

POPULATION-LEVEL AND FAMILY-LEVEL INBREEDING DEPRESSION IN A CLEISTOGAMOUS PERENNIAL

Christopher G. Oakley¹ and Alice A. Winn

Department of Biological Science, Florida State University, Tallahassee, Florida 32306, U.S.A.

Cleistogamous species constitute strong evidence for the stability of mixed selfing and outcrossing but have been largely overlooked as theoretical and empirical systems for studying the forces thought to influence mating-system evolution. Inbreeding depression is expected to play a major role in the maintenance of outcrossing, yet reliable estimates of inbreeding depression for cleistogamous species are virtually nonexistent. We estimated both population- and family-level inbreeding depression for all life cycle stages for a population of the cleistogamous perennial *Viola septemloba*. Our estimate of cumulative inbreeding depression of 43% is substantially greater than two previous estimates for cleistogamous species, but it is not sufficient by itself to explain the maintenance of potentially outcrossed chasmogamous flowers.

Keywords: cleistogamy, selfing, mixed mating, reproductive strategy, *Viola septemloba*.

Introduction

Inbreeding depression, the reduction in fitness caused by reproduction via inbreeding rather than outcrossing, plays a major role in the evolution of mating systems (Goodwillie et al. 2005). In hermaphroditic organisms, the average magnitude of inbreeding depression in a population strongly influences whether selection will favor increased self-fertilization or increased outcrossing. As a consequence of its pivotal role, inbreeding depression has been estimated for populations of many plant species (Husband and Schemske 1996; Armbruster and Reed 2005), but reliable estimates are virtually absent for cleistogamous taxa, an intriguing group of species that have been overlooked by most mating-system theory.

Individuals of cleistogamous species can produce both open chasmogamous (CH) flowers that are available for outcrossing and closed cleistogamous (CL) flowers that obligately self-fertilize. The ability of single individuals to produce both CH and CL flowers has been reported in 536 species in 41 different families (Culley and Klooster 2007). These taxa exemplify mixed mating, a combination of selfing and outcrossing, which constitutes a persistent puzzle for the theory of mating-system evolution (Goodwillie et al. 2005). The most general mating-system theory indicates that inbreeding depression $>50\%$ favors the evolution of complete outcrossing and that inbreeding depression of $<50\%$ favors complete selfing. A mixture of outcrossing and selfing is therefore not expected to be evolutionarily stable (Lloyd 1979; Lande and Schemske 1985; Charlesworth and Charlesworth 1987).

The repeated evolution and persistence of cleistogamy (Culley and Klooster 2007) are clearly at odds with the expectation that mixed mating is not stable, making these species attractive systems in which to study forces that affect the selective balance between self-fertilization and outcrossing.

¹ Author for correspondence; e-mail: coakley@bio.fsu.edu.

The threshold inbreeding depression necessary to favor outcrossing should be greater than 50% for cleistogamous species because the greater energetic economy and more reliable fruit set of CL flowers (Schemske 1978; Waller 1979) can mitigate negative effects of inbreeding. This scenario suggests that very strong inbreeding depression would be required to favor the maintenance of CH flowers (Oakley et al. 2007).

Theoretical models suggest that the production of both CH and CL flowers may be maintained as adaptive phenotypic plasticity, in which each flower type is favored in a different season (Schoen and Lloyd 1984), or as a way to avoid geitonogamy, in which CL flowers increase seed set and reduce selfing between costly CH flowers (Masuda et al. 2001). There is little empirical evidence to support or refute either of these models (Oakley et al. 2007), but both rely on a presumed advantage of CH reproduction to balance the energetic economy of CL selfing, and avoidance of inbreeding depression may be a component of this advantage.

Many studies of cleistogamous species report estimates of the fitness of progeny produced by CH and CL flowers. For 19 such studies of 14 species, the average fitness of progeny produced by CL flowers was 1.13 times that of progeny from CH flowers (Oakley et al. 2007), indicating that inbreeding depression would be negative, but these data do not provide an accurate assessment of inbreeding depression, for two reasons. First, CH flowers are self-compatible and are known to self at appreciable rates (Mitchell-Olds and Waller 1985; Waller and Knight 1989; Cole and Biesboer 1992; Lu 2000; Culley 2002). The inclusion of selfed offspring in the sample used to estimate the fitness of outcrossed progeny would downwardly bias estimates of inbreeding depression. Second, seeds produced by CH and CL flowers often differ in traits such as size and germination behavior (McNamara and Quinn 1977; Zeide 1978) that can influence offspring fitness and thereby confound inbreeding depression with the effects of nongenetic differences in seed phenotype. This second problem also compromises estimates of inbreeding depression based on

the fitness of progeny from hand-outcrossed CH flowers and progeny of CL flowers (McCall et al. 1989; Schmitt and Gamble 1990; Lu 2002).

Only two studies have estimated inbreeding depression from the fitnesses of offspring from selfed and outcrossed CH flowers of cleistogamous species. Trapp and Hendrix (1988) reported inbreeding depression for seed germination of *Amphicarpea bracteata* to be -41% . Culley (2000) reported inbreeding depression of 10% from seed set of hand crosses through adult progeny biomass for the perennial *Viola canadensis*. Estimates for both species are considerably less than 50%, indicating that selection would favor an increase in CL flowers at the expense of CH flowers, but neither estimate is likely to be accurate. The estimate for *A. bracteata* includes only one stage of the life cycle, and differences between inbred and outcrossed progeny expressed at later stages could affect the magnitude and even the direction of the estimate. The estimate for *V. canadensis* may be low because it is based on the relative fitnesses of progeny raised in a greenhouse, and on average, such estimates are lower than those for progeny grown in natural environments (Armbruster and Reed 2005).

In addition to population-level inbreeding depression, the rate and potentially the direction of mating-system evolution will be influenced by the amount of variation among lineages in the magnitude of inbreeding depression (Uyenoyama et al. 1993). Variation among families in the magnitude of inbreeding depression is common (Pico et al. 2004 and references therein) but has not been estimated for any cleistogamous species.

We estimated inbreeding depression from hand-selfed and outcrossed CH flowers for all life cycle stages from seed set through reproduction of the resulting progeny for the cleistogamous perennial *Viola septemloba*. We assessed the extent to which environmental conditions bias estimates by measuring inbreeding depression for both field- and greenhouse-grown parents and progeny. In addition to estimating population-level inbreeding depression, we also determined the pattern of variation in inbreeding depression among families.

Material and Methods

Study System

Viola septemloba (Violaceae) is a short-lived perennial cleistogamous herb found throughout the understory of southeastern coastal forests of the United States (Fernald 1970). It typically lives 8 yr or more. Adult *V. septemloba* produce CH flowers in the early spring and CL flowers in the late summer. In the field, large individuals produce an average of 1.5 flowers of each type per year (C. G. Oakley, unpublished data), and smaller plants may produce only CL flowers, indicating a minimum population selfing rate $>50\%$.

Experimental Design

In November 2001, we collected 30 adult *V. septemloba* from Saint Marks National Wildlife Refuge (St. Marks NWR) in Wakulla County, Florida, to serve as parents for self and outcross pollinations. We collected plants that were at least 5 m apart to minimize relatedness. Plants were transplanted into

4-in pots with a 3 : 1 mixture of potting soil and sand and grown in the greenhouse at Florida State University.

In January 2002, we performed controlled outcross and self-pollinations on each maternal plant that produced CH flowers (26 of the 30 plants). Unlike naturally occurring field plants, most of these greenhouse-grown plants produced three or more CH flowers. For outcross pollinations, recipient flowers were emasculated 2 d before anthesis, and a mesh bag was placed over the flower. Two days later, we applied pollen from a donor (chosen at random from among the remaining plants) to the stigma, using a dissecting probe, and rebagged the flower. For the self-pollinations, a mesh bag was placed over the flower 2 d before anthesis. At anthesis the flower was emasculated, pollen from the excised anthers was applied to the stigma, and the mesh bag was replaced. The same flower was used as dam and sire for the self-pollinations because the plants rarely produced more than one CH flower at a time. Pollinations were continued through March 2002, when CH flowering ceased, for a total of 74 pollinations on 26 maternal plants.

An additional 92 naturally occurring adults at St. Marks NWR were randomly chosen to serve as parents for crosses conducted in the field. For ca. 1 mo beginning in January 2002, controlled pollinations were conducted as described, for a total of 110 pollinations on 78 of these plants that produced CH flowers.

To determine whether the process of emasculation resulted in accidental self-pollination, we emasculated and bagged 62 flowers in the field and greenhouse in 2002. Because the self and outcross pollinations differed in the timing of emasculation, we conducted a separate assay to measure the effect of timing of emasculation on fruit set. We performed 74 controlled crosses in which approximately half of the flowers were emasculated and bagged 2 d before pollination and the other half were bagged 2 d before pollination and then emasculated immediately before pollination. These crosses were conducted in the field and the greenhouse over the course of two flowering seasons.

Fruit set was recorded for each hand-pollination, and we counted the number of seeds per fruit and determined the average seed weight per fruit. For maternal plants that produced at least one selfed and one outcrossed fruit (10 and eight maternal families from greenhouse and field crosses, respectively), we weighed each seed individually and germinated them in May 2002. We randomized the positions of seeds in wells of sand-filled flats in a seed germinator set at 10-h days with day and night temperatures of 20.5° and 18.5°C, respectively. When the cotyledons emerged, we scored germination, recorded the date, and then transplanted the seedlings into flats of field-collected soil in the greenhouse.

In October 2002, we recorded seedling survival and transplanted 65 seedlings from four maternal families (two to 29 seedlings of each cross type per maternal plant) into 4-in pots filled with field-collected soil in the greenhouse. All four maternal families were derived from the greenhouse crosses. We also transplanted four to 29 seedlings of each cross type from each maternal plant (360 total seedlings) from 14 maternal families (eight from the field and six from the greenhouse crosses) into the field site where parents had been collected at St. Marks NWR. The sets of families planted in the field and in the greenhouse did not overlap (fig. 1).

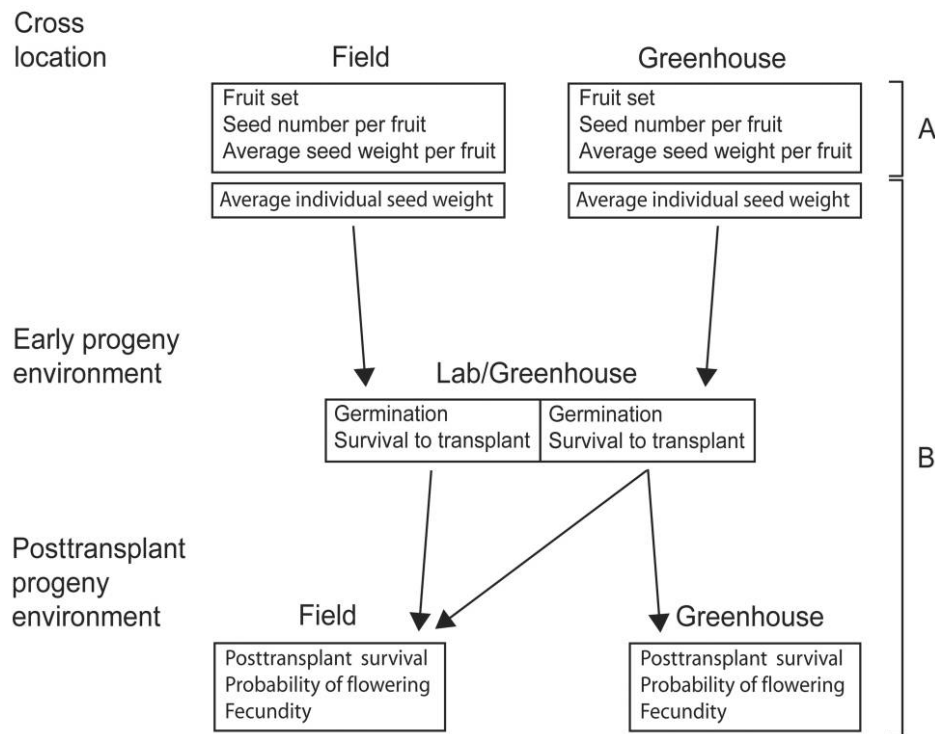


Fig. 1 Schematic diagram of the experimental design. Crosses were performed on maternal plants in the field and greenhouse. Seeds from maternal plants that produced at least one fruit per cross type were germinated in the lab and raised in the greenhouse for 5 mo. Seedlings from eight maternal plants crossed in the field and six maternal plants crossed in the greenhouse were transplanted in the field. Seedlings from the four remaining maternal plants crossed in the greenhouse were transplanted to pots and grown in the greenhouse. Survival, probability of flowering, and fecundity were measured in both progeny environments. Fitness components in *A* were estimated for all crosses; fitness components in *B* were estimated only for offspring from maternal plants that produced seeds for both cross types.

For each flowering season, we recorded whether plants survived and flowered. For each plant that flowered, we also recorded the number of seeds produced by each CL flower and the number of ovules produced by each CH flower. We monitored progeny grown in the greenhouse for 2 yr (2003 and 2004) and those grown in the field for 3 yr (2003–2005). We estimated fecundity for each individual as the sum of CL seed and CH ovule production. We used CH ovule number rather than seed number to estimate fecundity because low rates of pollination of CH flowers in the field (C. G. Oakley, personal observation) would have substantially reduced our sample sizes.

Data Analysis

We conducted two separate analyses of overlapping data sets to assess inbreeding depression at the population level and to test for variation in inbreeding depression among maternal plants. Within each level of analysis, we also describe inbreeding depression for progeny grown in two different environments. For the analysis of population-level inbreeding depression, we compared components of fitness for selfed and outcrossed pollinations with data from maternal families pooled. Fruit set, average seed number per fruit, and average seed weight per fruit were compared using data from all pollinations. All other progeny fitness components were compared using only data from maternal plants that produced at least one fruit from each cross type (fig. 1). To test for variation in the effects

of inbreeding among maternal plants, we conducted additional analyses comparing the fitness of selfed and outcrossed pollinations that included the maternal plant as a factor. These analyses necessarily included only data from maternal plants that produced at least one fruit from each cross type.

Unexpectedly, the location in which crosses were conducted had significant effects on some progeny traits (see “Results”). We therefore included cross location (greenhouse or field) as a factor in our analyses of both population- and family-level inbreeding depression and report differences between outcrossed and selfed progeny components of fitness separately for greenhouse and field crosses.

Population-Level Fitness Differences

For the analysis of population-level differences in the fitness of selfed and outcrossed pollinations, we tested the effects of cross location, cross type (self or outcross), and their interaction on probability of fruit set, seed germination, and survival of progeny until transplanting, using categorical models (Proc CATMOD). Posttransplant progeny survival and probability of flowering were analyzed in separate categorical models for progeny grown in the greenhouse and field. Effects of cross location, cross type, and their interaction on natural log-transformed average number of seeds per fruit, average seed weight per fruit, and progeny fecundity (CL seeds + CH ovules summed over the course of the experiment, for those plants

that produced flowers) were determined by ANOVA (Proc GLM). Analysis of fecundity of greenhouse progeny excluded the effects of cross location and the interaction between cross location and type because all plants were from crosses performed in the greenhouse.

Family-Level Fitness Differences

To test for variation in the effects of inbreeding among families, we analyzed individual seed weight, probability of seed germination, survival before and after transplanting, probability of flowering, and fecundity of transplanted progeny that produced flowers, including data for only the 18 maternal plants that produced both selfed and outcrossed seed. We were unable to test for differences among maternal plants for fruit set or number of seeds per fruit because most maternal plants produced only one fruit per cross type. We analyzed natural log-transformed seed weight and fecundity of progeny in separate mixed-model ANOVAs (Proc MIXED) in which cross location, cross type, and their interaction were treated as fixed effects, and maternal plant nested within cross location and maternal plant by cross type nested within cross location were treated as random effects. The significance of each random effect was tested with a χ^2 test of the difference in the -2 log-likelihood score between the full model and a model with one term removed, with 1 df (SAS 2000). For probability of germination, progeny survival to transplanting, posttransplant survival, and probability of flowering, the effects of cross location, cross type, maternal plant nested within cross location, and maternal plant by cross type interaction nested within cross location were examined in categorical models (Proc CATMOD). Posttransplant survival and probability of flowering were analyzed separately for progeny grown in the field and in the greenhouse. The analysis of posttransplant survival and probability of flowering in the greenhouse environment did not include the effect of cross location because all maternal plants were from the greenhouse (fig. 1). All analyses were performed with SAS, version 8.0 (SAS 2000).

Cumulative Fitness and Inbreeding Depression

Population-level cumulative fitness for progeny of each cross type was estimated separately for each cross location and progeny environment as the average total fecundity, including zero values for progeny that did not germinate, survive, and/or reproduce. This measure is based on data for the 18 maternal plants that produced seeds from both cross types and does not include differences in fruit set or seed number per fruit because the low per-plant flower production of this species made accurate estimation of these components infeasible. Population-level inbreeding depression was calculated for each progeny fitness component and for cumulative fitness, for each cross location, and for greenhouse- and field-grown progeny as $\sigma = 1 - W_{\text{self}}/W_{\text{outcross}}$, where W_{self} is the fitness of selfed progeny and W_{outcross} is the fitness of outcrossed progeny.

To describe the variation in the effects of inbreeding among families, we used the average fitness for offspring from each cross type for each maternal family to calculate the relative performance (RP) for each family as $RP_{\text{family}} = (W_{\text{family, outcross}} - W_{\text{family, self}})/\text{the larger of the two fitness estimates}$. This index ranges from -1 to 1 , giving equal weight to inbreeding and

outbreeding depression (Ågren and Schemske 1993; Pico et al. 2004). We also used our family-structured data to calculate confidence intervals around our population-level estimates of inbreeding depression, using the method of Johnston and Schoen (1994).

Results

Autogamy and Effects of Emasculation

Of the 62 flowers that underwent control emasculations, none set fruit, confirming that the fruits from outcross pollinations were not the result of accidental selfing. Fruit set for flowers subjected to the early emasculation treatment (35%) was considerably lower than for the late emasculation treatment (51%), although the difference was not statistically significant ($\chi^2 = 2.38$, $df = 1$, $P = 0.12$). Of the 92 flowers bagged for estimation of autogamous selfing, only one set fruit, indicating $<2\%$ autogamy of CH flowers.

Fitness Components for Selfed and Outcrossed Pollinations

Twenty-two plants in the greenhouse and 41 in the field produced seeds from at least one cross type. Self-pollinations were much more likely to set fruit than outcross pollinations for the field pollinations only (table 1), but neither cross location, cross type, nor their interaction had significant effects on fruit set (table 2). Likewise, the number of seeds per fruit was not significantly different between fruits produced by selfed and outcrossed pollinations ($F_{1,90} = 0.15$, $P = 0.70$; table 1), and it did not differ between the two cross locations ($F_{1,90} = 3.18$, $P = 0.08$). Cross location and cross type did not interact significantly ($F_{1,90} = 0.15$, $P = 0.70$). Average seed weight per fruit was significantly greater for crosses conducted in the greenhouse than for crosses conducted in the field ($F_{1,90} = 4.17$, $P = 0.04$; table 1), but neither cross type ($F_{1,90} = 0.13$, $P = 0.72$; table 1) nor the cross location by cross type interaction ($F_{1,90} = 1.27$, $P = 0.26$) had a significant effect. Cross location and the cross type by cross location interaction significantly affected probability of germination (table 2). Analyzed separately by cross location, the probability of germination for seeds from the greenhouse crosses was significantly greater for outcrossed than for selfed seeds ($\chi^2 = 7.56$, $df = 1$, $P = 0.006$; table 1), but the probabilities of germination for outcrossed and selfed seeds derived from crosses conducted in the field did not differ significantly ($\chi^2 = 0.23$, $df = 1$, $P = 0.63$; table 1).

Ninety-four percent of seedlings survived to the time of transplanting (table 1). Neither cross location nor cross type significantly affected survival to transplanting, but the interaction was marginally significant (table 2). Separate analyses for the two cross locations indicated no significant effect of cross type for the field crosses ($\chi^2 = 0.08$, $df = 1$, $P = 0.78$; table 1), but outcross progeny from the greenhouse crosses had a significantly greater probability of survival to transplanting than did selfed progeny ($\chi^2 = 5.87$, $df = 1$, $P = 0.02$; table 1). Only one transplant raised in the greenhouse failed to survive and reproduce, and selfed and outcrossed progeny did not differ significantly for either posttransplant survival or probability of reproducing (tables 1, 2). In the greenhouse,

Table 1
Population-Level Means (SD) for Fitness Components
of Outcross and Self-Progeny

Progeny environment and fitness component	Cross type	<i>n</i>	Mean (SD)	δ
Field location of cross:				
Field:				
Fruit set	Outcross	57	37% (49)	-.62
	Self	53	60% (49)	
Seed number per fruit	Outcross	21	13.6 (8.4)	-.05
	Self	32	14.3 (8.9)	
Average seed weight per fruit (mg)	Outcross	21	1.61 (.31)	.06
	Self	32	1.52 (.45)	
Posttransplant survival	Outcross	108	67% (47)	0
	Self	107	67% (49)	
Probability of flowering	Outcross	108	40% (49)	.35
	Self	107	26% (44)	
Fecundity	Outcross	43	52.0 (48.7)	.29
	Self	28	36.9 (26.8)	
Lab:				
Germination	Outcross	140	89% (32)	-.01
	Self	125	90% (30)	
Greenhouse:				
Survival to transplanting	Outcross	115	95% (22)	-.01
	Self	113	96% (21)	
Greenhouse location of cross:				
Greenhouse:				
Fruit set	Outcross	34	56% (50)	0
	Self	39	56% (50)	
Seed number per fruit	Outcross	19	11.3 (9.7)	.03
	Self	22	11.0 (8.3)	
Average seed weight per fruit (mg)	Outcross	19	1.83 (.55)	-.01
	Self	22	1.85 (.35)	
Survival to transplanting	Outcross	129	97% (17)	.09
	Self	101	88% (33)	
Posttransplant survival	Outcross	49	98% (14)	-.02
	Self	15	100%	
Probability of flowering	Outcross	49	98% (14)	-.02
	Self	15	100%	
Fecundity	Outcross	48	540 (143)	.11
	Self	15	480 (210)	
Lab:				
Germination	Outcross	147	88% (33)	.15
	Self	135	75% (44)	
Field:				
Posttransplant survival	Outcross	71	65% (48)	.15
	Self	73	55% (50)	
Probability of flowering	Outcross	71	31% (47)	-.10
	Self	73	34% (48)	
Fecundity	Outcross	22	50.1 (51.8)	.31
	Self	25	34.6 (44.5)	

Note. Means are presented separately by cross location. Population-level estimates of inbreeding depression (δ) are presented for each fitness component.

outcross progeny had, on average, 11% greater fecundity than did self-progeny, though this difference was not significant ($F_{1,61} = 2.61, P = 0.11$). For progeny raised in the field, cross location, cross type, and their interaction did not significantly affect posttransplant survival, probability of reproducing (table 2), or fecundity (results not shown), although, averaged across

cross locations, outcrossed progeny had 6% greater survival, were 24% more likely to reproduce, and had 44% greater fecundity than did self-progeny (table 1).

Variation among Families in Fitness Components of Selfed and Outcrossed Pollinations

For the 18 maternal plants that produced seeds from both cross types, cross type did not significantly affect average individual seed weight, but seeds produced by crosses made in the greenhouse were significantly larger than those produced in the field (table 3). The effect of maternal plant on seed weight was marginally significant, and there was a significant interaction between maternal plant and cross type (table 3). For germination, the effects of maternal plant nested within cross location and maternal plant by cross type interaction nested within cross location were highly significant (table 4). Survival to transplanting was not significantly affected by any variable (table 4). For the four maternal families grown in the greenhouse, maternal plant, cross type, and their interaction did not significantly affect posttransplant survival or probability of flowering (results not shown). For the progeny grown in the field, maternal plant nested within cross location was the only significant effect for both probability of flowering and posttransplant survival (table 4). Cross type, maternal plant, and their interaction did not significantly affect fecundity of plants grown in the greenhouse or in the field (results not shown).

Inbreeding Depression and RP

Stage-specific estimates for inbreeding depression were small and sometimes negative for all fitness components except progeny probability of flowering in the field and fecundity. Fecundity was the only stage at which estimates of inbreeding depression were consistently positive for all cross locations and progeny environments (table 1). Population-level inbreeding

Table 2
Effects of Cross Location, Cross Type, and
Their Interactions on Fruit Set and
Components of Progeny Fitness

Fitness component	Cross location	Cross type	Location by type
Fruit set	.99	2.55	2.33
Germination	5.72*	1.77	4.36*
Survival to transplanting	.38	2.17	3.52**
Posttransplant survival in the greenhouse	na	.36	na
Posttransplant survival in the field	1.86	.75	.99
Probability of flowering in the greenhouse	na	.36	na
Probability of flowering in the field	.00	1.06	2.79

Note. Table entries are χ^2 values from categorical analyses. Cross location is not relevant for survival in the greenhouse because only progeny from greenhouse crosses were raised in the greenhouse. na = not available.

* $P < 0.05$.

** $P = 0.06$.

Table 3
Results of ANOVA for Effects of Maternal Plant, Location of Cross, Cross Type, and Their Interactions on Natural Log-Transformed Individual Seed Weight

	χ^2	F	df	P
Random effects:				
Maternal plant (location)	3.7		1	.054
Maternal plant by type (location)	5.9		1	.015
Fixed effects:				
Location of cross		8.46	1	.010
Cross type		.03	1	.860
Location by type		2.92	1	.107

Note. Maternal plant was nested within cross location.

depression for cumulative fitness was estimated to be 48% for progeny derived from crosses performed in the greenhouse and raised in the greenhouse. For progeny raised in the field, population-level inbreeding depression was 25% for crosses performed in the greenhouse and 56% for crosses performed in the field. Ninety-five percent confidence intervals (CIs) for each of these estimates included 0, except for the plants from field crosses grown in the field (95% CI = 0.09–1.03).

Maternal families differed widely in cumulative RP (fig. 2). For plants from the greenhouse crosses grown in the greenhouse, estimates ranged from –0.23 to 0.67 (fig. 2, open bars). For plants from the greenhouse crosses grown in the field, estimates ranged from –0.64 to 0.89 (fig. 2, hatched bars). For plants from the field crosses grown in the field, the range was –1.0–0.97 (fig. 2, solid bars).

Discussion

We found evidence of moderately large cumulative inbreeding depression (average = 43%, range 25%–56%), although estimates for most life cycle stages were not significantly different from zero. This estimate is considerably larger than the two existing estimates of inbreeding depression for CL species of –41% (Trapp and Hendrix 1988) and 10% (Culley 2000) and greatly exceeds the negative value expected on the basis of the average 12% greater fitness of cleistogamous- than of chasmogamous-derived progeny based on data from 14 species (Oakley et al. 2007).

Germination and early survival for the greenhouse crosses were the only components of fitness for which selfed and outcrossed progeny differed significantly, but the larger estimates of inbreeding depression later in the life cycle match expectations for primarily selfing species (Husband and Schemske 1996). The observed absence of significant differences between self and outcross progeny at these later stages may be the result of reduced statistical power caused by diminishing sample sizes.

For a different perennial violet, Culley (2000) found no significant differences in components of fitness for selfed and outcrossed progeny except for 14% greater biomass of outcrossed progeny at 18 wk. Increasing magnitude of inbreeding depression through the life cycle in both her study and ours is consistent with the expected ability to purge early-acting lethal alleles but not later-acting and/or more mildly deleterious mu-

tations (Husband and Schemske 1996). Because *Viola septemloba* can live for eight or more years, inbreeding depression over the full lifetime may be greater than what we measured for 2–3 yr, both because later-expressed inbreeding depression may be stronger and because inbreeding depression can accumulate over the lifetime (Morgan et al. 1997). It should also be noted that our use of CH ovule number instead of CH seed production to estimate fecundity could bias our estimates of inbreeding depression if the rates of fertilization and seed set of CH flowers of plants derived from selfed seeds differ from those of CH flowers of plants derived from outcrossed seed.

Greater survival (greenhouse mean = 98%, field mean = 64%) and fecundity (greenhouse mean = 526, field mean = 44) of progeny raised in the greenhouse than of those raised in the field confirm that the greenhouse was a more benign environment. The qualitatively greater inbreeding depression we observed for probability of flowering and fecundity in the field relative to the greenhouse is therefore consistent with stronger expression of inbreeding depression in a more stressful environment (Dudash 1990; Armbruster and Reed 2005). One caveat to this conclusion is that, because different sets of maternal lineages were grown in the greenhouse and in the field, we cannot separate the effects of the environment from the effects of maternal lineage. We also note that, because all progeny were germinated in a growth chamber and started in the greenhouse, the comparison of inbreeding in the two environments does not include potential differences at these early life cycle stages.

Surprisingly, not only did the rearing environment of progeny influence the expression of inbreeding depression but the maternal environment appeared to as well. Significant differences between selfed and outcrossed progeny for both germination and survival to transplanting were found for progeny from greenhouse crosses but not for progeny from field crosses (tables 1, 2). It should be noted that when analyses included the effects of maternal plants, the cross location by cross type interaction was no longer significant, indicating that variation among maternal plants may be of greater importance than cross location with respect to the fitness of resulting progeny.

Table 4
Effects of Cross Location, Cross Type, Maternal Plant Nested within Cross Location, and Interactions on Components of Progeny Fitness

Effect	Germination	Survival to transplant	Probability of flowering	Posttransplant survival
Cross location	.02	.59	.69	.00
Cross type	.10	.12	.04	.40
Location by type	.85	.22	.51	.15
Maternal plant (location)	67.84**	19.94	23.99*	51.16**
Maternal plant by type (location)	46.37**	6.43	11.61	15.21

Note. Table entries are χ^2 values from categorical analyses. Posttransplant survival is for progeny grown in the field.

* $P < 0.05$.

** $P < 0.001$.

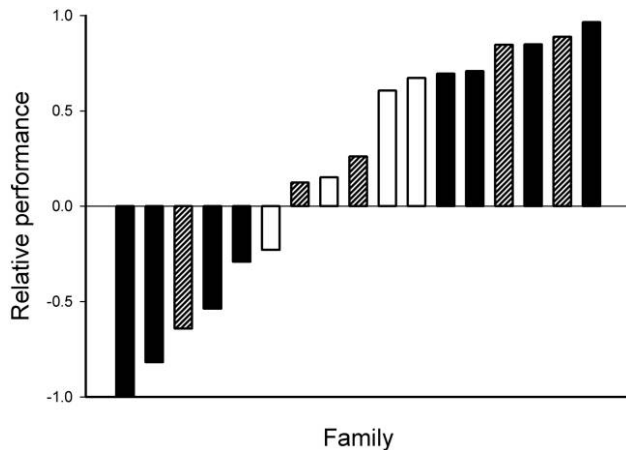


Fig. 2 Cumulative relative performance (the difference between outcross family fitness and self family fitness divided by the larger of the two fitness estimates) for maternal families. Each bar denotes a family. Open bars denote greenhouse pollinations grown in the greenhouse; hatched bars, greenhouse pollinations grown in the field; and solid bars, field pollinations grown in the field. One family was omitted because none of its progeny had fitness greater than zero.

We found evidence of significant family-level variation in the effect of cross type on some early components of progeny fitness (tables 3, 4, maternal plant by cross type interactions), and differences in cumulative RP among families was substantial (fig. 2). Differences among families in the effect of cross type on natural log-transformed fitness components have been demonstrated in several species that are not cleistogamous (Ågren and Schemske 1993; Pico et al. 2004) and are necessary, but insufficient, evidence for genetic variation for inbreeding depression (Johnston and Schoen 1994). Maternal effects, differences in maternal inbreeding history, and several different genetic mechanisms can result in differences in the effect of cross type among families and may be misinterpreted as genetic variation for inbreeding depression (Fox 2005; Kelly 2005; Moorad and Wade 2005). Only crossing designs that include pollen and ovule donors of known inbreeding history and that mate ovule parents with multiple pollen donors can resolve these uncertainties (Kelly 2005; Moorad and Wade 2005; Kelly and Tourtellot 2006).

Role of Inbreeding Depression in the Maintenance of CH Reproduction

Our overall estimate of inbreeding depression is below the 50% threshold necessary to maintain outcrossing in general

models of mating-system evolution. This threshold is likely to be even higher for cleistogamous species because selfing by means of CL flowers provides additional advantages from greater fruit set and greater energetic economy. In a review of cleistogamous species, Oakley et al. (2007) reported that CL flowers were three times more likely to set fruit than CH flowers and were only half as costly to produce. Undoubtedly, inbreeding depression contributes to maintenance of CH reproduction by balancing some of the advantages of CL selfing, but it is clearly not sufficient to maintain CH flower production on its own.

Lu (2002) has suggested that the production of CH flowers can be maintained despite the economy of CL reproduction and modest population-level inbreeding depression if lines that have purged deleterious mutations through several successive generations of CL selfing then benefit from heterosis upon outcrossing, which can be accomplished only by CH reproduction. Data for the cleistogamous annual *Impatiens capensis* indicating greater ovule fertilization rates for crosses between inbred parents than between those derived from recent outcrossing are qualitatively consistent with Lu's model (Lu 2002), but a quantitative evaluation would require estimating the full magnitude of heterosis in progeny from crosses between inbred lines relative to inbreeding depression in their progeny produced through CL selfing.

Although our estimate of inbreeding depression for *V. septemloba* exceeds estimates previously reported for cleistogamous species, it does not explain the maintenance of CH outcrossing. Empirical tests of Lu's (2002) hypothesis may provide evidence for an additional advantage of CH outcrossing that is missing from our current understanding of what maintains mixed mating in cleistogamous species. Additionally, because genetic variation in inbreeding depression and selfing rates can play important roles in mating-system evolution, future studies to quantify the extent of genetic variation for the expression of inbreeding depression among families (Kelly 2005) and to describe the genetic basis for variation in the proportion of flowers that are cleistogamous should further elucidate the forces that maintain CH outcrossing.

Acknowledgments

We thank K. S. Moriuchi for assistance and discussion throughout all phases of this research and J. H. Burns for assistance in the greenhouse. G. Clarke and E. Jones assisted in creating the figures. We thank two anonymous reviewers for comments that greatly improved the manuscript. This work was supported by National Science Foundation grant DEB-9903878 to A. A. Winn.

Literature Cited

- Ågren J, DW Schemske 1993 Outcrossing rate and inbreeding depression in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata*. *Evolution* 47:125–135.
- Armbruster P, DH Reed 2005 Inbreeding depression in benign and stressful environments. *Heredity* 95:235–242.
- Charlesworth D, B Charlesworth 1987 Inbreeding depression and its evolutionary consequences. *Annu Rev Ecol Syst* 18:237–268.
- Cole CT, DD Biesboer 1992 Monomorphism, reduced gene flow, and cleistogamy in rare and common species of *Lespedeza* (Fabaceae). *Am J Bot* 79:567–575.
- Culley TM 2000 Inbreeding depression and floral type fitness differences in *Viola canadensis* (Violaceae), a species with chasmogamous and cleistogamous flowers. *Can J Bot* 78:1420–1429.
- 2002 Reproductive biology and delayed selfing in *Viola pubescens* (Violaceae), an understory herb with chasmogamous and cleistogamous flowers. *Int J Plant Sci* 163:113–122.

- Culley TM, MR Klooster 2007 The cleistogamous breeding system: a review of its frequency, evolution, and ecology in angiosperms. *Bot Rev* 73:1–30.
- Dudash MR 1990 Relative fitness of selfed and outcrossed progeny in a self-compatible, protandrous species, *Sabatia angularis* L. (Gentianaceae): a comparison in three environments. *Evolution* 44:1129–1139.
- Fernald ML 1970 Gray's manual of botany. Van Nostrand, New York. 1632 pp.
- Fox CW 2005 Problems in measuring among-family variation in inbreeding depression. *Am J Bot* 92:1929–1932.
- Goodwillie C, S Kalisz, CG Eckert 2005 The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annu Rev Ecol Syst* 36:47–79.
- Husband BC, DW Schemske 1996 Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* 50:54–70.
- Johnston MO, DJ Schoen 1994 On the measurement of inbreeding depression. *Evolution* 48:1735–1741.
- Kelly JK 2005 Family level inbreeding depression and the evolution of plant mating systems. *New Phytol* 165:55–62.
- Kelly JK, MK Tourtellot 2006 The genetic analysis of family structured inbreeding depression studies. *Heredity* 97:346–354.
- Lande R, DW Schemske 1985 The evolution of self-fertilization and inbreeding depression in plants. 1. Genetic models. *Evolution* 39:24–40.
- Lloyd DG 1979 Some reproductive factors affecting the selection of self-fertilization in plants. *Am Nat* 113:67–79.
- Lu YQ 2000 Effects of density on mixed mating systems and reproduction in natural populations of *Impatiens capensis*. *Int J Plant Sci* 161:671–681.
- 2002 Why is cleistogamy a selected reproductive strategy in *Impatiens capensis* (Balsaminaceae)? *Biol J Linn Soc* 75:543–553.
- Masuda M, T Yahara, M Maki 2001 An ESS model for the mixed production of cleistogamous and chasmogamous flowers in a facultative cleistogamous plant. *Evol Ecol Res* 3:429–439.
- McCall C, T Mitchell-Olds, DM Waller 1989 Fitness consequences of outcrossing in *Impatiens capensis*: tests of the frequency-dependent and sib-competition models. *Evolution* 43:1075–1084.
- McNamara J, JA Quinn 1977 Resource-allocation and reproduction in populations of *Amphicarpum purshii* (Gramineae). *Am J Bot* 64:17–23.
- Mitchell-Olds T, DM Waller 1985 Relative performance of selfed and outcrossed progeny in *Impatiens capensis*. *Evolution* 39:533–544.
- Moorad JA, MJ Wade 2005 A genetic interpretation of the variation in inbreeding depression. *Genetics* 170:1373–1384.
- Morgan MT, DJ Schoen, TM Bataillon 1997 The evolution of self-fertilization in perennials. *Am Nat* 150:618–638.
- Oakley CG, KS Moriuchi, AA Winn 2007 The maintenance of outcrossing in predominantly selfing species: ideas and evidence from cleistogamous species. *Annu Rev Ecol Syst* 38:437–457.
- Pico FX, NJ Ouborg, JM Van Groenendael 2004 Evaluation of the extent of among-family variation in inbreeding depression in the perennial herb *Scabiosa columbaria* (Dipsacaceae). *Am J Bot* 91:1183–1189.
- SAS 2000 SAS/STAT user's guide, version 8. SAS Institute, Cary, NC.
- Schemske DW 1978 Evolution of reproductive characteristics in *Impatiens* (Balsaminaceae): significance of cleistogamy and chasmogamy. *Ecology* 59:596–613.
- Schmitt J, SE Gamble 1990 The effect of distance from the parental site on offspring performance and inbreeding depression in *Impatiens capensis*: a test of the local adaptation hypothesis. *Evolution* 44:2022–2030.
- Schoen DJ, DG Lloyd 1984 The selection of cleistogamy and heteromorphic diaspores. *Biol J Linn Soc* 23:303–322.
- Trapp EJ, SD Hendrix 1988 Consequences of a mixed reproductive system in the hog peanut, *Amphicarpaea bracteata* (Fabaceae). *Oecologia* 75:285–290.
- Uyenoyama MK, KE Holsinger, DM Waller 1993 Ecological and genetic factors directing the evolution of self-fertilization. *Oxf Surv Evol Biol* 9:327–381.
- Waller DM 1979 Relative costs of self-fertilized and cross-fertilized seeds in *Impatiens capensis* (Balsaminaceae). *Am J Bot* 66:313–320.
- Waller DM, SE Knight 1989 Genetic consequences of outcrossing in the cleistogamous annual, *Impatiens capensis*. 2. Outcrossing rates and genotypic correlations. *Evolution* 43:860–869.
- Zeide B 1978 Reproductive behavior of plants in time. *Am Nat* 112:636–639.