in PROC MIXED. 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. *P* values > 0.05 are shown in bold.

Pop	Life stage	*N*	Fixed effects				Random effects		
			Source of var	df	*F*	*P*	Source of var	χ^2	*P*
MAR	Sds/poll	32	Trtmt	1,27.9	416.2	**<0.0001**	Family(Morph)	1.8	0.180
			Morph	2,29.0	3.6	**0.041**	Trtmt×Fam(Morph)	166.2	**<0.0001**
			Trtmt×Morph	2,27.9	2.8	0.080			
	Pct emerge[1]	26	Trtmt	1,33.0	2.7	0.110	Family(Morph)	4.2	**0.040**
			Morph	2,31.0	0.4	0.693	Trtmt×Fam(Morph)	27.3	**<0.0001**
			Trtmt×Morph	2,29.7	0.1	0.866			
	N of flowers[2]	26	Trtmt	1,30.3	2.0	0.169	Family(Morph)	8.5	**0.004**
			Morph	2,30.3	1.9	0.175	Trtmt×Fam(Morph)	4.5	**0.034**
			Trtmt×Morph	2,29.0	4.5	**0.020**			
	Biomass (mg)	26	Trtmt	1,1600	82.5	**<0.0001**	Family(Morph)	13.2	**<0.001**
			Morph	2,31.1	0.5	0.588	Trtmt×Fam(Morph)	0	1.00
			Trtmt×Morph	2,1599	1.4	0.257			
CHIR	Sds/poll	39	Trtmt	1,35.1	75.1	**<0.0001**	Family(Morph)	4.8	**0.029**
			Morph	2,36.1	0.5	0.632	Trtmt×Fam(Morph)	334.7	**<0.0001**
			Trtmt×Morph	2,35.0	8.7	**<0.001**			
	Pct emerge[1]	20	Trtmt	1,34.5	0.2	0.687	Family(Morph)	1.1	0.294
			Morph	2,48.1	2.2	0.128	Trtmt×Fam(Morph)	12.8	**<0.001**
			Trtmt×Morph	2,31.9	0.2	0.845			
	N of flowers[2]	19	Trtmt	1,38.0	18.6	**<0.001**	Family(Morph)	2.7	0.100
			Morph	2,57.3	0.7	0.493	Trtmt×Fam(Morph)	2.8	0.094
			Trtmt×Morph	2,27.5	0.3	0.770			
	Biomass (mg)	18	Treatment	1,55.8	11.3	**0.001**	Family(Morph)	0.6	0.439
			Morph	2,55.8	0.2	0.814	Trtmt×Fam(Morph)	14.4	**<0.001**
			Trtmt×Morph	2,42.4	0.1	0.946			
PA	Sds/poll	36	Treatment	1,32.8	230.6	**<0.0001**	Family(Morph)	0.8	0.371
			Morph	2,32.8	11.1	**<0.001**	Trtmt×Fam(Morph)	277.6	**<0.0001**
			Trtmt×Morph	2,32.8	37.5	**<0.0001**			
	Pct emerge[1]	26	Treatment	1,27.6	14.6	**<0.001**	Family(Morph)	2.8	0.094
			Morph	2,43.1	0.2	0.815	Trtmt×Fam(Morph)	10.0	**0.002**
			Trtmt×Morph	2,26.4	2.7	0.084			
	N of flowers[2]	25	Treatment	1,24.3	0.9	0.349	Family(Morph)	19.9	**<0.0001**
			Morph	2,42.6	0.1	0.868	Trtmt×Fam(Morph)	0.8	0.371
			Trtmt×Morph	2,25.0	3.3	0.055			
	Biomass (mg)	25	Treatment	1,23.3	16.7	**<0.001**	Family(Morph)	13.5	**<0.001**
			Morph	2,44.9	2.9	0.066	Trtmt×Fam(Morph)	0.7	0.403
			Trtmt×Morph	2,23.9	2.6	0.097			

[1]Percent emergence was arcsine, square-root transformed.
[2]Dependent variables were square-root transformed.

greater for mid-styled relative to short- and long-styled morphs, because mid-styled morphs have two stamens whorls in close proximity to the stigmas (one whorl above and the other below), rather than only one close stamen whorl for the short- and long-styled morphs. Relative to short- and long-styled morphs, greater

SC of mid-styled morphs is expected to lead to greater selfing, greater expression of inbreeding depression, and ultimately result in loss of the mid-styled morph.

Although the Charlesworth's predictions for the relationship between morphology and compatibility have been observed
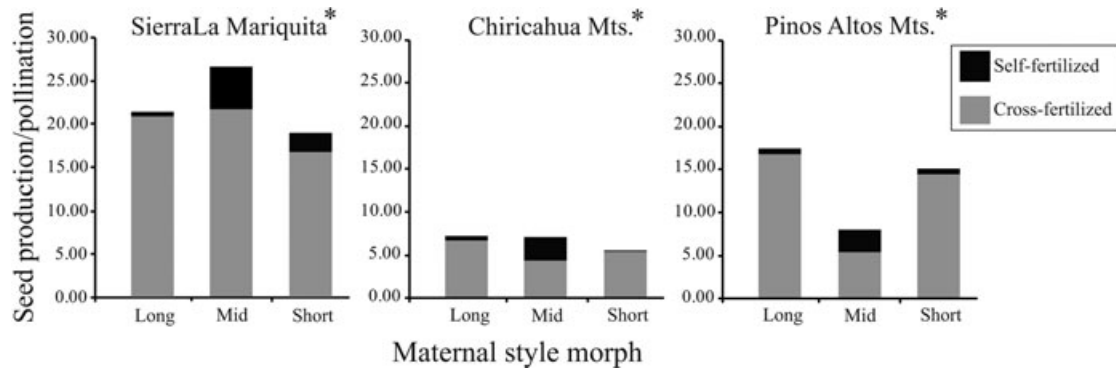
Figure 4. Seeds production per pollination from cross- and self-fertilization by style morph. Seed production from self-fertilized pollinations was significantly less than seed production from cross-fertilized pollinations in all populations (indicated by *; *P*<0.001 in all cases; Table 4). Mid-styled plants exhibited greater self-compatibility (as measured by seed production after self-pollination) than long- and short-styled plants in all populations.

Table 5. The index of self-compatibility (ISC) for mid-, short-, and long-styled maternal plants (sample size of maternal plants in parentheses), relative SC of mid-styled morphs (rISC) = (mid-styled ISC/[mean of short- and long-styled ISC]), population inbreeding depression [$\delta = 1 - w_s/w_o$) for three populations of *Oxalis alpina*. A higher ISC indicates a greater ability to produce seeds via self-fertilization; in all cases mid-styled plants exhibited relatively higher ISC than short- and long-styled plants. Cumulative relative fitness (w_s/w_o) for each family was calculated as the product of the relative fitness for percent emergence, number of flowers, and total biomass. Standard errors are provided in parentheses. *N* (S,M,L) indicates the number of families of each style morph used to calculate cumulative relative fitness (RF).

Population	ISC (M)	ISC (S)	ISC (L)	rISC	δ	*N* (S,M,L)
MAR	0.23 (10)	0.13 (11)	0.03 (11)	2.89	0.32 (0.10)	9,10,6
CHIR	0.62 (14)	0.04 (12)	0.07 (13)	11.67	0.61 (0.07)	2,12,2
PA	0.46 (15)	0.05 (11)	0.04 (10)	10.80	0.50 (0.04)	8,14,3

for tristylous species in different genera (*Oxalis*, Darwin 1877; Weller et al. 2007; *Lythrum*, Darwin 1877; *Pontederia*, Ornduff 1966; Barrett 1977), greater SC of the mid-styled morph did not appear to be a factor during the initial stages in the evolutionary transition from tristyly to distyly in populations of *O. alpina*. In the unmodified MAR population, mid-styled morphs were only marginally SC relative to the short- and long-styled morphs. In contrast, in CHIR and PA, two populations with highly modified incompatibility in the short- and long-styled morphs (high SI indices due to high seed production following SxmL and LxmS crosses), levels of SC for mid-styled morphs were very high compared to short- and long-styled morphs (higher rISC values). Our analysis of SC of the mid-styled morph across 13 Sky Island populations supported the results obtained for the three populations we studied in detail, as populations without detectable SC of mid-styled morphs had more typical tristylous incompatibility (much lower SI indices due to lower seed production following SxmL and LxmS crosses) than populations with significant SC of mid-styled morphs. Differences in SC among morphs within populations and of mid-styled morphs among Sky Island popula-

lations could have been overestimated if greater SC and greater selfing in the progeny of mid-styled morphs has led to more purging of early acting inbreeding depression. In unmodified populations, purging of early acting inbreeding depression is unlikely because levels of self-fertility were very low.

GREATER SC IN THE MID-STYLED MORPH IS ASSOCIATED WITH HIGHER SELFING LEVELS

Greater SC in mid-styled morphs was associated with more selfing, either in one or both of the years of the study. In populations where the mid-styled morph had significant SC (CHIR and PA), the greater level of selfing for the mid-styled morph may have resulted from the proximity of mid-level stigmas to two stamen whorls producing partially compatible pollen grains. Data from naturally pollinated plants show that the mid-styled morph has the greatest level of self-pollination (C. A. Domínguez and F. Baena-Díaz, pers. comm. 2011). In populations with more highly modified tristylous incompatibility (higher seed production of SxmL and LxmS crosses), adjustments in the positions of mid-level
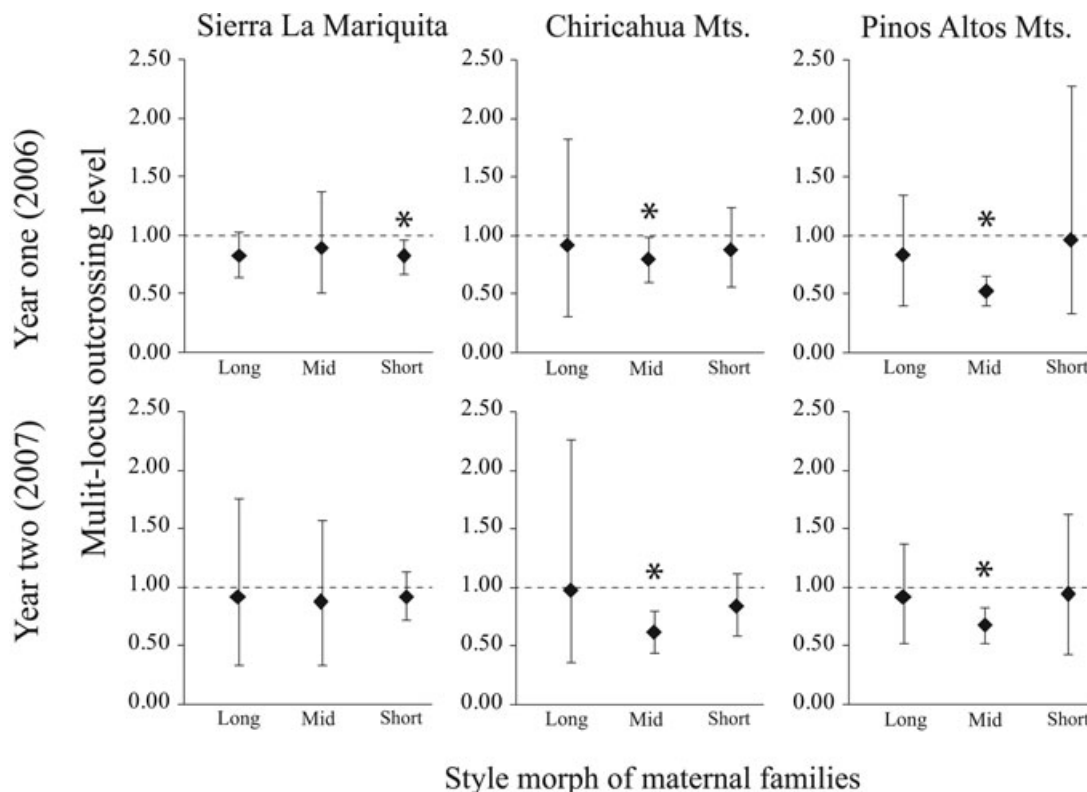
Figure 5. Multilocus (t_m) outcrossing levels and 95% confidence intervals for maternal families of three style morphs in three populations in two years. *indicates a value significantly different from unity ($t_m = 1$). Among floral morphs in each population, mid-styled families had lower outcrossing levels (higher selfing) than short- and long-styled families in both the Chiricahua Mts. (2007) and the Pinos Altos Mts. (2006 and 2007), based on the differences between randomly paired bootstrap outcrossing estimates for each pair of morphs.

anthers away from the position of mid-stigmas (Fig. 3, panels B and C; Sosenski et al. 2010) are likely to lead to even higher levels of self-pollination and self-fertilization of mid-styled morphs as fewer pollen grains from the mid-level anthers of short- and long-styled morphs are deposited by pollinators on mid-stigmas.

EXPRESSION OF INBREEDING DEPRESSION DIFFERS BY STYLE MORPH IN POPULATIONS WITH MODIFIED TRISTYLOUS INCOMPATIBILITY

Because substantial inbreeding depression could be detected in progeny produced from self- versus cross-pollinations, our results provide support for Charlesworth's hypothesis that significant selfing of the mid-styled morph will lead to greater expression of inbreeding depression in progeny and therefore selection against mid-styled morphs. In the three populations we studied (MAR, CHIR, PA), the expression of inbreeding depression would be expected only in populations with detectable selfing of the mid-styled morph (CHIR, PA). As mid-stamen whorls of short- and long-styled morphs shift in position with increasing modification of tristylous incompatibility, and self-pollination of the mid-styled morph increases, selection for higher levels of SC in the mid-styled morph is likely, as we observed in populations of

O. alpina with more highly modified tristylous incompatibility (greater seed production of SxmL and LxmS crosses). Shifts in anther positions should also decrease self-pollination of short- and long-styled morphs, as mid-anther whorls move away from stigmas in the same floral morph. The occurrence of significant self-fertilization of the short-styled morph in the unmodified MAR population, but not in modified populations, is consistent with these predictions and further supports the importance of positional effects in determining the extent of SC and self-pollination (Charlesworth 1979).

If inbreeding depression levels evolve in response to the evolution of selfing, predictions for the effects of selfing on loss of the mid-styled morph are likely to change. A long history of self-fertilization may result in purging of recessive deleterious alleles as they become exposed to selection in homozygous individuals (Crow 1948; Stebbins 1957; Wright 1977; Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Charlesworth et al. 1990). In *O. alpina*, the progeny of self-fertilizing mid-styled morphs may show reduced expression of inbreeding depression if purging of deleterious alleles has occurred. Traits that could promote selfing such as reduced SI and reduced stigma-anther separation in mid-styled plants have been observed in a number
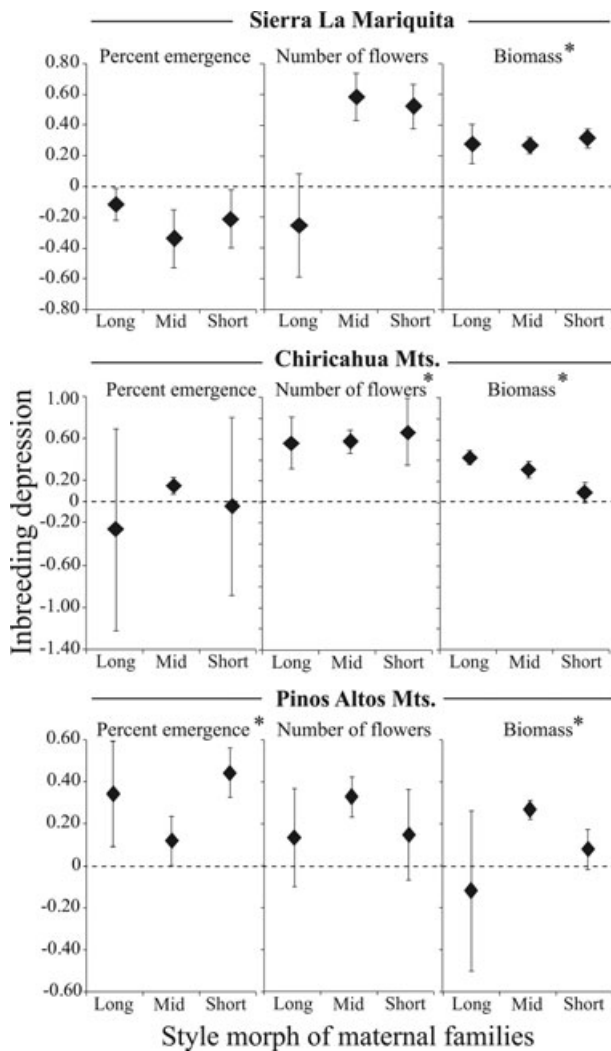
Figure 6. Inbreeding depression ($1 - [w_s/w_o]$) at percent emergence, number of flowers and biomass of long-, mid-, and short-styled families in three populations of *Oxalis alpina* (see Appendix for sample sizes). Values above zero indicate inbreeding depression, an * indicates significant population-level inbreeding depression at that life stage ($P<0.05$; Table 4: treatment effect). Error bars = ±1 SE.

of tristylous species (*Lythrum* [Lythraceae]; Stout 1925; *Oxalis* [Oxalidaceae]; Ornduff 1972; *Eichhornia* [Pontederiaceae]; Barrett 1988). If selfed offspring of mid-styled plants inherit traits that promote selfing (SC, reduced herkogamy), self-fertilizing lineages may evolve in heterostylous taxa, and mid-styled plants could be maintained or increase in frequency in tristylous populations. Opportunities for differential purging of inbreeding depression may explain populations of some *Oxalis* species of section *Corniculatae* where nearly homostylous, self-compatible mid-styled morphs have become common (Ornduff 1972), and might also occur in some populations of *O. alpina* (Weber et al. 2012). Among populations of *Eichhornia paniculata* (Pontederiaceae),

the mid-styled morph predominated in dimorphic populations, and was the only morph found in monomorphic populations (Barrett et al. 1989).

Inbreeding depression levels may have been underestimated in our greenhouse experiment compared to studies in natural habitats or in experiments carried out in competitive environments (Dudash 1990; Ramsey and Vaughton 1998; Daehler 1999). Additionally, polyploidy may reduce inbreeding depression because deleterious alleles are masked at multiple loci (Bever and Felber 1992; Otto and Whitton 2000). Comparisons of inbreeding depression in tetraploid versus diploid *Oxalis* populations or species would be necessary to test the relationship between polyploidy and inbreeding depression in *Oxalis*.

CONCLUSIONS: SELECTION FOR DISTYLY IN POPULATIONS OF *O. ALPINA*

The evolution of distyly in populations of *O. alpina* appears to result from both modifications of tristylous incompatibility of the long- and short-styled morphs and differential, morph-based expression of inbreeding depression. Initially, modifications of tristylous incompatibility favoring increased allele exchange between short- and long-styled morphs (Fig. 2, panel B) may initiate selection against mid-alleles. In the early stages of evolution of distyly, changes in incompatibility of the short- and long-styled morphs are not associated with increased SC of the mid-styled morph or modifications of anther positions likely to result in increased self-fertilization of this morph (Fig. 2, panel A; Kutaka et al. 2011). Selection against mid-alleles leads to decreased representation of mid-styled morphs, and decreased mid-styled morph representation in turn selects for modifications of anther positions leading to increased pollen exchange between short- and long-styled morphs (Fig. 2, panel C; Sosenski et al. 2010; Baena-Díaz et al. 2012). The increase in pollen exchange between the short- and long-styled morphs is also expected to result in increased self-pollination of mid-styled morphs, and the potential for expression of inbreeding depression in their progeny. This increase in self-pollination of mid-styled morphs may also select for the higher levels of SC in the mid-styled morph observed in more highly modified populations of *O. alpina*. Selection against mid-alleles eventually leads to greater expression of inbreeding depression in the progeny of mid-styled morphs, a key element at all stages of Charlesworth's model for evolution of distyly (Fig. 3, panels A–C). The genic selection model (Weller et al. 2007), which emphasizes selection against mid-alleles, may eventually lead to the evolution of SC of the mid-styled morph, and differential, morph-based expression of inbreeding depression, but initially does not depend on expression of inbreeding depression.

Our results suggest that evolution of distyly in populations of *O. alpina* results from complex interactions between modifications of incompatibility and morphology and the expression of

inbreeding depression. Breeding systems that possess three morphs may be inherently unstable, with slight asymmetries favoring loss of one of the morphs. Differences in morphology between floral morphs, as in the case of the mid-styled morph of tristylous species, may make one of these morphs particularly susceptible to selection. The diverse pathways of breeding system modification in tristylous species suggest, however, that predicting the outcome of selection on tristylous breeding systems is difficult. Additionally, stochastic responses related to fluctuations in the environment may be important drivers of breeding system modification (Pérez-Alquicira et al. 2010). The uniform loss of the mid-styled morph throughout the Sky Island region argues for selection against this morph, but the concentration of distylous populations in the northwest region of the Sky Islands, an area where repeated expansion and contraction of population sizes is likely to have occurred, suggests that stochastic events have been important as well (Pérez-Alquicira et al. 2010). In light of the many factors leading to loss of tristyly, retention rather than loss of tristyly may be the more remarkable occurrence within *Oxalis* and other plant families possessing tristyly.

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Appendix

Mean number of capsules, seeds, and transplanted plants per maternal family in each of three populations used in experiments of self-compatibility and inbreeding depression in *Oxalis alpina*. MAR = Sierra La Mariquita; CHIR = Chiricahua Mts.; PA = Pinos Altos Mts. *N* of maternal plants are provided in parentheses.

Population	Mean capsules/maternal plant			Mean seeds/maternal family		Mean transplanted plants/maternal family		
	Self-pollination	Cross-pollination	Control	Self-pollination	Cross-pollination	Self-pollination	Cross-pollination	Plants/population
MAR	17.1 (32)	29.6 (33)	0.13 (24)	147.5 (32)	619.8 (33)	16.0 (26)	51.9 (32)	1764
CHIR	14.2 (39)	25.6 (39)	0.03 (29)	70.2 (39)	186.3 (39)	20.5 (19)	53.4 (35)	1551
PA	14.0 (36)	26.2 (36)	0.06 (35)	94.8 (36)	351.9 (36)	20.9 (25)	70.5 (35)	2377