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AUTOGAMOUS SELFING ABILITY IN *COLLINSIA VERNA*

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THE EVOLUTION OF POPULATION DIFFERENTIATION OF AUTOGAMOUS SELFING ABILITY IN *COLLINSIA VERNA*

By

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ABSTRACT

THE EVOLUTION OF POPULATION DIFFERENTIATION OF AUTOGAMOUS SELFING ABILITY IN *COLLINSIA VERNA*

By

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Pollinator service may be one of the most important agents of selection on selfing. When pollinator service is relatively good, selection may decrease selfing ability to minimize the number of low quality selfed offspring that are produced. When pollinator service is poor, selection may increase selfing ability because many selfed offspring provide more fitness than producing no offspring, or very few outcrossed offspring. Therefore, differences in pollinator service across populations may lead to differences in selection on selfing ability, and produce adaptive differentiation of the trait. This hypothesis may explain why population differentiation in selfing ability is often observed in species that can reproduce through a mix of selfing and outcrossing. However, there are few empirical studies of this hypothesis.

In my dissertation research, I assayed differentiation of selfing ability across populations of the mixed-mating *Collinsia verna* from throughout its geographic range. I used three approaches to test the hypothesis that differences in selfing ability evolved as an adaptive response to differences in pollinator service across populations. I found a significant negative correlation between selfing ability and pollinator service, which was consistent with the hypothesis that adaptive differentiation of selfing ability arose from pollinator service. However, I discovered that genetic drift alone could have produced the amount of genetic differentiation in selfing ability across populations that I observed,

and therefore, differential selection may not have played a role in differentiation of selfing ability. Additionally, by measuring selection on selfing ability in a population under natural and experimentally reduced levels of pollinator service, I found no evidence that differences in pollinator service produce differences in selection on selfing ability though female seed production. There was strong positive selection on selfing ability in both pollination environments, which suggests that in many pollination conditions, populations may experience selection to improve selfing ability.

This research demonstrates that differences in selfing ability across populations of mixed-mating species may not always be adaptive. Further, this research highlights the importance of measuring selection on selfing ability through a measure of fitness that incorporates inbreeding depression and male fitness. Selection on selfing ability though female seed production may commonly be positive, but it remains untested whether differences in pollinator service would produce differences in selection on selfing ability if a more comprehensive measure of fitness were used.

This dissertation is dedicated to my family and my teachers

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INTRODUCTION

Background

The transition from outcrossing to selfing has occurred many times in plant evolutionary history (Stebbins 1974; Barrett et al. 1996; Schoen et al. 1997; Goodwillie 1999; Takebayashi and Morrell 2001). An outcrossing plant that acquires the ability to self while still siring offspring on other plants has two main advantages over other outcrossing plants in its population: 1) the selfer transmits two copies of its genes to selfed offspring, whereas outcrossers only transmit one copy of their genes to maternally produced offspring (Fisher 1941), and 2) the selfer can reproduce when pollinators are absent (Lloyd 1979). The first advantage suggests that when inbreeding depression is high, selection should disfavor the selfer; however, because inbreeding depression is probably due mostly to deleterious recessive alleles (Roff 2002) that can be purged with selfing, inbreeding depression can evolve with the mating system (Lande and Schemske 1985; Byers and Waller 1999; Crnokrak and Barrett 2002). The second advantage suggests that pollinator service is an important agent of selection on the mating system; when pollinator service is poor, selection should favor the selfer, but when pollinator service is good and inbreeding depression is high, selection should favor the outcrosser. Comparative evidence suggests that the transition to selfing has often been an adaptive response to pollinator service; selfing species are often found in habitats where pollinators are lacking (e.g. Vasek 1964, Solbrig and Rollins 1977, Rick 1966).

In species that regularly reproduce through both selfing and outcrossing, the direction of selection on selfing ability may be determined by pollinator service as well.

Increased selfing ability should always be advantageous when there is no trade-off between selfing and outcrossing proficiency, because only ovules that cannot be outcrossed are self-pollinated (Lloyd 1979). However, if the ability to self interferes with outcrossed seed production (Lloyd 1992), and inbreeding depression is relatively high, then the direction of selection on selfing ability would depend on the quality of pollinator service. When pollinator service is relatively good, selection may decrease selfing ability to minimize the loss of outcrossed offspring; when pollinator service is poor, selection may increase selfing ability because there are few outcrossed offspring to lose. Hence, long-term differences in pollinator service between populations may lead to adaptive differentiation of selfing ability.

Variation in autogamous selfing ability across populations of mixed-mating species is common (e. g. Schoen 1982; Belaoussoff and Shore 1995). However, in most species, the hypothesis that differentiation in selfing ability was an adaptive response to differences in pollinator service has not been directly tested (but see Fenster and Ritland 1994; Elle and Carney 2003; Moeller and Geber 2005). Since differentiation in selfing ability is so common, it is important to understand whether selection has created this pattern, and if so, which agents of selection are important. Further, tests of adaptive differentiation of selfing ability within species may inform the hypothesis that differentiation of mating system across species was adaptive. My dissertation research focuses on testing the hypothesis that population differentiation in selfing ability in the mixed mating species *Collinsia verna* was an adaptive response to different levels of pollinator service across populations.

Organization of the Dissertation

Chapter 2: In collaboration with Jeffrey Conner, I investigated how variation in autogamous selfing ability across populations of the mixed-mating species *Collinsia verna* corresponds to variation in floral morphology, the mating system, and one potential agent of selection on autogamous selfing ability, pollinator service. For 11 populations from most of the geographic range of the species, we measured autogamous selfing ability and floral traits in the greenhouse, and outcrossing rates and pollinator service in the field. We found significant variation in autogamous selfing ability, and that across-population correlations between selfing ability, herkogamy, and flower size were in the same direction as across-species correlations for the same traits. In contrast to studies in other mixed-mating species, there was no relationship between selfing ability and outcrossing rate across populations, suggesting that selfing ability does not have a deterministic effect on the mating system. Consistent with the hypothesis that differences in pollinator service across populations have driven adaptive differentiation of selfing ability, pollinator service was negatively correlated to selfing ability, but only during the early sampling time of the season.

Chapter 3: Because the negative correlation between pollinator service and selfing ability across populations of *C. verna* was consistent with the hypothesis that population differentiation in selfing ability is adaptive, in collaboration with Jeffrey Conner and Frank Shaw, I examined the degree to which differentiation in autogamous selfing ability across the 11 study populations was due to natural selection versus genetic drift. We compared genetic differentiation for autogamous selfing ability and correlated floral traits across populations (Q_{ST}) to differentiation at presumptively neutral microsatellite loci (F_{ST}). We found no evidence for adaptive differentiation of selfing ability or herkogamy, as genetic divergence for these traits was similar to divergence due to genetic drift. In contrast, differential selection has contributed substantially to divergence of daily flower production and flower size across populations. Our results suggest that if pollinator service is an important agent of selection on selfing ability, differences in pollinator service have not been consistent enough to create adaptive differentiation in the trait. Current selection on selfing ability, herkogamy, and flower size could result in an evolutionary response because we detected significant additive genetic variance in populations for these traits. We also estimated within population genetic correlations among selfing ability and floral traits that were correlated with selfing ability across populations. We found a strong negative genetic correlation between flower size and daily flower production, which provides evidence for a genetic size-number tradeoff. Genetic correlations of selfing ability with other floral traits were in the same direction as across-population correlations, but they were not significantly different than zero.

Chapter 4: To test the hypothesis that differences in pollinator service led to differences in selection on selfing, in collaboration with Jeffrey Conner, I measured selection on autogamous selfing ability under two levels of pollinator service: natural pollinator service, and natural pollinator service reduced by half. We estimated selection in a population of *C. verna* that was previously found to receive high levels of pollinator service. We also measured selection on herkogamy and floral traits that were correlated with selfing ability or herkogamy. Reducing pollinator visitation by half had no effect on selection on selfing ability or any of the other floral traits via female seed production. Selfing ability and flower production both had strong positive effects on fruit production, number of seeds per fruit, and seed production in both pollination treatments. The only other trait on which we found selection was flower size; flower size had a negative effect on number of seeds per fruit under reduced pollination. We infer that plants in both treatments were pollen limited, otherwise, we could not explain the increase in seed production that was produced by increased selfing ability. Our results demonstrate that large differences in pollinator service across populations would not necessarily lead to differences in selection on selfing ability or other floral traits through female seed production.

Chapter 5: I conclude my thesis with a summary of conclusions from the previous chapters. I also suggest directions for future research that would clarify results from the studies presented in my thesis as well as expand our understanding of adaptive differentiation of selfing.

CHAPTER 2

RELATING SELFING ABILITY TO FLORAL MORPHOLOGY, THE MATING SYSTEM, AND POLLINATOR SERVICE ACROSS POPULATIONS OF *COLLINSIA VERNA*

Abstract

Autogamous selfing ability often varies across populations of mixed-mating species. Understanding how this variation corresponds to variation in floral morphology and the mating system across populations may be important for understanding how the mating system evolves at the species level. Further, determining whether selfing ability is correlated with pollinator service across populations identifies a potential agent of selection that may have been responsible for differentiation of selfing ability across populations and species. For 11 populations of the mixed-mating *Collinsia verna* from across the geographic range of the species, we measured autogamous selfing ability and floral traits in the greenhouse, and outcrossing rates and pollinator service in the field. We found significant variation in selfing ability across populations. Selfing ability was negatively correlated with herkogamy and flower size across populations, similar to the pattern reported across species in this genus. In contrast to studies in other mixed-mating species, we found no correspondence between selfing ability and outcrossing rate across populations of *C. verna*. This suggests that selfing ability does not have a deterministic effect on the mating system, and that geitonogamous selfing or pollinator service may contribute significantly to the mating system. Pollinator service was negatively correlated to selfing ability during the early sampling time only. This result supports the hypothesis that differences in pollinator service led to adaptive differentiation in selfing

ability and suggests that pollinator service early in the season may have had greater impact on total selection on selfing ability than pollinator service late in the season.

Introduction

Evolutionary biologists have sought adaptive explanations for the diverse reproductive strategies employed by plants since Darwin (1876). The existence of primarily selfing and primarily outcrossing plant species inspired early work describing the relative advantages of selfing versus outcrossing as a function of both genetics and the environment (Knight 1799; Darwin 1859, 1876; Henslow 1879; Muller 1883). For example, selfing can automatically provide a two-fold transmission advantage because selfers may transmit two copies of their genes to selfed offspring and one copy to sired, outcrossed offspring, whereas outcrossers transmit only one copy of their genes to both maternal and sired offspring (Fisher 1941). However, selfing may also result in inbreeding depression (Charlesworth and Charlesworth 1987); in most outcrossing species, inbreeding depression is severe enough to outweigh the genetic transmission advantage of selfing (Husband and Schemske 1996).

The reproductive assurance hypothesis predicts that plants experience selection to self because doing so guarantees reproduction where pollinators are lacking (Lloyd 1979), outcross pollen is lacking (during colonization events or low population densities) (Baker 1955; Stebbins 1957; Pannell and Barrett 1998; Lloyd 1992), or interspecific pollen deposition interferes with cross-fertilization (Antonovics 1968; Levin 1972; Fishman and Wyatt 1999). The reproductive assurance hypothesis accurately predicts patterns of mating system variation observed across species. For example, comparative studies demonstrate that primarily selfing species predominate in marginal or island habitats where pollinators are rare (e.g. Vasek 1964; Solbrig and Rollins 1977). Further, animal-pollinated species are commonly observed to be pollen limited (reviewed by Burd 1994; Knight et al. 2005) and may be more prone to pollen limitation than wind-pollinated species, and mixed-mating species are more likely to by pollinated by animals than wind (Aide 1986; Vogler and Kalisz 2001). Therefore, even though inbreeding depression is common in mixed-mating species (Husband and Schemske 1996; Scofield and Schultz 2006), selfing can increase fitness by fertilizing ovules that would not be outcrossed.

The reproductive assurance hypothesis may also help explain the observation that autogamous selfing ability, or the rate at which flowers self-fertilize in the absence of pollinators, varies across populations of mixed-mating species (e. g. Schoen 1982; Belaoussoff and Shore 1995). High selfing ability may be adaptive in populations with low pollinator service because the benefit of reproductive assurance is greater. If selfing occurs after all opportunity for outcrossing has passed (delayed selfing), all populations in pollen-limited environments should experience selection to increase selfing ability regardless of the amount of inbreeding depression (Lloyd 1979). However, if selfing occurs before or during the period of outcrossing, ovules or pollen that would otherwise have been used in outcrossing may be usurped by selfing (seed and pollen discounting; Lloyd 1992; Harder and Wilson 1998). Gamete discounting can impose a high fitness cost to selfing if inbreeding depression is high. For example, Herlihy and Eckert (2002) demonstrated that the ability to self-fertilize lowered fitness in almost half of *Aquilegia canadensis* populations in their study because the maternal seed production benefit that selfing provided was more than counteracted by loss in fitness due to seed discounting and inbreeding depression. Therefore, in mixed-mating species, if inbreeding depression is high and gamete discounting occurs, the net fitness effect of selfing would depend on the frequency of cross pollination.

There is little empirical evidence for or against the hypothesis that variation in pollinator service results in adaptive differentiation of selfing ability. However, the plausibility of this hypothesis was supported by two studies that demonstrated that reduced pollinator service led to positive selection on autogamous selfing ability (Fenster and Ritland 1994; Moeller and Geber 2005). An important, but rarely tested, prediction of the adaptive hypothesis is that selfing ability is negatively correlated with average pollinator service across populations. Moeller (2006) found that increasing selfing ability was associated with decreasing specialist pollinator visitation rates across populations of *C. xantiana*, but Herrera et al. (2001) found no relationship between selfing ability and pollinator visitation rates across populations of *Helleborus foetidus*. Herrera et al. (2001) suggested that temporal variation in pollinator service within populations might have prevented adaptive differentiation of selfing ability in *H. foetidus*.

Variation in selfing ability across populations may correspond to variation in outcrossing rate. This relationship is clearly demonstrated by negative correlations between outcrossing rate and autogamous selfing ability (Schoen 1982), or traits that are expected to determine selfing ability, such as stigma-anther separation in time (protandry) or space (herkogamy) (Barrett and Husband 1990; Holtsford and Ellstrand 1992; Belaoussoff and Shore 1995). The simplest explanation for a negative relationship between selfing ability and outcrossing rate is that plants with low selfing ability cannot have low outcrossing rates unless pollinator service is extremely poor; whereas plants with high selfing ability can have the full range of outcrossing rates, but are more likely to have low outcrossing rates because plants with high selfing ability would produce more autogamously selfed seeds than plants with low selfing ability. Additionally, the difference in outcrossing rate between plants with high and low selfing ability would be more dramatic if selfing interferes with outcrossing (seed discounting). If seed discounting occurs, plants with high selfing ability would produce more autogamously selfed seed and fewer outcrossed seed than plants with low selfing ability. In addition to cases in which pollinator service is extremely poor, the correlation between selfing ability and outcrossing rate may not be found when differences in autogamously selfed seed production or seed discounting are minor relative to differences in pollinator service across populations. As a result, outcrossing rate would be correlated with pollinator service, but not selfing ability. Finally, geitonogamy (self pollination with pollen from different flower on the same plant) could obscure the relationship between autogamous selfing ability and outcrossing rate because the amount by which geitonogamy lowers the outcrossing rate is not expected to covary with selfing ability.

Covariation between selfing ability and outcrossing rate may produce a negative relationship between selfing ability and flower size across populations (e. g. Rick et al. 1978). Populations with low pollinator service may experience selection to increase selfing ability. Consequently, outcrossing rates would be lower in populations, and selection would minimize resources devoted to pollinator attraction traits. By this scenario, pollinator attraction traits and selfing ability may become negatively correlated across populations.

Across species in the genus *Collinsia*, higher levels of outcrossing are associated with more herkogamy, more delayed selfing, and increased flower size (Armbruster et al. 2002). Relative to other members in the genus, *Collinsia verna* has relatively large flowers, delayed selfing, high herkogamy, and high outcrossing rates (0.6-1.0 for populations in three states, Kalisz et al. 1999; Kalisz et al. 2004). The potential for selfing to provide reproductive assurance by increasing maternal fruit production was demonstrated in three Pennsylvania populations (Kalisz and Vogler 2003). However, because self-fertilization can occur while outcrossing is possible (Kalisz et al. 1999), selfing may result in gamete discounting. Therefore, we hypothesize that selection on selfing ability depends on pollinator service, and differences in pollinator service across populations should lead to adaptive differentiation of selfing ability. In *C. verna,* the degree of population differentiation in autogamous selfing ability and its correspondence with population outcrossing rates and pollinator service are unknown. Additionally, the floral traits that covary with selfing ability across populations have not been identified.

In this study, we used 11 populations of *C. verna* from across the species range to understand the relationship between selfing ability, floral morphology, and the mating system, and to test the hypothesis that variation in selfing ability is adapted to differences in pollinator service. Because the degree of variation in selfing ability was unknown in *C. verna*, we first assay selfing ability in the 11 populations. We then test the following four hypotheses: 1) selfing ability is negatively correlated with herkogamy and flower size across populations, 2) selfing ability is negatively correlated with outcrossing rates across populations, 3) selfing potentially provides reproductive assurance to *C. verna,* and 4) differentiation of autogamous selfing ability across populations resulted from differential levels of pollinator service. This study is the first to examine variation in the mating system of a mixed-mating species using populations from most of the geographic range.

Methods

Study Species and Populations

Collinsia verna (Plantaginaceae), blue-eyed Mary, is a winter annual that grows in woodlands in the eastern United States and flowers in the spring. Population outcrossing rates range from 60-100% (Kalisz et al. 1999, Kalisz et al. 2004). Plants have 3-7 flowers per whorl, 2-7 whorls per stem, and one to many stems. Flowers are bilaterally symmetrical, with five petals. One petal is folded into a keel that encloses the style and four stamens. During the 5-7 days that a flower is open, one stamen elongates and its anther dehisces per day (Kalisz et al. 1999). After Kalisz et al. (1999), we refer to stages in flower development by the number of dehisced anthers (i.e., Stage 1 flower has one dehisced anther). Large and small bees deliver most outcross pollen (Kalisz and Vogler 2003; F. Knapczyk, unpublished data).

Self pollination can result from contact between anthers and stigma. Most stigmas become receptive between Stage 3 and 4; by Stage 4, stigmas usually bear enough self-pollen (median of 20 grains) to fertilize all four ovules (Kalisz et al. 1999). Self pollination can also occur during corolla abscission if the stigma contacts self-pollen collected in the keel (Kalisz et al. 1999). Finally, geitonogamous selfing may occur, as plants usually have multiple flowers open at once; little is known about the frequency of geitonogamy in *C. verna*. Most autogamous self fertilization is inferred to take place between stage 4 and corolla dehiscence. However, observed variation in timing of stigma receptivity and contact between anthers and stigma in this species, and in *C. heterophylla*, a closely related species, suggests that self fertilization may also occur at the same time as outcrossing in some genotypes (Kalisz et al. 1999; Lankinen et al. 2007).

To study variation in the mating system of *C. verna*, we sampled populations spanning most of its geographic range (Table 2.1; Figure 2.1). Nine of 11 populations were very large in size (>10^5 individuals); MI2 consisted of ~5000 individuals and OH1 consisted of ~3000 individuals. All populations were in mesic woodlands near streams or rivers, but no two populations were located in the same drainage. The closest distance between two populations was 29 miles. PA is population EF in Kalisz and Vogler (2003) and Kalisz et al. (2004). The rank order of peak flowering across populations was consistent in the two years of this study, and spanned up to six weeks (first week of April to second week of May). Because of the large spatial scale of sampling, we expected populations to vary in pollinator and plant community composition as well as abiotic factors.

Measurement of Selfing Ability and Floral Traits

Fifty plants from each population were collected just prior to seed dispersal in May and June 2003. Sampled plants were located at evenly dispersed grid points that covered the entire population. The minimum distance between two plants in any of the populations was approximately 2 meters. Plants were dried in paper bags until seeds were released from fruits. We planted 20 randomly chosen seeds from each individual in Sunshine mix (Fafard, Agawam, MA) in seedling trays; planting was completed by the end of June. Seeds were kept in the greenhouse at Kellogg Biological Station at approximately 32C-day: 15C-night under ambient light, and were routinely watered until

Table 2.1. Location of 11 populations of *C. verna* surveyed in this study; populations are listed in geographic order from west to east.

Population	Location	County	Latitude/Longitude
MO	Graham Cave State Park	Montgomery, MO	38°55'N/91°35'W
IL	Allerton Park	Piatt, IL	39°59'N/88°40'W
TN	Taylor Hollow Preserve	Sumner, TN	36°30'N/86°54'W
MI1	Russ Forest	Cass, MI	42°01'N/85°54'W
MI2	Kalamazoo Nature Center	Kalamazoo, MI	42°21'N/85°33'W
KY	Raven Run Nature Sanctuary	Fayette, KY	37°54'N/84°24'W
OH1	Odell, OH	Guernsey, OH	39°36'N/83°54'W
OH3	Hockhocking-Adena Bikeway	Athens, OH	39°24'N/82°08'W
OH2	Salt Fork State Park	Clinton, OH	40°08'N/81°28'W
WV	Reader, WV	Wetzel, WV	39°35'N/80°45'W
PA	Enlow Fork Natural Area	Greene, OH	39°56'N/80°27'W

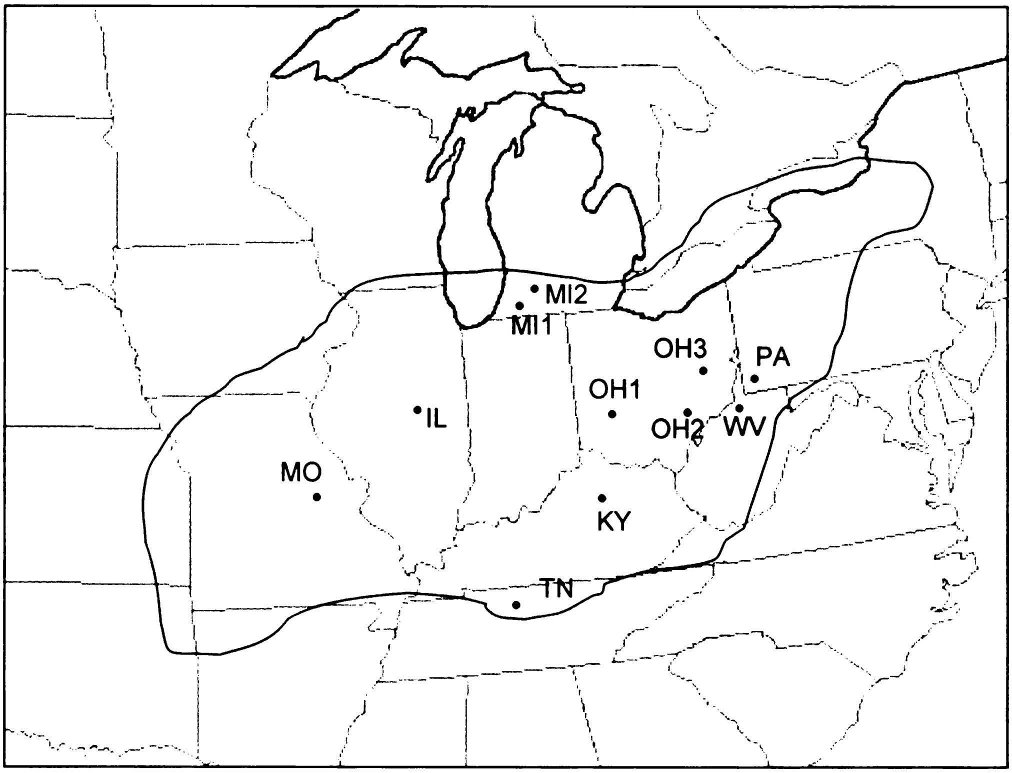


Figure 2.1. Geographic range of *C. verna* estimated from US county distribution maps at http://plants.usda.gov and Ontario, Canada distribution maps of Argus et al. (1982-1987). Location of 11 study populations are plotted.

September, when they were placed into environmental chambers for vernalization. Temperatures were ramped down from 20C-day: 14C-night to 8C-day: 4C-night over eight days. Germination began after two weeks of vernalization and continued for nine weeks. Two weeks after the last seedling germinated, we transplanted five seedlings from each family into 3-in pots filled with MetroMix 360 (Scotts-Sierra, Marysville, OH) mixed with 1/8 tsp of Osmocote Plus 15-9-12 (NPK) controlled release pellets (Scott's Miracle-Gro, Marysville, OH). One seedling was planted in each pot. Between 40 and 52 families from ten populations germinated; only 10 families germinated from TN. Plants were grown in a pollinator-free greenhouse maintained at 20C-day: 14C-night.

For one plant per family, we measured three traits that were correlated with outcrossing rate across *Collinsia* species (Armbruster et al. 2002): autogamous selfing ability (percent of flowers that set fruit in the absence of pollinators), herkogamy (distance between stigma and anther), and flower size. Floral traits were measured from digital photographs of stage 4-flowers; most stage 4-flowers have receptive stigmas, and therefore, are able to self fertilize (Kalisz et al. 1999). For one stage 4-flower from each plant, we photographed the front view of the corolla, then longitudinally bisected the corolla and photographed the profile. We were careful not to disturb the anthers and stigma during bisection. Using Object-Image 2.11 (Vischer et al. 1994), we measured herkogamy for all four anthers and keel area on the bisected flower image, and blue petal area and white petal area on the front image. The first principal component (PC1) of the four herkogamy measurements was used as the metric of herkogamy. PC1 for herkogamy accounted for 62% of variation in these traits (eigenvalue = 2.47; range of trait weightings on PC1: 0.35-0.58). The first principal component of keel area, blue petal area, and white petal area was the metric of flower size. PC1 for flower size accounted for 72% of variation in the three traits (eigenvalue = 2.17; range of trait weightings on PC1: 0.55-0.61).

In order to determine if the rank order of selfing ability across populations in the greenhouse accurately reflected the rank order of selfing ability across populations in the field, we assayed selfing ability in the field in seven populations (IL, MI1, MI2, MO, OH2, PA, WV) in 2005. We prevented pollinators from visiting a sample of plants in each population by placing 20 $(½\ \mathrm{m})^3$ sized cages over plants prior to flowering. Cages were constructed of PVC pipe and joints, and were tightly covered with Agribon+ AG-15 spun polypropylene (Polymer Group Inc., USA). Agribon+ AG-15 transmits 90% of ambient light and retains minimal heat. The cages were evenly dispersed throughout each population, and remained in the population until approximately one week after plants had ceased flowering. At that time, all plants under each intact cage were collected. In every population, at least one cage was destroyed before the end of the flowering season. Between three and 19 cages (median of 14) remained in each population, and four to 12 plants (median of 9) were collected from each cage. In total, between 14 and 168 (median of 94) plants were collected from each population. The number of flowers that fruited and failed to set fruit were counted for each plant. Selfing ability for each plant was estimated as described above. In each population, the mean selfing ability across all plants was not significantly different than the grand mean of mean selfing ability in each cage. Therefore, the mean selfing ability for each population was estimated across all plants.

We tested for overall differences among populations in selfing ability, herkogamy, and flower size with a MANOVA. Differences among populations for these traits were significant, so we used univariate ANOVAs to test for differences among populations in each trait. To test if herkogamy affects selfing ability, or if flower size is negatively related to selfing ability, we estimated Spearman rank correlations among population means of the three traits. To test for correspondence between selfing ability in the field and greenhouse, we estimated the Spearman rank correlation between population means of selfing ability measured in the greenhouse and field. Unless otherwise noted, all statistical analyses in this study were performed in JMP 5.0.1.2 (SAS Institute, 2003).

Estimation of Outcrossing Rates

For all 11 populations, we estimated the proportion of seeds sired by outcross pollen using microsatellite genotypes of field-sampled progeny arrays. We collected 35 plants just prior to senescence from each population in May and June of 2004; plants were located at evenly dispersed grid points that covered the entire population. We randomly selected six to 20 seeds from each field-collected mother to vernalize, as described above. After germination, three to ten seedlings from each mother were transplanted and grown in the greenhouse. Leaf tissue was collected and stored at -80C until extraction. DNA was extracted from each leaf tissue sample using FastDNA kits with the FastPrep Instrument (Bio101 Systems, Irvine, CA). For 4-5 individuals per mother, we amplified four microsatellite loci using fluorescently-labeled primers: CoveA134, CoveB105, CoveC8, and CoveA119 (Dunn et al. 2006). PCR conditions were similar to those in Dunn et al. (2006), except annealing temperatures were 54C, 54C, 54C, and 60C for each locus respectively, and we multiplexed primers of CoveA134 and CoveB105 in the same PCR reaction for each plant sample. We used acrylamide gel electrophoresis and the FMBio (MiraiBio, Alameda, CA) scanner and software to score allele size. Band size scores were divided into discrete alleles using Allelogram (http://592417348.onlinehome.us/software/allelogram/index.htm).

We estimated population-level multilocus and single locus outcrossing rates using Ritland (2002)'s maximum likelihood program, MLTR 2.2. Our sampling procedure (4-5 progeny per mother, genotypes from four loci) should yield estimates with low standard errors (Ritland and Jain 1981; Ritland 2002). Outcrossing rates estimated at a single locus (t_s) may be smaller than the outcrossing rate estimated using multilocus genotypes (t_m) if there is mating being close relatives, because close relatives are expected to have the same genotype at one locus more commonly than they have the same multilocus genotype. Accordingly, biparental inbreeding can be estimated as the difference between t_m and the average t_s across loci. Standard errors were calculated in MLTR as the standard deviation of estimates from 1000 bootstraps of the dataset, with progeny array (seedlings from one mother) as the resampling unit. Outcrossing rates were likely to be biased upward relative to outcross seed production because we sampled seedlings, and Kalisz (1989) found that outcrossed seeds germinated more frequently than selfed seeds (92% vs. 84%). However, it is likely that this bias affected populations similarly, as Kalisz et al. (2004) found that inbreeding depression for other early life cycle traits (emergence weight and early plant size) did not differ between populations.

Estimation of Potential for Reproductive Assurance and Pollinator Service

We assayed the potential for selfing to provide reproductive assurance in five populations (IL, MI1, MI2, MO, KY) in 2004 and eight populations (IL, MI1, MI2, MO, OH2, OH3, PA, WV) in 2005; these populations spanned the selfing ability range observed in the greenhouse. Reproductive assurance is the increase in fitness that the ability to self provides. In the study, we infer that selfing potentially provided reproductive assurance if flowers with the ability to self (unmanipulated flowers) produced fruit at a higher rate than emasculated flowers.

In 2005, we assayed pollinator service in the same eight populations in which the contribution of selfing to fruit set was measured. We measured pollinator service as the ratio of fruit set of emasculated flowers to fruit set of hand-pollinated intact flowers (Kalisz et al. 2004). Emasculated flowers only set fruit if visited by pollinators, and therefore, they assay pollinator service. However, in addition to lack of pollinator visitation, emasculated flowers may not set fruit if they are resource limited. Hand-pollinated flowers assay resource limitation in a population; these flowers are given ample pollen, so the only reason they do not set fruit is because they are resource-limited. In order to compare pollinator service across populations that might also vary in resource limitation, we standardized pollinator service by dividing the fruit set of emasculated flowers by the fruit set of hand-pollinated flowers in each population.

In 2004, we assayed the potential for reproductive assurance in the middle of the flowering season. In 2005, we assayed the potential for reproductive assurance and pollinator service just before and just after peak flowering in all populations except MO, where treatments were performed just before peak flowering only. The two treatments in 2005 were separated by six to thirteen days to sample pollination during separate periods.

In each population in each year, we performed treatments on ten evenly spaced plants located along each of five parallel transects evenly dispersed through the population. The 50 sampled plants were separated by at least one meter on all sides. In 2004, on each plant, we emasculated two flowers, and selected two control flowers. In 2005, on each plant, we emasculated two flowers, selected two control flowers, and hand-pollinated two flowers with pollen from plants located at least one meter away. Only flowers with pre-dehiscent anthers were chosen for emasculations, and only flowers with two or three dehiscent anthers were chosen for controls or hand-pollinations. We wanted hand-pollinated, control, and emasculated flowers to be similar in age, but wanted to avoid hand-pollinating too soon before stigma receptivity. Additionally, to determine if emasculation damaged the flower's ability to set fruit, one flower from all plants was both emasculated and hand-pollinated during the first sampling time of 2005. The pedicels of all flowers in the experiment were marked with non-toxic paint.

Plants were collected after fruits matured. Lost flowers or flowers on branches damaged prior to fruiting were excluded from analyses. 895 and 4210 treated flowers were collected in 2004 and 2005, respectively. Whether or not flowers set fruit and their relative whorl number were recorded (e.g. flower on the second highest whorl on a stalk with four whorls had whorl number of 0.75). Fruit set from selfing alone declines as whorl number increases (F. Knapczyk, unpublished data), perhaps because resource limitation of fruit production increases with plant age. Because average whorl number varied across treatments, we used whorl number as a continuous covariate to account for differences in resource limitation across treatments.

PROC Mixed (SAS v9.0), using restricted maximum likelihood estimation, was used for all analyses of floral treatments. To test if emasculation had a detrimental effect on fruit set of pollinated flowers, we performed an ANCOVA of fruit set on population, treatment (emasculated and hand-pollinated vs. hand-pollinated only), whorl number, and all pairwise interactions. In all ANCOVA models in this study, treatment was a fixed effect, whorl number was the continuous covariate, and all other factors were random effects. Emasculation did not affect fruit set of hand-pollinated flowers (treatment: $F = 0.10$, $P > 0.75$, N=970).

To test if selfing contributed to fruit set in 2004 or 2005, we performed separate ANCOVAs of fruit set for each year. In 2004, treatment (control vs. emasculated), population, plant (nested within population), whorl number, and all pairwise interactions were independent variables. In 2005, treatment, population, sampling time, plant (nested within time), whorl number, and all pairwise interactions were independent variables. Random effects were tested using chi-squared values. Chi-squared values (1 d.f.) were calculated for each effect as the difference in two times the log likelihood of each effect included versus excluded from the full model. Significance tests were one-tailed because variances cannot theoretically be negative (Littell et al. 1996). We removed interactions with whorl number that were not significant from the full model. In 2005, the time x treatment effect was significant, so for each sampling time, we performed separate ANCOVA models of fruit set on treatment, population, plant (nested within population), whorl number, and all pairwise interactions. The potential for selfing to provide reproductive assurance was inferred when treatment had a significant effect on fruit set and fruit set was greater in control flowers than emasculated flowers.

To test if pollinator service varied across populations in 2005, we performed a separate ANCOVA of fruit set on treatment (hand-pollinated vs. emasculated), population, sampling time, plant nested within sampling time, whorl number, and all pairwise interactions. A significant population x treatment interaction indicated variation in pollinator service across populations. Random effects were tested as described above. We removed interactions with whorl number that were not significant from the full model.

To correct for whorl number, pollinator service was estimated as least-square (LS) mean emasculated fruit set divided by LS mean hand-pollinated fruit set. LS mean fruit sets for both treatments were derived from ANCOVAs of fruit set on treatment (hand-pollinated vs. emasculated), whorl number and treatment x whorl number. To test the hypothesis that selfing ability is locally adapted to pollinator service across populations, we calculated Spearman rank correlations between selfing ability and pollinator service in early and late 2005 separately. Nonparametric correlations were used to minimize the influence of any one sample.

Results

Variation in Selfing Ability and Floral Traits

Selfing ability, herkogamy, and flower size varied significantly across populations, as indicated by the MANOVA and the individual ANOVA models (Table 2.2). Since all plants in this study were grown in a common greenhouse, we infer that there was significant among-population genetic variation for these traits (assuming no maternal effects).

Herkogamy was significantly negatively correlated with selfing ability (Table 2.3). Therefore, it is likely that herkogamy is an important determinant of selfing ability in *C. verna*. Flower size was positively correlated with herkogamy, but only at $P = 0.06$.

Table 2.2. MANOVA testing for overall difference in reproductive traits across populations ("all traits") and ANOVAs testing for differences in each trait across populations. Population was the only independent variable in the models. The proportion of variance explained is from the canonical correlation in the MANOVA and R^2s from the ANOVAs. Individuals from 11 populations were included in the analyses. $^{*}P<0.05$, $^{**}P<0.001$

Dependant variable	*N*	*F*	Proportion of variance explained
All traits (MANOVA)	451	9.71**	0.18
Herkogamy	465	1.92*	0.04
Flower size	477	15.41**	0.25
Selfing ability	488	6.12**	0.11

Table 2.3. Spearman rank correlations between population means of selfing ability, herkogamy, and flower size ($N = 11$). † $P = 0.06$, * $P < 0.05$

	Selfing ability	Herkogamy
Herkogamy	-0.69*	
Flower size	-0.20	0.58†

Mean autogamous selfing ability was quite high in all populations, ranging from 81% in MO to 65% in PA (Table 2.4). The selfing ability of individual plants within each population spanned a much larger range than the range of population means (Table 2.4). OH3 had the most variation in selfing ability; between 11% and 90% of flowers set fruit without pollinators. The least variable population was WV; plants from WV selfed between 44% and 93% of flowers produced.

There was a significant positive correlation between population mean selfing ability measured in the greenhouse and field (Rho = 0.79, $P = 0.04$). In all populations, mean selfing ability was higher in the greenhouse than it was in the field (Table 2.4).

Do Populations with Higher Selfing Ability Rely Less on Pollinators?

Multilocus outcrossing rates in 2004 were not significantly correlated with selfing ability (R=0.14, P=0.67). Multilocus outcrossing rates in the 11 populations ranged from 0.65 in OH1 to 0.90 in MI2 (Table 2.4). Between 14% and 24% of offspring in populations resulted from matings between close relatives; this proportion was significantly greater than zero in all populations (Table 2.4).

Does Selfing Increase Fruit Set?

The emasculation treatment had a significant effect on fruit set in 2004 and during the second sampling time in 2005 (Table 2.5). The ability of flowers to self increased fruit set by 13% in 2004 and 20% during the second sampling time in 2005 (Figure 2.2).

Is Selfing Ability Related to Pollinator Service?

Pollinator service provided between 63% and 111% of maximum fruit production in 2005 (Table 2.6); this across population variance was significant (population x treatment, Table 2.7). Selfing ability was significantly negatively correlated with

Table 2.4. Mean and range of selfing ability in the greenhouse (GH), mean selfing abililty in the field, outcrossing rates, and biparental inbreeding in 11 populations of *C. verna*. Standard errors of the mean are presented in parentheses; standard errors for multi-locus outcrossing rates (t_m), single locus outcrossing rates (t_s), and biparental inbreeding ($t_m - t_s$) are from 1000 bootstraps. Populations are listed in descending order of autogamous selfing ability measured in the greenhouse.

	Selfing ability					
Pop	GH Mean	GH Range	Field Mean	t_m	t_s	$t_m - t_s$
MO	0.81 (0.01)	0.57-0.97	0.55 (0.06)	0.72 (0.05)	0.48(0.04)	0.24 (0.02)
WV	0.79 (0.01)	0.44-0.93	0.44 (0.01)	0.62 (0.06)	0.44 (0.05)	0.19 (0.03)
IL	0.75 (0.02)	0.26-1.00	0.53 (0.03)	0.82 (0.04)	0.60 (0.04)	0.22 (0.03)
KY	0.75 (0.02)	0.31-0.95		0.84 (0.05)	0.63 (0.06)	0.21 (0.03)
TN	0.74 (0.05)	0.47-0.91		0.83 (0.04)	0.59 (0.05)	0.24 (0.03)
MI1	0.72 (0.02)	0.48-0.89	0.51 (0.01)	0.78 (0.05)	0.59 (0.04)	0.19 (0.02)
OH1	0.71 (0.02)	0.39-0.88		0.59 (0.05)	0.45 (0.05)	0.14 (0.02)
OH2	0.71 (0.02)	0.32-0.92	0.35 (0.01)	0.65 (0.06)	0.44 (0.05)	0.21 (0.03)
OH3	0.69 (0.03)	0.11-0.90		0.61 (0.06)	0.41 (0.05)	0.20 (0.03)
MI2	0.68 (0.02)	0.19-0.90	0.43 (0.02)	0.86 (0.04)	0.64 (0.04)	0.22 (0.02)
PA	0.65 (0.02)	0.23-0.87	0.38 (0.02)	0.64 (0.05)	0.47 (0.04)	0.16 (0.02)
Mean	0.73 (0.01)	0.58 (0.04)	0.46 (0.03)	0.72 (0.03)	0.52 (0.03)	0.20 (0.01)
Median	0.71	0.64	0.44	0.72	0.48	0.21

Table 2.5. Separate ANCOVA models of the benefit of selfing, measured as relative fruit set of emasculated and control flowers in 2004 and the two sampling times in 2005. Treatment is a fixed effect (*F*-value is shown), all other factors are random effects. Chi-squared values are the differences in two times the log likelihood of that factor included versus excluded from the model. Whorl number x population and whorl number x plant in 2004, and whorl number x treatment in 2005 were not significant, so they were excluded from the final model. * $P < 0.05$, ** $P < 0.001$

	2004		2005			
			Early		Late	
Effect	VarComp	χ^2 or *F*	VarComp	χ^2 or *F*	VarComp	χ^2 or *F*
Treatment		23.81**		0.13		42.29**
Population	0.007	3*	0.005	0	0	0
Plant	0.01	3.4*	0	0	0.01	0
Whorl number	1.21	1.2	0.37	0	0.47	0
Population x treatment	0.0006	0.3	0.0007	0	0.00008	0
Plant x treatment	0	0	0	0	0	0
Whorl x treatment	0.21	12**				
Whorl x population			0.02	4.9*	0.02	3.2*
Whorl x plant			0.09	24.1**	0.06	4.3*

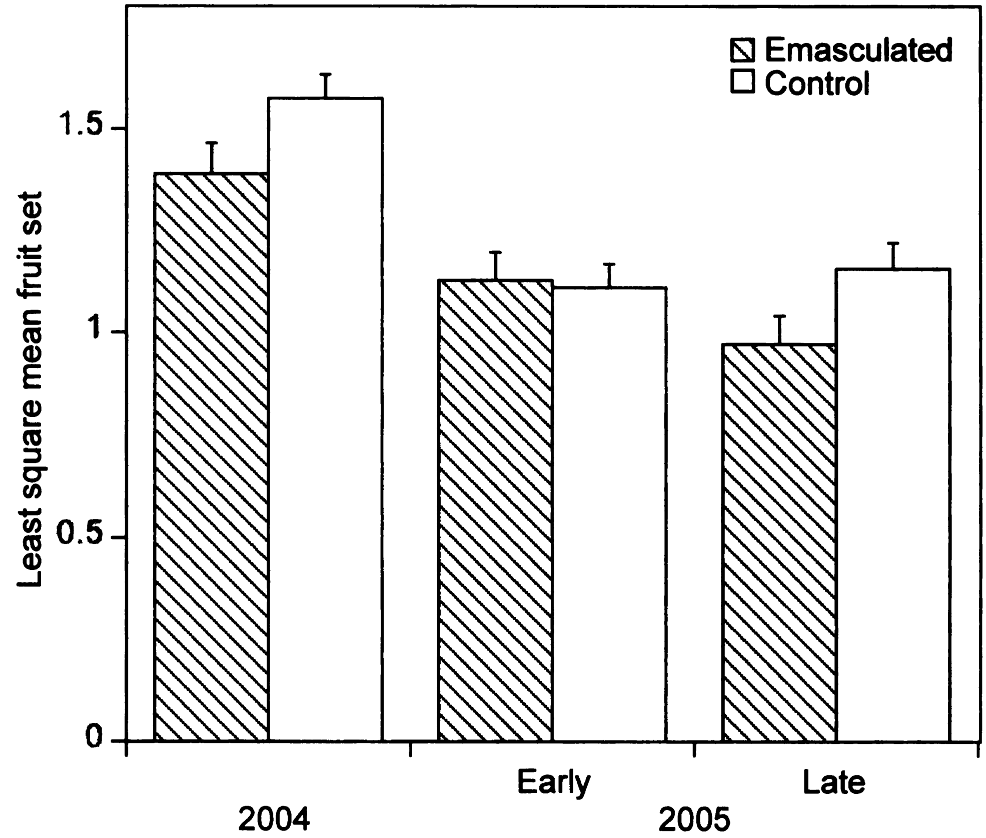


Figure 2.2. Least-square (LS) mean and standard error of fruit set of emasculated (filled) and control flowers (open) in 2004 and in the early and late sampling time in 2005. LS means in 2004 and the two sampling times in 2005 were estimated from the ANCOVA models of fruit set presented in Table 2.5.

Table 2.6. Raw ratios of the number of flowers that set fruit to the number of flowers that failed to fruit for emasculated and hand-pollinated flowers in two sample times in 2005. Least-square (LS) mean fruit sets with standard errors that correct for differences in whorl number are also presented (see Methods for details). Pollinator service is LS mean fruit set of emasculated flowers divided by LS mean fruit set of hand-pollinated flowers.

		Emasculated		Hand-pollinated		Pollinator
Pop	Time	Ratio	LS mean fruit set	Ratio	LS mean fruit set	service
MO	1	45:50	0.77 (0.14)	78:19	1.05 (0.12)	0.73
WV	1	28:26	0.70 (0.17)	60:24	0.86 (0.13)	0.81
	2	36:41	0.89 (0.18)	46:38	0.80 (0.15)	1.11
IL	1	70:27	1.15 (0.10)	97:2	1.31 (0.08)	0.88
	2	31:34	0.47 (0.06)	57:22	0.73 (0.06)	0.64
MI1	1	33:32	0.51 (0.06)	65:17	0.79 (0.05)	0.65
	2	18:34	1.08 (0.20)	52:11	1.35 (0.15)	0.80
OH2	1	69:13	0.88 (0.08)	66:12	0.87 (0.06)	1.01
	2	37:14	1.09 (0.18)	50:19	1.02 (0.14)	1.07
OH3	1	31:25	1.31 (0.24)	59:11	1.45 (0.20)	0.90
	2	27:43	0.62 (0.16)	64:23	0.93 (0.14)	0.67
MI2	1	37:25	0.95 (0.17)	54:24	0.94 (0.12)	1.01
	2	34:45	0.53 (0.13)	79:21	0.84 (0.10)	0.63
PA	1	80:7	0.92 (0.03)	86:8	0.92 (0.03)	1.00
	2	42:37	0.52 (0.06)	49:44	0.53 (0.06)	0.98

Table 2.7. ANCOVA model of pollinator service, estimated as relative fruit set of emasculated and hand-pollinated flowers in 2005. Treatment is a fixed effect (*F*-value is shown), all other factors are random effects. Chi-squared values shown are the differences in two times the log likelihood of that factor included versus excluded from the model. Whorl number x population and whorl number x time were not significant, so they were excluded from the final model. * $P < 0.05$, ** $P < 0.001$

	VarComp	χ^2 or F
Treatment		3.0
Population	0	0
Plant	0	0
Whorl number	0.13	0.3
Time	0.001	0
Population x treatment	0.006	18.8**
Population x time	0.006	14.8**
Plant x treatment	0.02	8.4*
Plant x whorl number	0.06	15.1**
Whorl number x treatment	0.10	12.2**
Time x treatment	0	0

pollinator service during the first sampling time in 2005 (Figure 2.3), but not during the second.

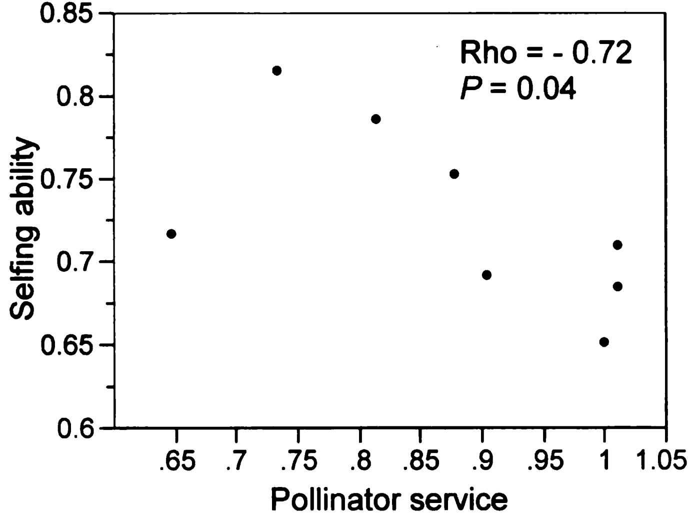
Discussion

The goal of this study was to test four hypotheses: 1) herkogamy and flower size are negatively related to selfing ability across populations of *C. verna* as they are across species in the genus, 2) higher selfing ability results in lower outcrossing rates across populations, 3) selfing increases fruit set, and therefore, may provide reproductive assurance and 4) differentiation in selfing ability arose from differential pollinator service. Understanding the evolutionary causes and ecological correlates of population differentiation in selfing ability in a mixed-mating species may lead to similar understanding of diversification of mating systems across plant species.

We observed significant variation in population mean selfing ability in *C. verna.* However, population mean selfing ability spanned a narrow range (0.65- 0.81) relative to other mixed-mating species in which similar surveys have been conducted. For example, in common garden surveys, population mean selfing ability ranged from ~0.25 to ~0.95 in *Gilia achilleifolia* (Schoen 1982) and from 0.07 to 0.94 in *Arenaria uniflora* (Wyatt 1984). In contrast to the narrow range of population mean selfing ability that we observed, the range of selfing ability within each of the populations in this study was wide. Similarly, Kalisz and Vogler (2003) found that selfing ability of individual plants in three Pennsylvania *C. verna* populations spanned almost the entire possible range of selfing ability. In Chapter 3, we found that there is a significant genetic component to the phenotypic variation in selfing ability within populations of *C. verna*, as was found in

Figure 2.3. The relationship between selfing ability measured in the greenhouse and pollinator service (A) early in the season in 2005 and (B) late in the season in 2005. Pollinator service is the ratio of the least-square (LS) mean fruit set of emasculated flowers to the LS mean fruit set of hand-pollinated flowers. LS means were estimated from ANCOVA models of fruit set on treatment, whorl number, and treatment x whorl number for each population in each sample time. We estimated pollinator service with LS means to correct fruit set for differences in average whorl height between treatments, as fruit set is negatively correlated with whorl height. Eight populations were measured early in 2005, seven populations were measured late in the season. Rho and its significance value were derived from Spearman rank correlations.

A. Early, 2005



B. Late, 2005

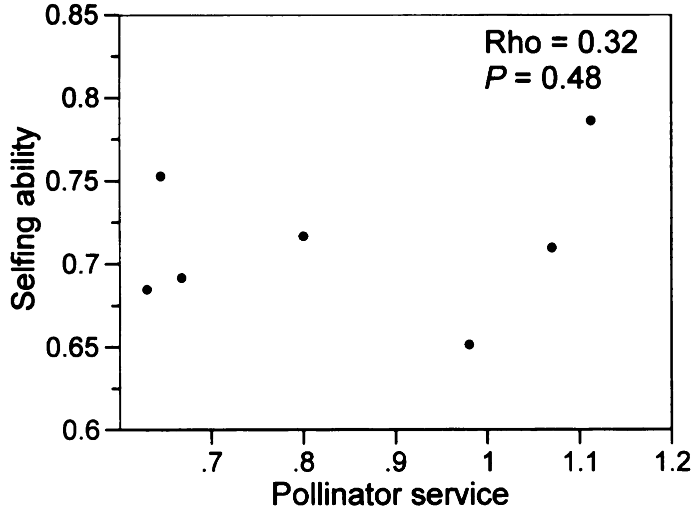


Figure 2.3

populations of other mixed-mating species (e.g. Holtsford and Ellstrand 1992; Carr and Fenster 1994). The presence of genetic variation for selfing ability indicates that selfing can evolve in response to selection.

Herkogamy and flower size were negatively correlated with selfing ability across populations of *C. verna* as they are across species in the genus *Collinsia* (Armbruster et al. 2002). Reduced herkogamy probably increases selfing ability because it increases self-pollen deposition. Herkogamy plays an important role in determining selfing ability of other mixed-mating species as well (e. g. Belaoussoff and Shore 1995; Brunet and Eckert 1998). A negative relationship between flower size and selfing ability across species is consistent with the hypothesis that flowers evolve to allocate fewer resources towards attracting pollinators in populations that rely less on pollinators for reproduction (Grant 1958). The match between the among-population correlations and the among-species correlations suggests that similar mechanisms have caused both population and species differentiation.

We found no evidence that differences in selfing ability across populations led to differences in reliance on pollinators for reproduction. We predicted that populations with high selfing ability would have lower outcrossing rates than populations with low selfing ability because plants with low selfing ability cannot have low outcrossing rates unless pollinator service is extremely poor. Additionally, plants with high selfing ability make more autogamously selfed seed, and therefore, have lower outcrossing rates than plants with low selfing ability. Detection of a true correlation between outcrossing rate and selfing ability may have been prevented if our estimate of the rank-order of selfing ability across populations in the greenhouse did not accurately represent the rank-order in the field. However, the correlation between selfing ability in the field and greenhouse was significantly positive. Because populations did not differ greatly in selfing ability, it is possible that differences in autogamously selfed seed production or gamete discounting across populations were small. In contrast, differences in pollinator service in 2004 may have been large enough to have more of an impact on outcrossing rates than did selfing ability. Additionally, geitonogamy may have contributed significantly to selfed seed production, because plants usually display multiple flowers with dehiscent pollen and a receptive stigma at the same time. Geitonogamy could obscure the predicted negative relationship between selfing ability and outcrossing rate because selfing ability should not affect the fraction of seeds produced through geitonogamy.

We found support for the hypothesis that differences in selfing ability are adapted to differences in pollinator service from a negative correlation between selfing ability and pollinator service just prior to peak flowering in 2005. However, there was no relationship between pollinator service and selfing ability just after peak flowering in 2005. These contrasting results are not inconsistent with the hypothesis of adaptive differentiation. We infer that fruit production in populations was more resource limited late in the season than it was early in the season (fruit set of hand-pollinated flowers was lower in the late sample than it was in the early sample in six of seven populations; Table 2.6). Resource limitation may increase during the flowering season of spring ephemerals as canopy cover increases and light availability decreases; in addition, fruit maturation of early flowers may limit resources of later flowers (e. g. McKenna and Houle 2005). If most ovules are fertilized just before or during peak flowering, then differences in total selection on selfing ability across population may be determined mostly by differences in pollinator service during this time. This scenario would result in a correlation between selfing ability and pollinator service early in the season, but not late in the season. In-depth studies of the timing of fertilization, as well as variation in pollinator service within seasons, between years, and across populations in *C. verna* would provide more data to test the hypothesis that differences in selfing ability are adaptive.

If pollinator service is commonly correlated with selfing ability across populations, the adaptive hypothesis would be strongly supported. There is already considerable evidence that selfing in *C. verna* is adapted to provide reproductive assurance when pollinator service is lacking. In this study, *C. verna*'s selfing ability increased average fruit production by 13% in five populations in 2004 and by 20% in seven populations during the late sampling time in 2005. Similarly, a multi-year study of three *C. verna* populations in Pennsylvania demonstrated that selfing increased fruit production in all populations in at least one sampling time (Kalisz and Vogler 2003). Therefore, it is possible that the ability to self increases plant fitness when pollinator service is poor. Pollen limitation in spring flowering species may be common, because the frequent rainfall and low temperatures that occur during the flowering period can prevent reliable pollinator service (Schemske et al. 1978; Motten 1986). Thus, selection to self-fertilize ovules that cannot be outcrossed may be responsible for those spring ephemerals that maintain selfing (Schemske et al. 1978; Motten 1986). Additionally, *C. verna*'s relatively delayed selfing mechanism suggests that the adaptive value of selfing for this species comes from fertilizing ovules that outcrossing does not.

However, the rank-order of pollinator service across populations may vary from year to year. If selection to improve selfing ability is stronger with decreasing pollinator service in *C. verna*, as was found in other mixed-mating species (Fenster and Ritland 1994; Moeller and Geber 2005), considerable variation in pollinator service between years could lead to annual changes in the direction of selection on selfing ability. If there are no long term differences in pollinator service between populations, then adaptive differentiation of selfing ability would be prevented. We would then conclude that the observed population divergence in selfing ability arose from genetic drift or differential selection from an unmeasured agent of selection.

Plant populations experience heterogeneity in pollinator service for various reasons, including differences in weather (Vicens and Bosch 2000), the surrounding plant community (Moeller 2005), flowering time (Goodwillie 2001), and herbivory (Steets and Ashman 2004); some of these factors may lead to inconsistent differences in pollinator service between populations across years. Multi-population, multi-year studies of pollinator service are rare, but in general, they have found that the rank order of pollinator service across populations changes between consecutive years (Baker et al. 2000; Goodwillie 2001; Vanhoenacker et al. 2006). Long-term studies of pollinator service would be especially useful in populations of species in which a strong relationship between selfing ability and outcrossing rate has been demonstrated (e.g. Schoen 1982; Belaoussoff and Shore 1995), to determine if differences in pollinator service are consistent from year to year.

In general, support for adaptive differentiation of selfing ability in mixed-mating species is minimal. It is too early to make conclusions about what types of species are more or less likely to undergo local adaptation for selfing ability, or how temporal variation in pollinator service affects local adaptation. There are several lines of evidence that would aid understanding of differentiation of selfing ability. For example, reciprocal transplant experiments can provide evidence that trait differentiation is adaptive. The only study we know to use this approach to study differentiation in selfing ability measured seed production of small- (high selfing ability) and large-flowered (low selfing ability) populations of *Collinsia parviflora* transplanted into both habitats (Elle and Carney 2003). Small-flowered plants produced the most seed in both habitats, suggesting that selfing ability may not be locally adapted; however, measurements of outcrossing rates and inbreeding depression are needed to make this conclusion, as the proportion of selfed seeds produced by the two plant types probably differed. Comparing quantitative genetic differentiation (Q_{ST}, Spitze 1993) for selfing ability and genetic differentiation at neutral loci across populations is another way to test the hypothesis that differentiation of selfing ability was adaptive (Chapter 3). Finally, it is possible that differentiation of selfing ability may result from differential selection by agents other than pollinator service, or from selection on genetically correlated traits. These alternative hypotheses should be investigated more seriously.

CHAPTER 3

HAS SELECTION PROMOTED POPULATION DIVERGENCE OF FLORAL TRAITS RELATED TO SELFING IN A MIXED-MATING SPECIES? COMPARISONS BETWEEN QUANTITATIVE AND NEUTRAL GENETIC VARIATION IN *COLLINSIA VERNA*

Abstract

Autogamous selfing ability in mixed-mating plant species may be under strong selection by pollinator service. If pollinator service varies across populations, then adaptive differentiation in selfing ability could result. *Collinsia verna* is a mixed-mating species in which there is evidence that selfing is adaptive, and in which autogamous selfing ability varies across populations. We tested for adaptive differentiation in selfing ability across 11 populations from across the geographic range of the species by comparing the degree of genetic differentiation for autogamous selfing ability and correlated floral traits across populations (Q_{ST}) to the degree of differentiation at presumptively neutral microsatellite loci (F_{ST}). We also estimated genetic correlations between selfing ability and floral traits that were phenotypically correlated to selfing ability in a previous study to determine if selection on other traits might influence differentiation of selfing ability. We found no evidence for adaptive differentiation of selfing ability or herkogamy, as genetic divergence for these traits was similar to divergence at neutral loci due to genetic drift. In contrast, differential selection has contributed substantially to divergence of daily flower production and flower size across populations. Further, we found a strong negative genetic correlation between flower size and daily flower production within populations, which provides evidence for a genetic

size-number tradeoff. Our results suggest that if pollinator service is an important agent of selection on selfing ability in *C. verna*, differences in pollinator service have not been consistent enough through time to create adaptive differentiation in the trait.

Introduction

Most species are not panmictic; rather, they are subdivided into populations. Once gene flow is reduced between populations, genetic differences may arise from differential selection, genetic drift, and mutation. Population differentiation is one source of genetic diversity within species, and is the first step in speciation. Therefore, understanding the relative contribution of selection and neutral processes in creating differences in traits across populations may provide insight into the importance of selection for speciation and the maintenance of genetic variation.

There is a long history of testing the hypothesis that differential selection and local adaptation drive population divergence, particularly in plants (reviewed in Langlet 1971; Briggs and Walters 1997). Support for selection as a differentiating force has come from reciprocal transplant experiments (Clausen et al. 1948; Joshi 2001), correlations of habitat or resource to presumably adaptive traits across populations (reviewed in Linhart and Grant 1996), and divergent selection gradients across populations that match differences in the average trait values (Caruso 2000; Totland 2001). Studies of adaptive differentiation commonly include multiple traits, as genetic correlations between traits can contribute to population differentiation. For example, differential selection on one trait may lead to differentiation of that trait and also genetically correlated traits.

Evidence that differential selection in the past created the pattern of divergence observed in the present also comes from the common result that genetic differentiation of quantitative traits (Q_{ST}) is greater than differentiation of neutral genetic markers (F_{ST}) across populations (reviewed by Merila and Crnokrak 2001; McKay and Latta 2002). This relatively new approach takes advantage of the fact that quantitative genetic variation in a trait is directly analogous to variation at a single locus (Lande 1992). Quantitative traits and neutral marker genes experience identical levels of genetic drift, migration, and inbreeding, whereas only quantitative traits are potentially under differential selection (assuming the genetic markers are truly neutral). Therefore, any difference in population level genetic variance between a trait and neutral markers is the result of either divergent ($Q_{ST} > F_{ST}$) or convergent ($Q_{ST} < F_{ST}$) selection in the past. Although comparisons between Q_{ST} and F_{ST} are less direct tests of adaptive differentiation than reciprocal transplant studies, they facilitate tests for adaptive differentiation across more populations than reciprocal transplant studies.

In flowering plants, divergence of traits associated with the mating system is observed across species in many families, and across populations in mixed-mating species (species that regularly self and outcross) (reviewed in Jain 1976; Barrett et al. 1996; Barrett 2003; Goodwillie et al. 2005). How selection could lead to differentiation in the mating system is a key focus of plant mating system theory. Most models have weighed the costs of selfing (inbreeding depression and gamete discounting, i. e., using gametes that could otherwise be outcrossed) against the transmission benefit of selfing. Fisher (1941) demonstrated that selfers pass two copies of their genes to selfed offspring and one copy to sired offspring, whereas obligate outcrossers pass only one copy of their genes to both maternal and sired offspring. Models have shown that variation in inbreeding depression (reviewed in Husband and Schemske 1996; Byers and Waller 1999) or gamete discounting can produce variation in selection on selfing, and subsequently result in selfing, outcrossing, and mixed-mating populations (e.g. Uyenoyama 1986; Holsinger 1991; Latta and Ritland 1993). Given the ubiquity of pollen limitation (reviewed in Burd 1994; Knight et al. 2005) and spatial variation in pollinators (e.g. Baker et al. 2000), selfing may commonly provide the additional benefit of fertilizing flowers that cannot by cross-pollinated. Theory has demonstrated that variability in pollinator service can also produce variability in selection for selfing (e.g. Schoen and Brown 1991; Sakai and Ishii 1999; Morgan and Wilson 2005). The hypothesis that adaptive differentiation of selfing ability is a response to differences in pollinator service assumes that gamete discounting and inbreeding depression occur. This assumption is fair in most cases; otherwise, all populations would experience selection to increase selfing ability (Lloyd 1979), and we would not predict the genetic variability in selfing ability that we observe across populations (e. g. Schoen 1982; Belaoussoff and Shore 1995).

Thus far, empirical investigation of the role of selection in differentiation of mating system traits lags behind that for other traits, such as physiological, morphological, or life history traits (Linhart and Grant 1996; Barrett 2003). Mating system traits are those that are functionally related to selfing or outcrossing proficiency, such as autogamous selfing ability (measured as the number of seeds produced per ovule by a plant from which pollinators have been excluded), anther-stigma separation in space or time (herkogamy or dichogamy), flower size, and floral display. Correlations of mating system traits to pollinator service across populations provide indirect evidence for adaptive differentiation. For example, Moeller (2006) showed that greater specialist bee abundance was associated with greater herkogamy (lower selfing ability) across populations of *Clarkia xantiana* ssp. *xantiana*. Similar evidence for adaptive differentiation of mating system traits in response to pollinator service has been found in *Arenaria uniflora* (Fishman and Wyatt 1999), but not in other species in which the hypothesis has been tested (Herrera et al. 2001; Molina-Freaner et al. 2003).

Two key requirements for adaptive differentiation of traits is that traits affect plant fitness, and that the effect is environment dependent; however, tests for these requirements in mating system traits are rare. For example, we know of one reciprocal transplant experiment that compared fitness between populations that differ in mating system traits across sites. Elle and Carney (2003) did not find evidence that differences in autogamous selfing ability among populations of *Collinsia parviflora* were adaptive; plants from populations with high selfing ability produced more seed than plants from populations with low selfing ability in both types of populations. In contrast, two studies that estimated selection gradients on selfing ability in different environments found support for adaptive differentiation. Fenster and Ritland (1994) found selection to reduce anther-stigma separation in a pollen-limited population of *Mimulus guttatus*, but no selection on this trait in populations with sufficient pollen. Additionally, Moeller and Geber (2005) found selection to reduce anther-stigma separation in isolated experimental populations of *C. xantiana*, but found no selection in populations placed with congeners, where pollinator visitation was greater. Finally, we know of no comparisons between Q_{ST} of mating system traits with F_{ST} of neutral markers. For most traits that have been examined, Q_{ST} is larger than F_{ST}, indicating that among-population divergence is commonly adaptive (McKay and Latta 2002; Merila and Crnokrak 2001). However, most of the few estimates of Q_{ST} for floral morphological traits were not different than F_{ST} (Podolsky and Holtsford 1995; Waldmann and Andersson 1998; Widen et al. 2002; Jorgensen et al. 2006). Therefore, the frequency of adaptive differentiation of mating system traits is unclear.

In a previous study, we found substantial population differentiation in autogamous selfing ability, correlated floral traits, and outcrossing rates in *Collinsia verna*, a mixed-mating plant species (Chapter 2). Two lines of evidence are consistent with the hypothesis that the observed differentiation of mating system traits is adaptive. First, by comparing fruit production between intact flowers and flowers whose anthers had been removed, we demonstrated that the ability to self provided a fitness benefit via female reproductive success to plants during some sampling times (Chapter 2). This result corroborated a previous study of *C. verna* populations (Kalisz and Vogler 2003). However, pollinator service varied significantly across populations (Chapter 2). Therefore, the relationship between selfing ability and fitness probably varies across populations, which could result in adaptive differentiation in selfing ability if the underlying traits are heritable. Second, we found that high levels of pollinator service were associated with low selfing ability across eight populations during one season (Chapter 2).

In this study, our primary goal was to test the hypothesis that selection contributed to differentiation of autogamous selfing ability across populations of *C. verna*. We compared Q_{ST} for selfing ability and traits correlated with selfing ability to F_{ST} for neutral markers across 11 populations distributed throughout the species range. This is a novel approach to studying adaptive differentiation in the mating system, and we apply it in a system in which there is already some evidence for adaptive divergence. The second goal of this study was to estimate genetic correlations between selfing ability and phenotypically correlated traits to determine if selection on other traits may affect divergence of selfing ability. We know of no other estimates of genetic correlations between autogamous selfing ability and phenotypically correlated traits.

Methods

Study Species and Populations

Collinsia verna (Plantaginaceae), blue-eyed Mary, is a winter annual that flowers with the spring ephemeral community in woodlands of the eastern United States. Large and small bees probably carry out most cross-pollination (Kalisz and Vogler 2003; F. Knapczyk, pers. obs.); self-pollination can occur via direct contact between the stigma and four anthers, or between the stigma and dehisced pollen that remains in the keel of the corolla (a folded petal that encloses the style and stamens) (Kalisz et al. 1999). Autogamous selfing commonly occurs at the end of the period during which the stigma is receptive, but observed variation in timing of stigma receptivity and relative positioning of anthers and stigma in this species, and in *C. heterophylla*, a closely related species, suggest that the timing of selfing is variable across individuals (Kalisz et al. 1999; Lankinen et al. 2007).

To study genetic variation of traits related to the mating system, we sampled 11 populations spanning most of the geographic range of the species (Table 3.1). All of the populations and some of the individuals in this study were part of a previous study of

Table 3.1. Locations of populations and sample sizes for the neutral ($N_{genotypes}$) and quantitative genetic ($N_{parents}$ and $N_{offspring}$) analyses. The number of genotypes assayed in each population varied among the seven loci used in this study because not all loci were scoreable in all individuals. Populations are listed in geographic order from west to east.

Pop	Location	County	Latitude/Longitude	N_{geno}	N_{par}	N_{off}
MO	Graham Cave State Park	Montgomery, MO	38°55'N/91°35'W	27-32	48	184
IL	Allerton Park	Piatt, IL	39°59'N/88°40'W	24-32	49	186
TN	Taylor Hollow Preserve	Sumner, TN	36°30'N/86°54'W	28-32	10	31
MI1	Russ Forest	Cass, MI	42°01'N/85°54'W	24-30	46	131
MI2	Kalamazoo Nature Center	Kalamazoo, MI	42°21'N/85°33'W	22-31	48	120
KY	Raven Run Nature Sanctuary	Fayette, KY	37°54'N/84°24'W	18-31	50	164
OH1	Odell, OH	Guernsey, OH	39°36'N/83°54'W	19-31	38	80
OH3	Hockhocking-Adena Bikeway	Athens, OH	39°24'N/82°08'W	25-32	48	155
OH2	Salt Fork State Park	Clinton, OH	40°08'N/81°28'W	19-31	45	121
WV	Reader, WV	Wetzel, WV	39°35'N/80°45'W	26-31	47	131
PA	Enlow Fork Natural Area	Greene, OH	39°56'N/80°27'W	25-32	46	111

among-population variation in floral traits and outcrossing rates. Descriptions of the populations can be found in Chapter 2. In a common greenhouse, population mean selfing ability (fruit set per flower in pollinator-free greenhouse) ranged from 65% to 81%. In a 2004 survey, the field outcrossing rates of these populations ranged from 59-86%.

Differentiation of Neutral Markers

We measured F_{ST} using microsatellite genotypes of 18-32 plants per population. Plants were collected from the field as seeds in 2004 and grown in the greenhouse at Kellogg Biological Station. Sampling methods, plant growth conditions, and DNA extraction procedures for these plants were described in Chapter 2. Using total genomic DNA from 32 individuals (from 32 different field-collected families) from each of the 11 populations, we amplified seven microsatellite loci: CoveA134, CoveB105, CoveC8, CoveA119, CoveA125, and CoveB116. We amplified the loci using PCR with conditions similar to those described in Dunn et al. (2006) with the following exceptions: the reaction volume was 10 μl, the annealing temperature was 60C for Cove B116 and CoveA119 and 54C for the remaining loci, and we combined primers (multiplexed) for CoveA134 and CoveB105 in all reactions for these two loci. The methods used to visualize and genotype the PCR products were described in Chapter 2. We were unable to amplify all loci in all individuals, so the number of genotypes at some loci is less than 32 in some populations (Table 3.1).

We estimated Wright (1951)'s F_{ST} for *C. verna* using Weir and Cockerham's (1984) θ, which estimates the degree of heterozygote deficiency across populations without any assumptions about sample sizes or heterozygote frequencies. We used

FSTAT 2.9.3 to estimate multilocus F_{ST}; the 95% confidence interval was estimated in the program through bootstrapping across all seven loci (Goudet 1995). We examined loci for lack of neutrality by estimating F_{ST} for each locus to look for outliers. Our genotype sampling effort should be adequate to estimate F_{ST} and compare it to Q_{ST}; Whitlock and McCauley (1999) showed in a simulation that estimating F_{ST} with genotypes from five loci from each of 50 individuals in each of ten populations yielded an estimate of F_{ST} with a 95% confidence interval <0.02. Estimates of F_{ST} from microsatellite markers have been shown to be comparable to estimates from other types of putatively neutral nuclear markers, and should not bias our comparison of F_{ST} and Q_{ST} (Allendorf and Seeb 2000; Nybom 2004).

Differentiation of Quantitative Traits

As part of a previous study, we measured autogamous selfing ability, herkogamy, flower size, and daily flower production (number of flowers produced divided by the number of days of flowering) on 32 plants from each population grown from seed in the greenhouse. Each measured plant was collected from a different mother from the natural populations in 2003 (measured plants were not the same plants that were used for F_{ST}). Methods for seed collection and measurement of traits were described in Chapter 2.

To estimate additive genetic variance for these traits, within populations, measured individuals were mated to one another such that each individual was mated as a female to one plant and a male to a second plant, with no reciprocal crosses. Otherwise, mating pairs were randomly chosen. The resulting seeds were collected from plants just prior to dehiscence, then planted and grown as described in Chapter 2. The same floral traits were measured in the offspring that were measured in the parental generation. In total, between 10 and 50 full-sib families were created in each population, on average, each family had 3.7 offspring (Table 3.1).

We calculated Q_{ST} for each trait using the equation defined by Spitze (1993):

$$Q_{ST} = \frac{V_{A(b)}}{V_{A(b)} + 2V_{A(w)}} .$$

where $V_{A(b)}$ is the between-population additive genetic variance and $V_{A(w)}$ is the average within-population additive genetic variance. Between-population additive genetic variance was calculated as the variance among population means of parent and offspring trait values, and is expected to be composed mostly of additive genetic variance among populations. Restricted maximum-likelihood (REML) estimates of the average within-population additive genetic variance ($V_{A(w)}$) for each trait were estimated using the nf6.p program in Quercus (Shaw 1991). We derived 95% confidence intervals for the Q_{ST} estimates through direct parametric bootstrapping. Quercus generated 1000 data sets using the maximum likelihood estimates of the variance components. Q_{ST} was calculated from each simulated data set, and the 95% confidence interval was taken empirically from their distribution. Like F_{ST}, Q_{ST} ranges from zero to one; Q_{ST} close to zero indicates minimal population genetic differentiation in the trait, and Q_{ST} close to one indicates a large amount of genetic differentiation. We inferred that Q_{ST} was significantly different from F_{ST} when the 95% confidence intervals of Q_{ST} and F_{ST} did not overlap. Additionally, we estimated mean within population narrow sense heritability for each trait by dividing $V_{A(w)}$ by the mean of within-population phenotypic variance of parents and offspring.

In a previous study of the parental generation population means, we found that selfing ability was negatively correlated to herkogamy (Chapter 2) and positively correlated to daily flower production (Rho = 0.60, $P = 0.05$, $N = 11$), and that herkogamy was positively correlated to flower size (Chapter 2). To determine whether these relationships reflect genetic correlations within populations, we estimated the average genetic correlation between selfing ability, herkogamy, flower size, and daily flower production within populations. We derived the average genetic covariance for each pair of traits within populations from the nfp.6 program in Quercus; covariances were derived from a single model containing all measurements of all traits in both the parents and offspring in all populations. The correlation between each pair of traits was estimated by dividing the covariance for the two traits by the square root of the product of the additive genetic variances for the same two traits. The average within-population additive genetic variance for each trait was estimated as described above. Significance tests were based on likelihood ratio tests -- chi-squared values were calculated for each pairwise correlation as the difference in two times the log likelihood of the full model minus a model in which each covariance was constrained to zero. The full genetic model included: among-population variance and covariance components, within-population additive and maternal variance and covariance components, and environmental variance and covariance components. Chi-square tests had one degree of freedom and were one-tailed; one-tailed test were appropriate because the full genetic model always explains as much, or more variance that a model with one covariance constrained to zero.

Results

Differentiation of Neutral Markers

Genetic differentiation among populations (F_{ST}) was similar across loci. Confidence intervals overlapped for all pairwise comparisons of F_{ST} between loci except for one (Table 3.2). Therefore, we considered all of the microsatellite markers to be neutral, or nearly so. The multilocus estimate of F_{ST} was 0.09. All loci were polymorphic in all populations, and several populations had unique alleles. The mean (± standard error) number of alleles within a population ranged from 5.9 ± 0.9 to 10.0 ± 1.0 across the seven loci. Across all 11 populations, between 10 and 20 alleles were found at each locus.

Differentiation of Quantitative Traits

Mean additive genetic variance within populations for selfing ability, flower size, and herkogamy were all significantly greater than zero, with population mean heritabilities for these three traits ranging from 0.11 to 0.28 (Table 3.3). All traits were genetically differentiated across populations, as all Q_{ST} values were significantly greater than zero (Figure 3.1). Daily flower production had the largest Q_{ST}, and herkogamy had the smallest. Q_{ST} for daily flower production and flower size were significantly greater than F_{ST}, suggesting that divergent selection has contributed to the divergence of daily flower production and flower size across the 11 populations in our study. Q_{ST} for selfing ability and herkogamy were not significantly different than F_{ST}. Therefore, this analysis does not provide evidence that selection contributed to the divergence of selfing ability or herkogamy.

The genetic correlations between selfing ability and the other three floral traits were in the same direction as correlations among population means that we estimated in a previous study. The only correlation that was significantly different than zero was the

Table 3.2. Population differentiation (F_{ST}) for each microsatellite locus. The multilocus estimate of F_{ST} is also presented. Standard errors for each single locus estimate of F_{ST} were derived from jackknifing over populations; the standard error of the multilocus estimate was produced through bootstrapping across loci.

Locus	F_{ST}
CoveA119	0.084 (0.018)
CoveA125	0.089 (0.018)
CoveB105	0.163 (0.040)
CoveA134	0.124 (0.061)
CoveC8	0.032 (0.014)
CoveB116	0.085 (0.018)
CoveA107	0.065 (0.022)
Multilocus mean	0.091 (0.014)

Table 3.3. Mean within-population additive genetic variance ($V_{A(w)}$) and its confidence interval, among population additive genetic variance ($V_{A(b)}$), mean within-population phenotypic variance (V_P), and population mean narrow sense heritability (h^2) for each trait.

Trait	$V_{A(w)}$	$V_{A(w)}$ CI	$V_{A(b)}$	V_P	h^2
Herkogamy	0.63	0.40-0.88	0.07	2.24	0.28
Flower size	0.52	0.32-0.74	0.40	1.98	0.26
Daily flower production	0.06	-0.22-0.34	0.36	3.46	0.02
Selfing ability	0.002	0.001-0.003	0.0008	0.02	0.11

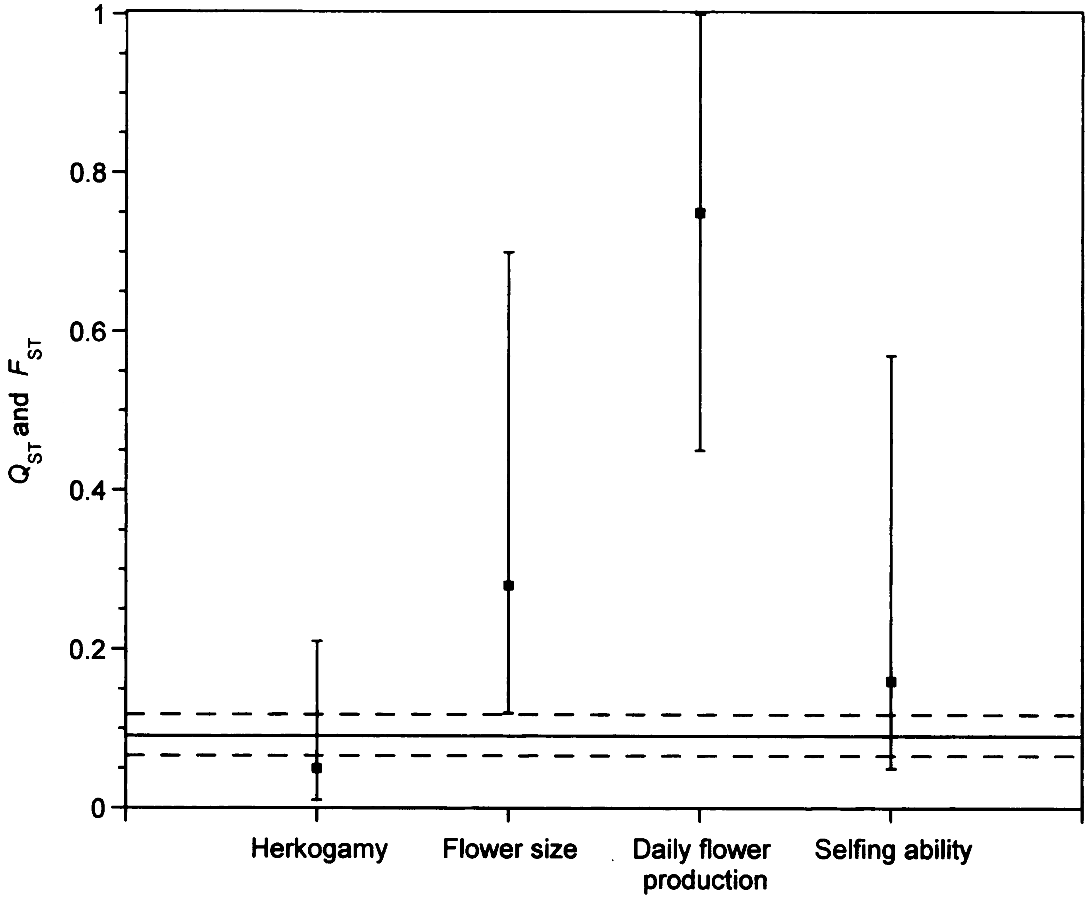


Figure 3.1. Q_{ST} and its 95% confidence interval for each trait are plotted with F_{ST} (solid line) and its 95% confidence interval (dashed lines). Q_{ST} was estimated using within- and among-population additive genetic variance components for each trait. F_{ST} was estimated using allele frequencies at seven microsatellite loci.

strong negative genetic correlation between flower size and daily flower production (Table 3.4).

Discussion

Determining whether trait divergence across populations is adaptive is an important research goal; studying this question with a variety of traits and organisms may help determine the importance of adaptation in speciation and the generation of biodiversity. Understanding the role of selection in population differentiation of mating system traits in mixed-mating species is particularly important because population differentiation may be a good model for the transition from outcrossing species to selfing species, perhaps the most frequent evolutionary transition in plant families (Stebbins 1974; Barrett et al. 1996; Schoen et al. 1997; Goodwillie 1999; Takebayashi and Morrell 2001). This study expands our limited knowledge of adaptive differentiation of mating system traits; to our knowledge, it is the first to compare Q_{ST} and F_{ST} for such traits, and the first to examine differentiation in the mating system of a species using populations from almost the entire geographic range. An additional strength of this study is that we derived estimates of Q_{ST} from estimates of additive genetic variance, whereas most other studies of Q_{ST} in outcrossing plants estimated Q_{ST} with full-sib family mean variance (but see Petit et al. 2001; Koskinen et al. 2002; Widen et al. 2002; Palo et al. 2003). Estimating Q_{ST} with additive genetic variance removes the upward or downward bias that nonadditive genetic effects can impose on full-sib family mean variance (Whitlock 1999; Lopez-Fanjul et al. 2003).

We found that genetic differentiation of autogamous selfing ability was not different than differentiation at presumptively neutral markers. We also found no Table

3.4. Mean within-population additive genetic correlations between floral traits for all 11 populations in the study. Chi-squared values were calculated for each correlation as the difference in two times the log likelihood of each covariance included versus excluded from the full genetic model (see Methods for details). Significance of chi-squared values was tested with one-tailed tests, with one degree of freedom. * $P < 0.05$

	Daily flower production	Herkogamy	Flower size
Selfing ability	0.33	-0.29	-0.05
Daily flower production		0.07	-0.74*
Herkogamy			0.01

evidence for adaptive differentiation of herkogamy, which likely partially determines selfing ability (supported by a negative correlation across populations means, and trend towards a negative genetic correlation). Therefore, we cannot rule out the possibility that neutral processes were responsible for the observed divergence of selfing ability and herkogamy across populations. This result was unexpected because results from previous experiments were consistent with adaptive divergence. For example, average selfing ability was negatively correlated to pollinator service across a subset of these study populations (Chapter 2) and differences in pollinator service have been demonstrated to create differences in selection on selfing in other mixed-mating species (Fenster and Ritland 1994; Moeller and Geber 2005).

The large confidence intervals associated with Q_{ST} may have hampered our ability to detect significant differences between Q_{ST} and F_{ST}. However, because the point estimates of Q_{ST} for selfing ability and herkogamy were close to that for F_{ST}, any adaptive divergence or convergence that may have occurred in these traits was probably minimal. Additionally, simulation and experimental evolution studies have demonstrated that parametric bootstrapping, the method used in this study, or Bayesian approaches yield the most accurate estimates of confidence intervals for Q_{ST} (Morgan et al. 2005; O'Hara and Merila 2005). Alternative approaches for calculating the Q_{ST} confidence interval, such as nonparametric bootstrap or the delta method, tend to underestimate the confidence interval.

Adaptive differentiation of selfing ability and herkogamy would be prevented if agents of selection on these traits were not consistently different across populations. We hypothesized that pollinator service is the most important agent of selection on selfing ability; however, if pollinator service varies within populations across seasons, and long-term average pollinator service does not vary across populations, then adaptive divergence or convergence of selfing ability might not occur. Although there are no data to address whether annual variation in pollinator service is greater than spatial variation across populations of *C. verna* in the long term, across the four populations in which we measured pollinator service in two years, the rank order of pollinator service changed between years (F. Knapczyk, unpublished data). Annual variability in pollinator abundance in populations of other species is often observed (e.g. Goodwillie 2001; Vanhoenacker et al. 2006), and may be especially strong in habitats where weather is unpredictable, but has a strong influence on pollinators, like spring woodlands (Schemske et al. 1978).

Evidence for adaptive differentiation of selfing ability in other mixed-mating species is scarce. Q_{ST} and F_{ST} comparisons in other mixed-mating species in which population differentiation in selfing ability has been observed would clarify the generality of our results. Additionally, reciprocal transplant experiments should also be used to test whether differentiation in selfing ability is adaptive. The only study we know of to use this approach to study differentiation in selfing ability measured seed production of populations of *Collinsia parviflora* with high and low selfing ability transplanted into both habitats (Elle and Carney 2003). Results from the experiment suggested that selfing ability may not be locally adapted; however, the proportion of selfed seeds produced by the two plant types probably differed, so estimates of outcrossing rates and inbreeding depression are needed to make this conclusion. Reciprocal transplants and Q_{ST}/F_{ST} comparisons are complimentary approaches: Q_{ST}/F_{ST} studies can examine differentiation across more populations than reciprocal transplant experiments, but reciprocal transplant experiments provide more direct and detailed evidence concerning the effects of traits on fitness in different habitats. If differences in pollinator service cause differences in selection on selfing ability, as shown by Fenster and Ritland (1994) and Moeller and Geber (2005), then adaptive differentiation of selfing ability may be found among populations that have fairly consistent differences in pollinator service over time.

We found evidence for adaptive divergence of flower size across populations of *C. verna*. Although flower size varies across populations in a multitude of species (e.g. Schwaegerle et al. 1986; Podolsky and Holtsford 1995; Caruso et al. 2003), most Q_{ST} values for floral morphology traits have not differed from F_{ST} (Podolsky and Holtsford 1995; Waldmann and Andersson 1998; Widen et al. 2002; Jorgensen et al. 2006). In the few cases in which spatial variation in selection on flower size is well documented, differential selection was the result of a wide variety of selective agents: temperature (Totland 2001), soil moisture (Caruso et al. 2003), pollinators (Gilbert et al. 1996), or some combination of abiotic and biotic factors (Galen 1999). Closer examination of the eleven populations of *C. verna* in this study is needed before we can identify candidate selective agents that are responsible for the observed genetic differentiation in flower size.

We also found evidence for adaptive divergence of daily flower production. Daily flower production is highly positively correlated to floral display size across populations in *C. verna* (Pearson's R=0.76, P=0.006, N = 11), so differentiation of daily flower production may have resulted from differential selection on floral display. Evolution of floral display size may result from the combination of positive selection to attract more pollinators (reviewed in Ohashi and Yahara 2001), diminishing fitness gains of large displays that result if single pollinators visit many flowers on a plant (Kudo and Harder 2005), and negative selection to reduce inter-flower self-pollination by pollinators (geitonogamy) (Klinkhamer and deJong 1993). Therefore, variation in pollinator service (including pollinator abundance and composition) across populations may result in differential selection on display size (Kudo and Harder 2005). We know of one other measure of Q_{ST} for floral display; in contrast to our study, Widen et al. (2002) found Q_{ST} for floral display was similar to F_{ST} in the self-incompatible *Brassica cretica.*

Genetic differences in daily flower production may also result from differences in the duration of the flowering window between populations. The life spans of spring ephemerals are probably limited by low temperatures early in the season, and low light levels (due to canopy closure) and lack of water late in the season (Rackham 1966; Schemske et al. 1978; McKenna and Houle 2000). If the average time-span between temperature limitation and light/water limitation varied across *C. verna* populations, selection for daily flower production may have varied as a result, such that populations with long flowering seasons produce flowers more gradually, and have smaller floral displays than populations with short seasons.

The negative correlation between daily flower production and flower size is the first evidence for a size-number trade-off in *C. verna*. Size-number trade-offs between traits that positively affect fitness may be maintained because resources are fixed. There is evidence for the general occurrence of some size-number trade-offs in plants, like seeds per fruit (Roff 1992) or pollen per flower (Vonhof and Harder 1995); however, trade-offs between floral display and flower size were detected in less than half of studies reviewed by Worley and Barrett (2000). Because the genetic correlation between daily flower production and flower size within populations was large, and in the same direction as the correlation across populations means of the traits (Rho = -0.56, *P* = 0.07, *N* = 11), it is not surprising that we found evidence for adaptive differentiation for both traits. Adaptive divergence of one of the traits could produce divergence of the other.

Our study tested the hypothesis that divergence in autogamous selfing ability and related mating system traits across the geographic range of *C. verna* resulted from selection. We hypothesized that differential selection from differences in pollinator service across populations would lead to the signature of differential selection on selfing ability. Although we found evidence of adaptive divergence in flower size and daily flower production, two traits that were phenotypically related to selfing ability, we found no evidence for the hypothesis that divergence of selfing ability was adaptive. Adaptive differentiation of selfing ability may have been prevented because differences in selection on selfing ability across populations were not consistent. Measures of selection on selfing ability in individual populations, and across populations that differ in ecological factors, are needed to understand why adaptive differentiation was not responsible for the observed differentiation in *C. verna*, and how general that result is for mixed-mating species.

CHAPTER 4

SELECTION ON SELFING ABILITY IN CONTRASTING POLLINATION ENVIRONMENTS

Abstract

A dominant pattern in flowering plant evolution is the transition from outcrossing to selfing. The acquisition of selfing may be adaptive if selfing provides plants with reproductive assurance in the face of unreliable cross pollination. One tractable way to understand how selection might result in selfing species is to study selection in species that regularly reproduce through both selfing and outcrossing. In this study, we tested the hypothesis that differences in pollinator service lead to differences in selection on selfing. In a population of the mixed-mating *Collinsia verna* that was previously found to receive high levels of pollinator service, we measured selection on autogamous selfing ability under two levels of pollinator service: natural pollinator service and natural pollinator service experimentally reduced by half. We also measured selection on herkogamy and floral traits that were correlated with selfing ability or herkogamy in a previous study. Reducing the number of days during which plants received pollinators by half had no affect on selection on selfing ability or any of the floral traits via female seed production. Selfing ability had a strong positive effect on fruit production, number of seeds per fruit, and seed production in both pollination treatments. From these results, we infer that during our study, the unmanipulated natural population was strongly pollen limited. Our results demonstrate that large differences in pollen limitation across populations would not necessarily lead to differences in selection on selfing ability or other floral traits through female seed production.

Introduction

The transition from outcrossing to selfing species is common, and can be observed at all taxonomic scales of angiosperm evolution (Stebbins 1974; Barrett et al. 1996; Schoen et al. 1997; Goodwillie 1999; Takebayashi and Morrell 2001). A major prediction in evolutionary biology is that transitions from outcrossing to selfing were often adaptive. This prediction is supported by studies that have shown that the relative fitness of selfing and outcrossing can differ according to genetic and ecological context. For example, Fisher (1941) identified a fitness advantage of selfing that results when hermaphroditic plants self all of their own ovules and sire ovules of other plants with equal success to other plants in the population. However, selfing usually results in inbreeding depression (Charlesworth and Charlesworth 1987; Husband and Schemske 1996), which can more than counteract this automatic advantage of selfing. Therefore, in Fisher's (1941) simple scenario, selfing should be favored when the fitness of selfed individuals is more than half the fitness of outcrossed offspring (Lloyd 1979). If the ability to self interferes with outcross siring success, because less pollen is available for export (pollen discounting), then the threshold level of inbreeding depression below which selfing is favored decreases (Lloyd 1979).

The abundance and quality of pollinators also affect the relative advantage of selfing and outcrossing. Where pollinators are lacking (Lloyd 1979), outcross pollen is lacking (during colonization events or low population densities) (Baker 1955; Stebbins 1957; Pannell and Barrett 1998; Lloyd 1992), or interspecific pollen deposition interferes with cross fertilization (Antonovics 1968; Levin 1972; Fishman and Wyatt 1999), selfing may be advantageous, even if inbreeding depression is severe. Because selfing species are often found in habitats that are unfavorable for cross-pollination (e.g. Vasek 1964, Solbrig and Rollins 1977, Rick 1966), selfing may commonly evolve as an adaptation to provide reproductive assurance in such conditions (Stebbins 1974).

Theoretical models have also shown that pollen limitation and variability in pollinator service may be important for maintaining a mixed-mating strategy (i.e., reproducing through both selfing and outcrossing; Schoen and Brown 1991; Morgan and Wilson 2005). This hypothesis is corroborated by the observations that 49% of animal-pollinated species versus 10% of wind-pollinated species are mixed mating, and that animals are less reliable pollinators than wind (Vogler and Kalisz 2001). Selfing can provide reproductive assurance, or increase fitness, when inbreeding depression is severe (commonly observed in mixed-mating species; Scofield and Schultz 2006) by fertilizing ovules that would not otherwise be fertilized. Therefore, in general, a selfing mechanism that is delayed until most opportunity for outcrossing has passed increases fitness the most, but selfing prior to outcrossing may result in decreased fitness if selfed pollen or ovules would otherwise be outcrossed (gamete discounting; Lloyd and Schoen 1992).

Population differentiation in autogamous selfing ability (e. g. Schoen 1982; Belaoussoff and Shore 1995) in mixed-mating species may be the first step toward the evolution of selfing across species. Autogamous selfing ability is usually measured as the number of seeds produced per ovule, or fruits produced per flower, by plants from which pollinators are excluded. Selection should always improve selfing ability if selfing is perfectly delayed because delayed selfing only fertilizes ovules that would not otherwise be fertilized (Lloyd 1979); however, completely delayed selfing mechanisms are probably rare (but see Klips and Snow 1997). Therefore, we predict that the direction of selection on selfing ability in a population is a function of outcross probability (pollinator service). Differences in pollinator service across populations may lead to differences in selection on selfing ability, and over time, adaptive differentiation. Gamete discounting and inbreeding depression are expected to influence selection on selfing ability, but they may also be targets of selection by pollinator service.

The effect of pollinator service on selection on selfing ability in natural populations is not well studied. However, pollen limitation and variation in pollinator service are widespread (Burd 1994; Knight et al. 2005) because they can arise from variation in weather (Vicens and Bosch 2000), plant community composition (Moeller 2005), plant density (Kunin 1993), and other factors. One way to test if pollinator service affects selection on selfing ability is to measure selection in populations that differ only in pollinator service (Wade and Kalisz 1990). If selection differs, then the importance of variation in pollinator service in leading to adaptive differentiation in selfing ability would be supported. This approach has been employed to identify potential selective agents for other traits (phenology and plant size, Stewart and Schoen 1987; plant height, Dudley and Schmitt 1996), but has seldom been used to understand selection on selfing ability. However, recently, Moeller and Geber (2005) found that decreases in bee visitation rates resulted in stronger selection to decrease herkogamy (distance between stigma and anthers, an important determinant of autogamous selfing ability) across experimental populations of *Clarkia xantiana*. Bee visitation rate was manipulated in populations by growing plants with and without congeners. The only other study to measure selection on selfing ability across differing pollination environments similarly found that pollinator service may influence selection on selfing ability; selection to increase selfing ability was found in the one population that was pollen limited, but not in the other two study populations that were not pollen limited (Fenster and Ritland 1994).

Collinsia verna is a mixed-mating annual plant in which selfing has been shown to increase maternal seed production when pollinators are limited (Kalisz and Vogler 2003; Chapter 2). The mechanism of selfing is relatively delayed, such that, in three populations, outcrossing rates increased with pollinator service over a two-year period (Kalisz et al. 2004). Therefore, variable pollinator service may be important in the maintenance of the mixed-mating system. In a previous study of 11 populations of *C. verna* from throughout its geographic range, we found that selfing ability varied significantly across populations, and was negatively correlated with pollinator service in one year (Chapter 2). We hypothesized that differential selection by pollinator service created the observed differentiation in selfing ability. However, quantitative genetic variation across populations (Q_{ST}) for selfing ability was not different than genetic variation for neutral genetic markers (F_{ST}), which indicated that differentiation in selfing ability was probably not caused by sustained differential selection in the past (Chapter 3). It is possible that the differences in pollinator service that we observed in Chapter 2 did result in differences in selection on selfing ability between populations during that season, but annual variation in pollinator service is such that populations do not differ in long term mean pollinator service.

The goal of this study was to determine if spatial or temporal variation in pollinator service results in variation in selection on autogamous selfing ability. By definition, in populations that are pollen limited, the addition of self or outcross pollen should increase seed production. Therefore, we predicted that increases in selfing ability would improve seed production when plants were pollen limited, and that the effect of selfing ability on seed production would be stronger as pollen limitation increased. In contrast, we predicted that selfing ability would have no effect on seed production in populations that were not pollen limited.

To test these predictions, we measured selection on selfing ability in plants that received natural pollination versus plants that received half of natural pollination. We performed this study in a population of *C. verna* that was not pollen limited in a 2005 study; unmanipulated flowers set fruit at the same rate as hand-pollinated flowers. Further, the population received relatively high levels of pollinator service in 2005, as emasculated flowers set fruit at the same rate as hand-pollinated flowers. Therefore, we predicted that there would be no selection on selfing ability under natural pollination, but that selection on selfing ability would be positive under reduced pollination because plants would be pollen limited. We also measured selection on three floral traits that may be related to selfing and outcrossing success: herkogamy, flower size, and flowering duration.

Methods

Study Species and Population

Collinsia verna (Plantaginaceae), or blue-eyed Mary, is a winter annual that grows in woodlands in the eastern United States and flowers in the spring. Populations are mostly outcrossing (rates range from 60-100%, Kalisz et al. 1999; Kalisz et al. 2004; Chapter 2), and individual autogamous selfing ability ranged from 11% to 100% of flowers setting fruit in a pollinator-free greenhouse (11 populations measured, Chapter 2). Plants have 3-7 flowers per whorl, 2-7 whorls per stem, and one to many stems.

Flowers have bilateral symmetry with five petals. One petal is folded into a keel that encloses the style and four stamens. During the 5-7 days that a flower is open, one stamen elongates and its anther dehisces per day (Kalisz et al. 1999). After Kalisz et al. (1999), we refer to stages in flower development by the number of dehisced anthers (i.e., Stage 1 flower has one dehisced anther).

Self-pollination can result from contact between anthers and stigma. Most stigmas become receptive between Stage 3 and 4; by Stage 4, stigmas usually bear enough self-pollen (median of 20 grains) to fertilize all four ovules (Kalisz et al. 1999). Self-pollination can also occur during corolla abscission if the stigma contacts self pollen collected in the keel (Kalisz et al. 1999). Finally, geitonogamous selfing (pollen transfer between flowers on one plant) is possible, but has not been studied.

We measured phenotypic selection on selfing ability and related floral traits of *C. verna* in a population at the Kalamazoo Nature Center (Kalamazoo Co., MI 42°21'N/85°33'W) in southwest Michigan in spring 2007. This population was labeled MI2 in previous studies investigating adaptive population differentiation in the mating system of *C. verna* (Chapters 2 and 3). The population is large (~5000 individuals) and consists of two locations of plants separated by approximately 10 m. The lower location of the population is adjacent to a stream that drains into the Kalamazoo River, and covers approximately 75 m^2. The upper location of the population is located up-slope from the lower section, and covers approximately 200 m^2. Plant density in the lower location was approximately twice that of the upper location. Fruit set of emasculated flowers was not pollen limited in this population in a previous study (Chapter 2). The population had the highest outcrossing rate (0.86 ± 0.04) of 11 populations from across the species range, and had the second lowest selfing ability of 11 populations grown in a greenhouse common garden (mean fruit set was 0.68 ± 0.02) (Chapter 2). Most pollinators observed in this population were large and small bees; we occasionally observed flies and butterflies as well.

Experimental Design and Data Collection

100 pairs of *C. verna* seedlings were chosen for the selection experiment in mid-April. 60 pairs were chosen to span the entire upper section of the population and 40 pairs were chosen to span the entire lower section. Plants within pairs were chosen to be similar in size, and located less than one meter apart. Plant pairs were separated by at least one meter. One plant per pair was randomly chosen to receive natural levels of pollination, the other plant received approximately 50% reduced pollination. We reduced pollination by surrounding each plant in the treatment with a separate wire peony cage. Cages were not placed around plants in the natural pollination treatment. We placed a tightly fitting cover made from Agribon+ AG-15 spun polypropylene (Polymer Group Inc., USA) over the cage on every other day in which pollination was likely. Agribon+ AG-15 transmits 90% of ambient light and retains minimal heat. We assumed that pollination was not likely during days in which the high temperature was less than 54 degrees, or in which rainfall and heavy cloud cover persisted for most of the day. During the 29 days that spanned the bulk of the flowering season (in which >5% of plants had open flowers), flowering plants in the reduced pollination treatment were covered during ten pollination days and uncovered during ten pollination days and nine cold and/or rainy days.

We measured the following floral traits on each plant: selfing ability, distance between stigma and each of the four anthers, area of one blue petal, area of one white petal, area of keel, flowering duration, daily flower production, and total flower production. Population mean autogamous selfing ability was negatively correlated with herkogamy (Chapter 2) and positively correlated with daily flower production (Chapter 3), and herkogamy was positively correlated with flower size (Chapter 2). Selfing ability was also correlated with flowering duration ($R = -0.61$; $P = 0.05$). Herkogamy is probably an important determinant of autogamous selfing ability; flower size, daily flower production, and flowering duration may be related to pollinator attraction. Although estimating selection on selfing ability was the main goal of this study, estimating selection on traits that are functionally related to selfing and outcrossing may improve understanding of selection on the mating system. Total flower production was also measured because in most species, flower production is highly correlated with female fitness (e. g. Johnston 1991; Conner et al. 1996; Caruso et al. 2003). Among plants in this experiment, daily flower production was highly correlated with total flower production ($R = 0.94$; $N = 199$), so daily flower production was not included in the selection analyses.

To measure selfing ability for each plant, we prevented pollinator visitation to one flowering branch per plant by covering the inflorescence on the branch with a bag made from bridal veil material. The bag was placed on the first side branch to flower on each plant just before the first bud opened, and removed after the branch ceased flowering. Fruits on the branch were collected individually, just prior to seed dispersal, and the whole branch was harvested at the end of the experiment. Pedicels of flowers that fail to become fruit remain on the plant after flower senescence and plant harvesting, allowing us to count the number of nonfruiting flowers in the lab. Selfing ability for the branch was estimated as the number of seeds divided by the total number of available ovules (the number of flowers multiplied by four ovules per flower). Selfing ability was not estimated for the six individuals that produced one or no flowers on the selfing ability branch. On three additional plants, fruits on the selfing ability branch were lost, presumably to frugivores.

Floral morphological traits were measured from digital photographs of stage 4-flowers. We chose to measure stage 4-flowers because most flowers at this stage have receptive stigmas, and therefore, are able to self-fertilize (Kalisz et al. 1999). We photographed the intact corolla of two stage 4-flowers, then photographed the longitudinally bisected corollas of the flowers. We took care not to disturb the positioning of the anthers and stigma during the bisection. We measured floral traits from the digital photographs using ImageJ (Vischer et al. 1994). The areas of one blue and one white petal were measured on the corolla image. We measured herkogamy (distance between stigma and anther) for all four anthers, and the area of the keel on the bisected profile view of the stage 4-flower. We averaged the two measurements of each floral morphological trait for each individual. The first principal component of the four herkogamy measurements was used as the metric of herkogamy (60% variation explained; eigenvalue = 2.39; range of trait weightings on PC1: 0.36-0.58). The first principal component of blue petal area, white petal area, and keel area were used as the metric of flower size (74% variation explained; eigenvalue = 2.22; range of trait weightings on PC1: 0.54-0.62). Flowering duration was calculated as the number of days during which plants had open flowers. Fruits on all plants were harvested just prior to seed dispersal. Total flower production was determined by adding the number of fruits from each plant and the number of nonfruiting flower pedicels on the remaining plant harvested at the end of the experiment.

We estimated female fitness using all branches other than the branch used to estimate selfing ability. For each plant, we estimated lifetime female fitness as the total number of seeds produced. We also estimated two multiplicative components of female fitness: number of fruits produced and average number of seeds produced per fruit.

Data Analysis

We used a two-way MANOVA to test for differences in selfing ability, herkogamy, flower size, flowering duration and total flower production between pollination treatments or locations (upper vs. lower) within the population. We estimated correlations among the traits separately for the upper and lower locations because we found significant differences between the two locations ($F_{1,188} = 46.92$; $P < 0.0001$), but not between the natural and reduced pollination treatments ($F_{1,188} = 0.96$; $P = 0.33$).

To determine if surrounding plants with cages affected flower production or selfing ability, while controlling for possible differences between locations in those traits, we performed two-way ANOVAs of flower production and selfing ability with treatment, location, and the treatment by location interaction as independent variables. A significant treatment effect would indicate an effect of caging.

We also tested the prediction that reducing and eliminating pollinator visitation increased pollen limitation. First, to determine if reducing pollinator visitation decreased the number of fruits per flower or seeds per fruit, we performed two-way ANOVAs of each of the fitness components with treatment, location, and the treatment by location interaction as independent variables. We divided fruit and seed production by flower and fruit production, respectively, to control for differences among plants in flower or fruit number. Second, to determine if eliminating pollinator visitation to the selfing ability branch decreased the number of seeds per fruit or fruits per flower, we used paired *t* tests to compare the fitness components between whole plants and selfing ability branches. We tested whether differences in number of seeds per fruit and fruits per flower between whole plants and selfing ability branches were greater in the natural pollination treatment. We subtracted seeds per fruit and fruits per flower of the selfing ability branch from seeds per fruit and fruits per flower of the whole plant for each plant and performed two-way ANOVAs of the difference in seeds per fruit and fruits per flower with treatment, location, and the treatment by location interaction as independent variables.

We estimated phenotypic selection gradients for each trait. We relativized fitness by dividing each individual's fitness by mean fitness (Lande and Arnold 1983). We standardized each trait to a mean equal to zero and variance equal to one (Sokal and Rohlf 1995). Fitness components were relativized and floral traits were standardized separately for each treatment in each location.

In order to determine if fitness or selection gradients differed between locations within the population, we performed ANCOVA models of fruit production, number of seeds per fruit, and lifetime female fitness (seed production) with location, selfing ability, herkogamy, flower size, flowering duration, total flower production, and interactions of location and all floral traits as independent variables. Location or interactions of location with floral traits were not significant in any of the models, and removing location did not alter the significance of the remaining effects.

We used separate ANCOVA models to determine if selection gradients differed between the two treatments. Independent variables in the model were treatment (natural vs. reduced pollination), the five floral traits, and interactions of treatment with all floral traits. Separate models were performed with lifetime female fitness, fruit production, and number of seeds per fruit as dependent variables. A significant treatment by trait interaction indicated that selection for the trait differed between treatments. All ANCOVA models in this study were repeated with raw trait values to determine if standardizing the traits affected the results. The use of raw trait values, as opposed to standardized trait values, did not affect the outcome of significance tests for any of the effects.

When there were no significant interactions in the ANCOVA, we combined data from the two treatments and estimated standardized linear selection gradients for each trait with multiple regression of all five traits on relative female fitness. When there were significant interactions in the ANCOVA, we estimated standardized linear selection gradients for each trait in each treatment with multiple regressions of all five traits on relative female fitness. We estimated standardized quadratic selection gradients for each trait in each treatment by regressing all five traits and their quadratic terms on relative lifetime female fitness. The quadratic selection gradient is equal to twice the regression coefficient of the quadratic term for a trait (Lande and Arnold 1983). In each treatment, we also estimated quadratic selection gradients with each of the two multiplicative fitness components, relative number of fruits and number of seeds per fruit, as the dependent variable. We detected significant curvature in selection gradients via at least one of the fitness components for flower production, flower size, and herkogamy. However, in each case, removing two high leverage points (different data points in each case) eliminated the significance of the curvature. Therefore, we do not present quadratic selection gradients. Residuals of all regression analyses were normally distributed, and multicollinearity among the traits was low as indicated by variance inflation factors (all VIFs were <2) (Neter et al. 1990).

All statistical analyses in this study were performed in JMP 5.0.1.2 (SAS Institute, 2003).

Results

In general, pairwise correlations between traits were fairly low ($r \leq 0.35$) (Table 4.1); however, there were significant positive correlations between flower size and herkogamy, total flower production and flowering duration, and selfing ability and total flower production in one location, and a significant positive correlation between flower size and flowering duration in both locations.

The pollination treatment did not affect selfing ability or total flower production (Table 4.2); therefore, caging had a minimal effect on plant reproduction and flowering. Selfing ability and total flower production differed between the upper and lower locations in the population (Table 4.2). Selfing ability was higher in the upper location than in the lower location (0.20 ± 0.01 vs. 0.14 ± 0.01), as was flower production (36.1 ± 1.7 vs. 21.4 ± 1.2).

Contrary to our expectations, reducing pollinator visitation to plants did not affect the number of seeds per fruit and fruits per flower (Table 4.2). These fitness components

Table 4.1. Pairwise correlations between all floral traits measured on individuals in both treatments. Correlations among plants in the upper location of the population are in the upper right hand corner of the table, correlations for plants in the lower location are in the lower left hand corner. *N* = 115 and 76 for the upper and lower locations, respectively. Significant correlations ($P < 0.05$) are in bold.

	Duration	Herkogamy	Flower size	Total flower production	Selfing ability
Duration		**0.20**	**0.23**	**0.31**	-0.03
Herkogamy	0.06		**0.31**	0.001	0.05
Flower size	**0.35**	0.07		0.009	0.13
Total flower production	0.15	0.14	0.09		-0.02
Selfing ability	0.13	-0.15	-0.02	**0.32**	

Table 4.2. Two-way ANOVA models to test for effect of reducing pollination by caging (treatment) on measures of reproduction, including selfing ability, flower production, number of fruits per flower, and number of seeds per fruit. Plants in the experiment were sampled from two locations within the population. $N = 191$

	Selfing ability		Flower production		Fruits/flower		Seeds/fruit	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Location	**13.97**	**0.0002**	**40.46**	**<0.0001**	0.01	0.91	1.66	0.20
Treatment	0.07	0.79	0.18	0.67	1.20	0.28	3.28	0.07
Location x Treatment	0.89	0.35	0.45	0.50	0.09	0.77	0.08	0.78

were also similar between plants in the two locations within the population. However, selfing did not completely compensate for the loss of seed production by pollinators on the selfing ability branch. Preventing pollinator visitation altogether caused a reduction in number of seeds per fruit and fruits per flower, indicated by the comparison between selfing ability branches and whole plants (Figure 4.1). The difference in number of seeds per fruit between the selfing ability branch and the whole plant was not affected by location or pollination treatment (Figure 4.1, Table 4.3); across all plants in the experiment, the selfing ability branch produced fewer seeds per fruit than the whole plant ($t = 9.7$, $P < 0.001$, $N = 191$). The difference in number of fruits per flower was affected by location, but not pollination treatment. In both locations, selfing ability branches produced fewer fruits per flower than the whole plant (upper: $t = 11.6$, $P < 0.001$, $N = 115$; lower: $t = 10.6$, $P < 0.001$, $N = 76$), but this difference was greater in the lower location than in the upper location (Figure 4.1).

Linear selection gradients for selfing ability, flower production, flowering duration, and herkogamy via all fitness measures were similar between the reduced and natural pollination treatments (trait x pollination interaction terms in the ANCOVA; Table 4.4). Plants with higher selfing ability produced significantly more fruits, seeds per fruit, and seeds. Plants that produced more flowers produced significantly more fruits and seeds, but significantly fewer seeds per fruit. Herkogamy had a negative effect on the number of seeds per fruit, but no effect on fruit or seed production. Flowering duration did not influence any component of female fitness.

Selection on flower size via number of seeds per fruit differed between treatments, but selection through fruit and seed production did not differ between

Figure 4.1. Mean and standard error of number of A) fruits per flower and B) seeds per fruit of whole plants (shaded bars) and selfing ability branches (open bars) in the reduced and natural pollination treatments in each of the locations (upper vs. lower) in the population in which the experiment was conducted.

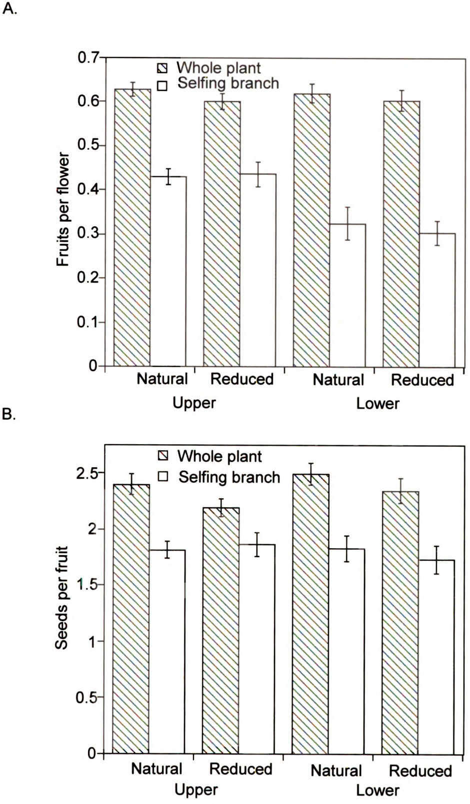

A.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fruits per flower
▧ Whole plant
☐ Selfing branch
Natural
Reduced
Natural
Reduced
Upper
Lower
B.
2.5
2
1.5
1
0.5
0
Seeds per fruit
▧ Whole plant
☐ Selfing branch
Natural
Reduced
Natural
Reduced
Upper
Lower

Figure 4.1

Table 4.3. Two-way ANOVA models to test for effect of eliminating pollinators to selfing ability branches. The dependent variables in the models were the differences in the number of fruits per flower and seeds per fruit between the whole plant and the selfing ability branch on the same plant. Plants in the experiment were sampled from two locations within the population. $N = 191$

	Difference in fruits/flower		Difference in seeds/fruit	
	F	*P*	*F*	*P*
Location	**4.62**	**0.03**	1.21	0.27
Treatment	1.30	0.26	1.68	0.20
Location x Treatment	0.14	0.71	0.87	0.35

Table 4.4. ANCOVA models to test for differences in selection on traits between the natural and reduced pollination treatments. The same model was tested with lifetime female fitness (seed production) and two multiplicative female fitness components as dependant variables. All traits were standardized to a mean of zero and standard deviation of one. $N = 191$

	Fruit production		Seeds per fruit		Seed production	
Factor	Slope	*P*	Slope	*P*	Slope	*P*
Pollination	0.001	0.94	-0.001	0.95	-0.001	0.96
Duration	-0.02	0.33	0.003	0.87	0.008	0.74
Herkogamy	-0.004	0.81	**-0.04**	**0.05**	-0.03	0.16
Selfing ability	**0.06**	**<0.001**	**0.08**	**<0.001**	**0.15**	**<0.001**
Flower size	0.02	0.28	-0.02	0.30	-0.007	0.79
Flower production	**0.42**	**<0.001**	**-0.05**	**0.02**	**0.36**	**<0.001**
Duration x pollination	-0.009	0.55	0.03	0.18	0.03	0.28
Herk. x pollination	-0.009	0.57	0.01	0.51	0.001	0.96
Selfing ability x pollination	-0.005	0.78	-0.02	0.34	-0.02	0.44
Flow. size x pollination	-0.001	0.94	**-0.05**	**0.03**	-0.04	0.08
Flower production x pollination	-0.01	0.33	0.008	0.69	-0.03	0.19

pollination treatments (Table 4.4). Plants with smaller flowers produced significantly more seeds per fruit in the reduced pollination treatment, whereas flower size had no effect on number of seeds per fruit in the natural pollination treatment (Table 4.5).

Discussion

The results presented here show that *C. verna* individuals with higher selfing ability produced more fruits, more seeds per fruit, and more seeds under both natural and reduced pollinator service. It is possible that inbreeding depression has led us to overestimate the strength of selection on traits in this study; Willis (1996) demonstrated that, in mixed-mating populations, the strength of selection on a trait that experiences inbreeding depression may be biased upward as a function of the strength of inbreeding depression on that trait. The only trait in this study for which inbreeding depression has been estimated is flower production. Inbreeding depression for this trait was quite low in three populations of *C. verna* ($\delta < 0.10$ in a greenhouse assay; Kalisz et al. 2004), suggesting that selection gradients for this trait may be minimally biased by inbreeding depression. Furthermore, selection on flower production and selfing ability through seed production was so strong that even after correcting for a 50% upward bias, the most extreme bias demonstrated by Willis (1996) in a population with substantial inbreeding depression, selection on these traits would still be significantly positive. For all traits, inbreeding depression should not bias our comparisons of selection between pollination treatments, because inbreeding depression should not have differed between treatments.

Direct selection on selfing ability through female fitness components did not differ between the two pollination treatments. Our results suggests that large differences in pollinator service between years or across populations may not produce similarly large

Table 4.5. Standardized linear selection gradients (*β*) and their standard errors via fruit production, number of seeds per fruit, and seed production. Selection gradients via seeds per fruit were estimated separately for each of the two pollination treatments (natural vs. reduced) because selection on flower size significantly differed between treatments (Table 4.4). $N = 191$ for selection gradients through fruit production and seed production; $N = 95$ and 96 for selection gradients through number of seeds per fruit in the reduced and natural pollination treatments, respectively. Selection gradients in bold are significantly different than zero ($P < 0.05$).

		Seeds per fruit		
	Fruit production	Reduced	Natural	Seed production
Duration	-0.02 ± 0.02	0.03 ± 0.03	-0.02 ± 0.03	0.004 ± 0.02
Herkogamy	-0.004 ± 0.01	-0.03 ± 0.03	-0.05 ± 0.03	-0.03 ± 0.02
Selfing ability	**0.06 ± 0.02**	**0.06 ± 0.03**	**0.10 ± 0.03**	**0.15 ± 0.03**
Flower size	0.02 ± 0.02	**-0.07 ± 0.03**	0.03 ± 0.03	-0.007 ± 0.02
Flower production	**0.42 ± 0.02**	-0.04 ± 0.03	-0.05 ± 0.03	**0.37 ± 0.02**

differences in the direction or strength of selection on selfing ability; in *C. verna*, increases in selfing ability generally increased female fitness. Therefore, we did not find support for the hypothesis that spatial or annual variation in pollinator service produces variation in selection on selfing ability. There are three factors that were not accounted for in this study, which might result in total selection on selfing ability being weaker with increased pollinator service. First, pollinator service in 2007 seemed to be low, so both our natural and reduced pollination treatments were pollen limited. We predicted that selfing ability would not affect seed production when plants were not pollen limited, but we did not measure selection in this context. Second, correcting our estimate of female fitness for inbreeding depression may have resulted in weaker selection on selfing ability in the natural pollination treatment because seed discounting (selfing ovules that would otherwise be outcrossed) was probably stronger under natural pollination. Third, if pollen discounting was more severe in the natural pollination treatment than in the reduced pollination treatment, selection on selfing ability through male fitness may have been weaker in the natural pollination treatment.

We predicted that selfing ability would have no effect on seed production in the natural pollination treatment because in a previous study in 2005, the population in this study was not pollen limited, and pollinator service was high enough that emasculated flowers set fruit at the same rate as hand-pollinated flowers. However, this study suggests that in 2007 the population was pollen limited because selfing ability had a strong linear relationship with fruit production, seeds per fruit, and seed production under natural pollination conditions. It seems likely that selection on selfing ability would be weaker than we observed under natural pollination in a year with higher pollinator service (like 2005) or in a supplemental pollen treatment.

The net effect of increasing selfing ability on female fitness depends on the relationship between selfing ability and seed discounting, the probability of outcrossing, and the strength of inbreeding depression. Unless self-pollination is perfectly delayed, the ability to self can affect female fitness in two ways: selfing can cause some ovules to be selfed that would have been outcrossed (seed discounting), and selfing can increase seed production if pollinators are not capable of providing maximum seed set to plants. Therefore, the increase in fitness via seed production that results from increases in selfing ability may be counteracted or outweighed by inbreeding depression if increases in selfing ability also lead to a greater trade-off between outcrossed and selfed seed production. The effect of seed discounting on fitness has rarely been estimated in empirical studies. In a rare example, Herlihy and Eckert (2002) demonstrated in the mixed-mating *Aquilegia canadensis* that relative female fitness, after accounting for inbreeding depression, declined as autogamous selfing ability increased because seed discounting increased with selfing ability. Furthermore, the cost of seed discounting outweighed the benefit of selfing to female seed production in seven of 13 comparisons within populations.

A trade-off between selfing ability and outcross seed production may occur in *C. verna*. On average, self-pollen was present on 85% of stigmas at the time when most stigmas become receptive (Kalisz et al. 1999), so self-pollination could occur while outcrossing is still possible. The cost of seed discounting in *C. verna* is not trivial: selfing results in offspring that are 50% as fit as outcrossed offspring (median for 11 populations across the species range, as measured by changes in the inbreeding coefficient; Knapczyk, unpublished data). In our study, we presume that seed discounting was more severe and the relationship between seed discounting and selfing ability was stronger in the natural pollination treatment than in the reduced pollination treatment. We presume this because the probability that an ovule would be outcrossed was greater under natural pollination, when plants were exposed to pollinators for twice as many days. If seed discounting and inbreeding depression were taken into account in our measure of female fitness, the selection gradient for selfing ability might have been smaller in the natural pollination treatment than in the reduced pollination treatment. However, further studies of seed discounting in *C. verna* are needed before we can make this prediction with confidence.

Thus far, studies of selection on autogamous selfing ability have not accounted for selection through male fitness. However, selection on traits through male and female fitness usually differs in studies that have measured both components of fitness (Kobayashi et al. 1999; Elle and Meagher 2000; Morgan and Conner 2001; van Kleunen and Ritland 2004; Wright and Meagher 2004). In our study, selfing ability had a positive affect on seed production, but it is plausible that selfing ability negatively affected siring success in both the natural and reduced pollination treatments if increased selfing ability led to the availability of less pollen for cross-pollination (pollen-discounting). Although a negative relationship between selfing ability and outcross siring success has not been tested in *C. verna*, it has been demonstrated with the use of experimental arrays in other mixed-mating species (e.g. Kohn and Barrett 1994; Chang and Rausher 1998; Fishman 2000). The cost of the trade-off between selfing ability and siring success may have been higher in the natural pollination treatment where pollinators were more abundant. Therefore, selection on selfing ability via siring success may have been weaker in the reduced pollination treatment than in the natural pollination treatment.

Flower production, herkogamy, and flower size were the only other traits on which we detected selection. It was not surprising that selection on flower production through seed production was so strong ($\beta>0.3$) in both pollination treatments, given that flower production is commonly a close determinant of lifetime female fitness in other species (e.g. Johnston 1991; Conner et al. 1996; Caruso et al. 2003). Flower production affected seed production mostly through its effect on fruit production, as selection on flower production via fruit production was strongly positive, and selection through number of seeds per fruit was negative. Flower production can increase fruit production in *C. verna* in two ways: it increases the number of ovaries that can develop into fruit, and it may increase the number of pollinators that visit and deposit outcross pollen. The negative relationship between flower production and number of seeds per fruit suggests that less self or cross pollen was deposited per flower on plants with more flowers and/or individual flowers were more resource limited on plants with more flowers. Similarly, the negative relationship between herkogamy and seeds per fruit suggests that herkogamy affected pollen deposition or resource limitation. Correlations between herkogamy and selfing ability were low in this study, so herkogamy probably did not strongly affect self-pollen deposition. However, it is possible that pollinators deposited more pollen per stigma in flowers in which the stigma was closer to the anthers.

Pollination treatment significantly affected selection on flower size via number of seeds per fruit. We detected significant negative selection on flower size via number of seeds per fruit in the reduced pollination treatment, but there was no selection on flower size in the natural pollination treatment. This result was surprising; it suggests that flower size negatively affected pollen deposition or resource allocation per flower, but only when pollinator visitation was reduced. Additionally, we did not detect selection on flower size in the reduced pollination treatment via seed production, presumably because flower size did not affect fruit production, and fruit production had a stronger influence on seed production than number of seeds per fruit. Moeller and Geber (2005) found negative selection on flower size through number of seeds per ovule in *C. xantiana* when pollination was experimentally reduced, but no selection when pollination was greater. Because they manipulated pollination by growing plants with and without congeners, they hypothesized that plants with smaller flowers produced more seeds per ovule because they received less interspecific pollen. In our study, plants in both treatments were interspersed throughout the population, so this mechanism is unlikely to have produced the difference in selection on flower size that we observed. Additional studies of selection on flower size in *C. verna* are needed to understand why flower size negatively affected the number of seeds produced per fruit under reduced pollination.

The advantage of maintaining the ability to self for *C. verna* was clear in this study. Plants that received 10 days of pollination produced the same number of fruits per flower and seeds per fruit as plants that received 20 days of pollination. Although selfing could not entirely make up for the absence of pollinators, as the selfing ability branch produced fewer fruits per flower and seeds per fruit than the whole plant, self fertilization appeared to compensate for the difference in outcross pollination between the two pollination treatments. Because we did not estimate outcrossing rates of plants in the two treatments, we cannot quantify the maternal fitness benefit that selfing provided plants in either treatment. However, in Chapter 2, we found that the ability to self could increase fruit production of intact flowers by as much as 20% over emasculated flowers in natural populations. From our 2005 survey of pollinator service in eight populations of *C. verna*, we infer that a lack of pollinator service may be common: the ratio of fruit set between emasculated and hand-pollinated flowers was significantly less than one in five out of eight populations in the middle of the flowering season (Chapter 2). More generally, the evidence that mixed-mating species maintain selfing because it provides reproductive assurance in the face of inadequate pollinator service is growing (reviewed in Goodwillie et al. 2005).

In conclusion, we showed that selfing ability positively affected female seed production in *C. verna* similarly in both natural and reduced pollination conditions. Although our results suggest that large differences in pollinator service may not lead to differentiation of selfing ability across populations, there are too few studies of selection on selfing ability in contrasting pollination conditions to understand general patterns of selection on selfing ability. In order to determine whether annual or spatial variation in pollination may create variation in selection on selfing ability, future studies should directly estimate pollen limitation and measure selection on selfing ability via male fitness, as well as the trade-off between selfing ability and outcross seed production.

CHAPTER 5

CONCLUSION

Summary

My thesis research had two main objectives. The first objective was to document population differentiation of selfing ability in *Collinsia verna* and identify patterns of covariation between selfing ability, floral traits, and the mating system. The second objective was to test three major predictions of the hypothesis that differentiation of selfing ability is adaptive, and has been caused by differences in pollinator service across populations: 1) pollinator service is negatively correlated with selfing ability across populations, 2) differential selection has contributed significantly to genetic differentiation in selfing ability, and 3) the strength and direction of selection on selfing ability depend on the level of pollinator service.

Chapter 2 demonstrated that population differentiation in selfing ability in *C. verna* spanned a narrower range than in other mixed-mating species, despite the fact that I sampled populations across a larger proportion of the species range than previous studies. Across populations, selfing ability was negatively correlated with herkogamy and flowering duration, and positively correlated with daily flower production. Additionally, selfing ability was unrelated to outcrossing rates across populations. In support of the hypothesis of adaptive differentiation, pollinator service and selfing ability were negatively correlated across populations. However, Chapter 3 demonstrated that differences in selfing ability across populations could be sufficiently explained by genetic drift. Additionally, Chapter 4 failed to support pollinator service as a cause of adaptive differentiation in selfing ability; we demonstrated that differences in pollinator service do not necessarily lead to differences in selection on selfing ability through female fitness.

Future Directions

The importance of pollinator service in driving evolution of selfing at the species scale is fairly well accepted, but the importance of pollinator service in driving population differentiation is mostly untested. This is one of only a few studies to test the hypothesis that differences in selfing ability across populations are due to local adaptation. Additionally, few empirical studies have investigated how differences in pollinator service lead to differences in selection on selfing ability.

In order to determine whether adaptive differentiation in selfing ability across populations is common, or what ecological contexts promote adaptive differentiation of selfing ability, more reciprocal transplant studies and comparisons of F_{ST} and Q_{ST} for selfing ability are needed. Using the F_{ST}/Q_{ST} approach, Chapter 3 of my thesis did not find support for the hypothesis that differentiation in selfing ability across populations of *C. verna* was adaptive. However, the *C. verna* populations in my thesis also spanned a narrow range of mean selfing ability across populations relative to other mixed-mating species that have been investigated (e. g. Schoen 1982; Wyatt 1984). It would be interesting to compare Q_{ST} for selfing ability to F_{ST} across populations that span a broader range of selfing ability and for which there is some evidence that differentiation in selfing ability is adaptive. Kelly (1999) showed theoretically that inbreeding may reduce the response to selection in mixed-mating species. Therefore, we may discover that adaptive differentiation of traits in mixed-mating species is rarer than it is in outcrossing species.

Additional selection studies are needed to test the hypothesis that differences in pollinator service can lead to differences in selection on selfing ability. Surprisingly, Chapter 4 demonstrated that experimentally reducing pollinator service by half had no effect on selection on selfing ability through female fitness. Moeller and Geber (2005) found that selection on herkogamy was correlated to pollinator visitation rates; selection to reduce herkogamy was weaker when pollinator visitation rates were greater. One explanation for the difference in results between Chapter 4 and Moeller and Geber (2005) is that we did not measure selection on selfing ability in plants that were not pollen-limited, whereas Moeller and Geber (2005) may have (they did not measure pollen limitation in their treatments). Studies that measure selection on selfing ability across a broad range of pollinator service and directly quantify pollen limitation in all contexts would advance our understanding of how pollinator service affects selection on selfing ability through female fitness.

Our understanding of selection on selfing ability would also benefit from measurements of gamete discounting, inbreeding depression, and male reproductive success. In Chapter 4, we were unable to estimate selection on selfing ability through total (male and female) fitness; however, we predicted that we would have found a difference in selection on selfing ability between our pollination treatments if we corrected female fitness for inbreeding depression and measured selection through male fitness. More generally, there are very few estimates of seed or pollen discounting in the literature. From the few available estimates, gamete discounting can be extremely strong (Herlihy and Eckert 2002) and the strength of pollen discounting tends to vary depending on the floral phenotypes of the surrounding population (Kohn and Barrett 1994; Fishman

2000). Theory has demonstrated that gamete discounting may be important for the maintenance of the mixed-mating strategy (reviewed in Goodwillie et al. 2005), but additional estimates of gamete discounting in natural populations are needed to confirm the importance of gamete discounting in the evolution of selfing.

The trade-off between selfing ability and seed and pollen discounting in *C. verna* could be estimated with controlled experiments. For example, artificial selection could be used to create lines of plants with high, moderate, and low selfing ability. Following the methods of Fishman (2000), siring success of plants from the three lines could be estimated by placing plants in arrays of multiple *C. verna* plants, and genotyping the seeds produced by the array. The result that siring success is negatively correlated with selfing ability would provide support for a relationship between selfing ability and pollen discounting. The effect of selfing ability on gamete discounting could be determined by hand cross-pollinating plants and then testing for a difference in the outcrossing rate of the resultant seeds between plants from the different lines.

A large gap in our knowledge of population differentiation of selfing ability lies in our understanding of the most likely agent of selection on selfing ability: pollinator service. Chapter 2 demonstrated that pollinator service varied across populations of *C. verna* spanning the geographic range of the species, and was negatively correlated with selfing ability in the early part of the flowering season. However, we do not know if the differences we observed reflect the long-term differences that are necessary to create adaptive differentiation. If the rank order of pollinator service across populations varies from year to year, as was demonstrated by Goodwillie (2001) and Vanhoenacker et al. (2006), adaptive differentiation of selfing ability may be slow, or impossible. A common hypothesis for the evolution of a mixed-mating strategy is that selfing provides reproductive assurance when pollinators are unreliable. If differences in pollinator service between populations of mixed-mating species are similarly unreliable, then adaptive differentiation of selfing ability in mixed-mating species may be uncommon.

A thorough understanding of adaptive differentiation of most types of traits is rare. However, it may be of particular value to understand adaptive differentiation of selfing, for two reasons. First, selfing appears to have evolved from outcrossing repeatedly, so understanding the dynamics of population differentiation would contribute understanding of this dominant pattern in plant evolution. Second, because the mating system of a population can determine how traits respond to selection, population differentiation of selfing may have consequences for differentiation of other types of traits as well.

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