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THE REPRODUCTIVE BIOLOGY, MATING SYSTEM, AND GENETIC CONSEQUENCES OF CHASMOGAMOUS AND CLEISTOGAMOUS FLOWER PRODUCTION IN VIOLETS (Viola)

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The production of open, chasmogamous (CH) flowers and closed, cleistogamous (CL) flowers on the same individual is an unusual mating system found in several plant species. CL flowers are automatically self-pollinated, while CH flowers are often assumed to be outcross-pollinated. Despite longstanding recognition of this mating system, not much is known about why certain plant species produce these two flower types. The goal of my dissertation research was to study the genetic consequences and the factors that help maintain the CH/CL mating system in violets. Two species, *Viola pubescens* and *V. canadensis* were chosen because they are important components of the forest understory and despite their common nature, little is known about their reproduction.

I first examined the reproductive biology of a central Ohio population of *Viola pubescens* to determine the relative importance of CH and CL flowers in overall reproduction. Flowering phenology was monitored over two years, and the fates of each flower and capsule (abortion, damage, or successful seed dispersal) were recorded. Seed mass and percent seedling emergence were measured for CH and CL progeny derived from these capsules. A pollination experiment was performed to determine if CH flowers could also self-pollinate. CH flowers were produced first during the season, followed by CL flowers, with a distinct switch point that corresponded with tree leaf-out. Although
similar numbers of CH and CL flowers were produced per plant, CH flowers were nearly
twice as likely to mature and disperse seeds as CL flowers. Both capsule types had
similar numbers of seeds of comparable mass, with no difference in emergence of CH
and CL seedlings. CH flowers could outcross-pollinate under field conditions, but
unvisited flowers could also self via a delayed selfing mechanism. Although both CH
and CL flowers are important in reproduction, CH flowers may contribute
disproportionately more seeds.

Because delayed selfing could occur, the amount of self-pollination in CH flowers
was measured over a two-year period in the Ohio population of _Viola pubescens_. Selfing
rates were derived from multi-locus outcrossing rates and were measured using isozyme
electrophoresis of field-collected seeds. Correlated mating parameters were also
calculated for one year in which a large number of maternal families were available. The
selfing rate in CH flowers was substantial and significantly higher in 1996 (0.60) than in
1997 (0.07), with low biparental inbreeding in both years. Correlated mating parameters
indicated that siblings within a maternal family resulted from a mixture of selfing and
outcrossing ($r_t = 0.56$), and that the majority of outcrossed progeny were sired by one
paternal parent ($r_p = 0.98$). The large year-to-year variation in selfing rates may have
resulted from fluctuations in pollinator availability or the limited number of maternal
families sampled in 1997. Together with studies of other CH/CL species, this
investigation shows that CH flowers may be capable of substantial selfing rates.

One consequence of high selfing is a reduction in fitness of selfed progeny
(compared to outcrossed progeny) - this is known as inbreeding depression. The level of
inbreeding depression was measured in _Viola canadensis_ in such a way that fitness
differences could be attributed to either cross type (self versus outcross) or to floral type (CH versus CL). *Viola canadensis* was used instead of *V. pubescens* because low seed germination of the latter made it intractable for the study. Hand-pollinations were carried out to produce self- and outcross-pollinated CH progeny, and CL seeds were also collected. In a greenhouse, selfed and outcrossed CH flowers produced similar numbers of seeds, and both types of the resulting progeny had similar survival rates and comparable numbers of CH flowers, although outcrossed CH progeny had 14% greater biomass than selfed CH progeny. The level of inbreeding depression in *V. canadensis* was low (0.10), indicating that there may be few fitness disadvantages to selfing in this species. A comparison of CL and selfed CH progeny showed that although there were differences in CH flower number and number of days to first flower, overall fitness differences were minimal.

One potential consequence of selfing is reduced genetic variation. To determine if this occurred in *V. pubescens*, the genetic structure of six populations was measured using isozyme and inter-simple sequence repeat (ISSR) markers. Results from eight isozyme loci showed that there was considerable genetic variation in the species, and population structuring was indicated by unique alleles and a $\theta (F_{ST})$ value of 0.29. High genetic variation was also found using ISSR markers, and population structuring was again evident with unique bands. Overall, isozyme and ISSR techniques yielded similar results, indicating that ISSR markers show potential for use in population genetic studies. *Viola pubescens* appears to have a true mixed mating system in which selfing through CL and CH flowers contributes to population differentiation, and outcrossing through CH flowers may increase variation and gene flow among populations.
Dedicated to my husband.
Robert Culley
and my parents.
Ronald and Nancy Minear
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CHAPTER 1

INTRODUCTION

Background

Unlike many plant species in which individuals only produce one type of flower, some species produce two different floral types - open chasmogamous (CH) and closed cleistogamous (CL) flowers. Chasmogamous flowers typically are showy and are often visited by insects, potentially resulting in outcross-pollinations. In contrast, inconspicuous cleistogamous flowers resemble small buds that never open and are automatically self-pollinated. The production of both floral types is widespread (Darwin 1877), occurring in at least 56 angiosperm families, and comprising 287 species (Lord 1981). Species that posses this mating system are known as cleistogamous, or CH/CL species.

Despite longstanding recognition of the CH/CL mating system, not much is known about why certain plant species produce these two types of flowers or what the genetic consequences are of this mating system. Darwin (1877) believed that the purpose of CL flowers was to ensure seed production with as little expenditure of energy as possible, during times when conditions were unfavorable for pollination of CH flowers. Although Darwin only had a small amount of evidence, he believed that CH flowers were produced for occasional cross-fertilization, resulting in “invigorated” offspring. At the
time, Darwin was not aware of the genetic basis for variation, but it is now known that high levels of heterozygosity found in many outcrossing populations are associated with increased offspring vigor (Mitton 1993).

Many researchers since Darwin have attempted to explain the dual occurrence of CH and CL flowers. This work has focused almost exclusively on four members of the genus Impatiens that inhabit temperate deciduous forests. Because the CH/CL system is considered evolutionarily stable, the relative costs and benefits of chasmogamy and cleistogamy were assumed to balance each other over time. Schemske (1978) and Waller (1979) were the first to measure the relative energy spent in CH and CL seed production. They found that CH seeds were energetically more costly to produce, requiring at least one and a half times as much material and energy as CL seeds. Waller (1979) concluded that to offset this higher cost of CH seed development, there must be some type of CH fitness advantage.

Other scientists tried to document the occurrence and extent of the CH fitness advantage in early life history stages (Waller 1984: Bennington and McGraw 1995; Mitchell-Olds and Waller 1985; Schmitt et al. 1985; Antifinger 1986; Schmitt and Ehrhardt 1987, 1990; McCall et al. 1989; Schmitt and Gamble 1990). In general, these investigations found that CH and CL fitness components were very similar to one another, and that any significant differences were in the direction of a CH advantage. CL fitness advantages were detected on occasion, but always in CH/CL species other than Impatiens that had dimorphic seed forms (e.g., subterranean and aerial). In some cases, the documented CH advantage was considered high enough to offset CH production costs.
(e.g., Waller 1984), but in other studies, additional factors may have been responsible in maintaining the balance between chasmogamy and cleistogamy.

Several different explanations were presented. Schemske (1978) proposed that because many species are subjected to high degrees of temporal or spatial variation (e.g., decline in light levels from the forest edge to the center), strong selective pressures for reproductive plasticity jointly favors the production of CH and CL flowers. Pollinator unpredictability may also promote a CH/CL system; Lloyd (1979) noted that outcrossing will be favored if pollinators are present, while selfing will be favored when pollinators are scarce. Stewart (1994) suggested that inbreeding depression constrained greater CL reproduction, while CH reproduction was limited by a correlation between geitonogamy (within-plant selfing) and CH flower production. Sibling competition could also affect the maintenance of the CH/CL mating system if outcrossed CH progeny are genetically variable because they would be more likely to include the most favorable genotype suitable in different areas of a spatially variable environment (Schmitt and Ehrhardt 1987). However, evidence for the sib-competition model has not been detected (Mitchell-Olds and Waller 1985; Schmitt and Ehrhardt 1987; McCall et al. 1989).

Inherent in many of these studies was the assumption that chasmogamy and cleistogamy were equivalent to outcrossing and selfing, because CH flowers were considered outcross-pollinated (CL flowers are selfed, by definition). This was not surprising given that CH flowers typically have features attractive to pollinators (nectar, showy petals, fragrance), which may promote visitation rates and hence outcrossing. In addition, many investigations of CH/CL species focused on Impatiens, in which automatic selfing within CH flowers is structurally impossible (Schmitt et al. 1985; Lloyd
and Schoen 1992; Stewart 1994). However, the assumption of outcrossing in CH flowers may not hold true for all CH/CL species. For example, CH flowers were recently found capable of self-pollination in at least nine different species, all representing different genera (Kadono and Schneider 1989; Schoen and Brown 1991; Bryan 1993; Momose and Inoue 1993; Ruiz de Clavijo and Jimenez 1993; Ruiz de Clavijo 1997; Novosyelova 1998; Porras and Álvarez 1999; Culley, in review).

**Purpose of the Study**

My research focused on various factors that could influence the maintenance of CH and CL flowers, as well as the genetic consequences of this mating system. My first goal was to determine if a CH fitness advantage was present, and if it was high enough to explain the stability of the CH/CL system in a particular species. If not, I planned to explore other factors that may be important.

The violet species, *Viola pubescens* Aiton and *V. canadensis* L. (Violaceae) were chosen as study subjects for the following reasons. Both *V. pubescens* and *V. canadensis* are a significant part of the understory of beech-maple forests throughout eastern North America. They are among the first plants to flower in the early spring and they remain important components of the forest understory until early autumn - in contrast to spring ephemerals that die back once the trees begin to leaf out. Both violet species reproduce throughout the spring and summer, during which time light availability declines drastically as the forest canopy forms. Both stemmed species are nonclonal, making it easy to identify genetic individuals in the field (in contrast to stemless violet species). Despite their common nature, the reproductive biology of the two species was relatively
unknown and therefore in need of study. In fact, the Viola genus has received little attention since several ecological studies were conducted in the 1970s and 1980s.

As a first step in studying the CH/CL mating system in Viola, I investigated the reproductive biology of a central Ohio population of V. pubescens (Chapter 2). The goal of this study was to determine the importance of both CH and CL flowers in overall reproduction and to find whether a CH fitness advantage was present. The flowering phenology was measured to ascertain if CH and CL flower production corresponded with pollinator availability or different light levels. A pollination experiment was also performed to verify that CH flowers could outcross-pollinate and to determine if they were capable of autogamy (selfing within a flower). A CH fitness difference was not found, but other factors such as temporal variability of light levels and pollinator unpredictability were present. CH flowers were only produced in the early spring before leaf-out, when light was most available and pollinators were most frequent. CL flowers then appeared after the forest canopy formed and when pollinators were most scarce. Surprisingly, CH flowers were capable of delayed selfing, a mechanism of self-pollination in older flowers left unvisited by pollinators.

To quantify the amount of self-pollination occurring in CH flowers, I measured the rate of selfing over a two-year period in the Ohio population of V. pubescens (Chapter 3). Selfing rates were derived from multi-locus outcrossing rates and were measured using isozyme electrophoresis of field-collected seeds. I found that the selfing rate in CH flowers could be quite substantial in that over half of the seeds collected from plants in the field resulted from self-pollination.
Because substantial selfing occurred in *V. pubescens*, it was important to determine if self-pollination was associated with any negative fitness effects, such as inbreeding depression. Defined as the reduction in fitness in the progeny of closely related individuals, inbreeding depression may be important in maintaining the CH and CL flower production (Stewart 1994). However, most studies that measured inbreeding depression have done so by comparing the fitness of CH and CL progeny (i.e., assuming that chasmogamy and cleistogamy are equivalent to selfing and outcrossing). The problem with this approach is that it is not possible to distinguish between fitness differences due to cross type (self versus outcross) and to floral type (CH versus CL). To address this problem, inbreeding depression was measured in CH progeny of *V. canadensis*, with floral type differences quantified separately by comparing the performance of CL and selfed CH progeny (Chapter 4). *Viola canadensis* was used instead of *V. pubescens* because it has a higher rate of seedling emergence (~ 50% compared to 2-21%). In general, selfed and outcrossed CH progeny performed similarly in early life history stages, and the resulting level of inbreeding depression was very low. Floral type differences were also minimal because of similar performances of CL and self-CH progeny. The low level of inbreeding depression indicates that there may be few disadvantages to selfing — not surprising considering that self-pollination normally occurs in CL flowers.

Last of all, the genetic consequences of the CH/CL mating system are known in only a few species (Schoen 1984; Knight and Waller 1987; Lesica et al. 1988; Cole and Biesboer 1992; Sun 1999). By themselves, selfing and outcrossing have different effects on the genetic structure of plant populations. Selfing promotes population substructuring
through the loss and/or fixation of certain alleles, resulting in lower overall genetic diversity (Wright 1946). In contrast, outcrossing species may have higher levels of gene flow, resulting in elevated levels of genetic variation with populations sharing a number of alleles. Together in a mixed mating system involving CH and CL flowers, the relative contributions of selfing and outcrossing to reproduction within the population may influence population genetic structure. Using isozymes and inter-simple sequence repeat (ISSR) markers, genetic variation was measured in six populations of *V. pubescens* (Chapter 5). ISSR markers are a new technique that shows much promise in population genetic studies because they are more variable and less sensitive to degradation than isozymes. This was one of the first studies to use ISSR markers to measure genetic variation in natural populations. For both isozymes and ISSR markers, considerable genetic variation was detected in the species, and population structuring was also indicated by the presence of unique bands and alleles. This is consistent with the mixed mating system of *V. pubescens* in which selfing through CL (and CH) flowers contributes to population differentiation, and outcrossing through CH flowers may increase variation and gene flow among populations.
CHAPTER 2

THE REPRODUCTIVE BIOLOGY OF THE CLEISTOGAMOUS SPECIES,
Viola pubescens (VIOLACEAE)

When beechen buds begin to swell
And woods the blue-bird's warble know.
The yellow violet's modest bell
Peeps from the last year's leaves below.

William Cullen Bryant

Introduction

Unlike many plants that produce only one type of flower, some species produce two floral types - open chasmogamous (CH) and closed cleistogamous (CL) flowers. The showy CH flowers are often attractive to pollinators and have the potential to be outcross-pollinated, while CL flowers resemble small buds that self-pollinate and develop directly into seed capsules. In some species, CH flowers are able to self-pollinate in the absence of pollinators (Kadono and Schneider 1989; Schoen and Brown 1991; Bryan 1993; Momose and Inoue 1993; Ruiz de Clavijo and Jimenez 1993; Ruiz de Clavijo 1997; Novosyelova 1998; Porras and Álvarez 1999). Both flower types are usually produced on the same individual, but they may appear at different times or on plants in different microsites (e.g., sun versus shade). These CH/CL (or cleistogamous) species occur in at least 56 angiosperm families, comprising 287 species (Lord 1981).
The CH/CL mating system is of interest because it can enhance reproductive success under variable environmental conditions (Darwin 1877). In optimal situations of resource and pollinator availability, outcrossing in CH flowers can lead to greater genetic diversity, potentially increasing the population's ability to adapt to new environments (Stebbins 1950; Briggs and Walters 1997). On the other hand, CL flowers provide reproductive assurance when pollinators are scarce (Kerner von Marilaun 1902; Redbo-Torstensson and Berg 1995). CL flowers also facilitate reproduction under less than optimal availability of light, water, or nutrients (Le Corff 1993), because CL flowers are less costly to produce than CH flowers (Schemske 1978; Waller 1979). However, the relative contributions of each flower type to seed production and recruitment in a population is unknown for the majority of CH/CL species.

Most mating system studies of CH/CL species have concentrated on the Impatiens genus as a model system (Schemske 1978; Waller 1979; Waller 1984; Bennington and McGraw 1995; Mitchell-Olds and Waller 1985; Schmitt et al. 1985; Antifinger 1986; Schmitt and Ehrhardt 1987, 1990; McCall et al. 1989; Schmitt and Gamble 1990). Many of these studies compared CH and CL progeny and found either no fitness difference or only a slight CH fitness advantage. While these investigations have contributed greatly to our knowledge of CH/CL reproduction, it would be beneficial to examine additional species to determine if the reproductive biology of Impatiens is typical of most CH/CL species. Only a few other dicotyledon CH/CL genera have been studied (e.g., Gara and Muenchow 1990; Le Corff and Horovitz 1995; Le Corff 1996).

The Viola genus provides an ideal opportunity to study CH/CL reproductive biology because it has a worldwide distribution with many species producing both CH
and CL flowers. The pollination biology of several Viola species has been well-documented (Darwin 1877; Beattie 1971), as well as seed dispersal (Beattie and Lyons 1975; Culver and Beattie 1978; Trunbull and Culver 1983), floral development (Mayers and Lord 1983a,b), physiology and photosynthesis (Curtis 1984; Curtis and Kincaid 1984), and population biology (Cook 1980; Solbrig et al. 1980; Solbrig 1981a,b; Solbrig et al. 1988).

One Viola species in which the reproductive biology is relatively unknown is the downy yellow violet, V. pubescens. This species is commonly found in deciduous forests of eastern North America, often occurring in the same habitat as Impatiens. Viola pubescens is a nonclonal, stemmed species consisting of two varieties - var. pubescens (including V. pensylvanica Michx.) and var. scabriuscula (previously V. eriocarpa Schwein.). These varieties are morphologically different (Brainerd 1921; Baird 1942; Lévesque and Dansereau 1966; Cain 1967; Ballard 1994), and genetically different in at least one area in which they co-occur (Culley and Wolfe. in review). This perennial species overwinters as rhizomes and in the early spring, produces bright yellow CH flowers (mid-April to May), which are pollinated by bees and other insects (Beattie 1974). Small, closed CL flowers that appear in late May resemble small buds that are produced in the axils of the uppermost leaves. CH capsules are typically formed on longer peduncles than CL capsules (T. Culley, personal observation), and seeds from both capsule types are ballistically dispersed as the capsule splits into three valves that dry and slowly squeeze shut. Dispersal of seeds away from an unobstructed maternal plant has been measured up to 5.4 m with a mean distance of 1.2 m (V. pubescens, formerly known as V. pensylvanica in Culver and Beattie 1978 and V. eriocarpa in
Beattie and Lyons 1975). Both CH and CL seeds have elaisomes, and ant dispersal has been documented (V. pensylvanica in Culver and Beattie 1978).

The goal of this study was to investigate the reproductive biology of Viola pubescens by addressing the following questions: (1) What is the CH and CL flowering phenology? (2) What are the relative contributions of CH and CL flowers to seed production? (3) Are there any differences between CH and CL progeny in early life history stages? (4) Are CH flowers able to self-pollinate automatically?

Materials and Methods

Field experiments and observations were carried out in 1996, 1997, and 1999 at Ohio Wesleyan University's Bohannan Scientific Preserve, a 40.5 ha area in Delaware and Morrow Counties, Ohio (40°21' N and 82°55' W). The preserve is nearly surrounded by agricultural fields and consists of mature, second growth forest that is ~109 to 142 years old (Wallace 1982). Deciduous trees, such as beech, maple, oak, hickory, elm, ash, and sycamore, are common (Vadas and Sanger 1997). Individuals of V. pubescens var. scabriuscula are found throughout the preserve, especially in drier sites that are slightly disturbed, such as along trails.

A. Flowering Phenology

In April 1996, I marked 40 newly emerged plants with flags and metal identification tags. Every 2-7 days during the flowering season, the numbers of new and old CH and CL flowers were counted on each plant, so that the total numbers per season could be calculated. At the end of the season, I marked 46 additional plants to increase
the sample size for 1997. Four individuals did not survive the winter and were excluded from further observations. Beginning in mid-April, 1997, 82 plants were monitored as before.

Leaf-out of the forest canopy appeared to correspond to the switch to CL flowers in 1996, so I took light measurements throughout the 1997 flowering season to measure the amount of photosynthetically active radiation (PAR) available to the plants. A LI-1000 datalogger (LI-COR Inc., Lincoln, NE) with two quantum sensors (LI-190SA; LI-COR Inc.) was used to measure PAR during a 24-hour period on five, clear days during tree leaf-out (April 20, April 26, May 7, May 11, and May 21). Two quantum sensors were used to provide replication and these were placed near two Viola plants approximately eight meters apart. I chose clear days so that any decrease in radiation would be due to leafing out of the forest canopy and not to changes in cloud cover. This method of selecting either completely cloudless or uniformly cloudy days for light measurements is not unusual (Schemske et al. 1978; Motten 1986). I also visually estimated the progress of canopy closure.

B. Relative Fruit Production in CH and CL Flowers

During the 1997 season, I monitored the fate of each flower on each of the 82 plants used to measure flowering phenology. Over the course of the season, each flower was observed to determine if it aborted or developed into a seed capsule; each capsule was then examined as to whether it aborted, was damaged, or successfully dispersed seeds. Nonparametric tests had to be used to compare fruit production in CH and CL flowers because data were non-normally distributed, even after several transformations.
Sign tests on paired data (PROC UNIVARIATE; SAS 1989) were employed to compare on a per plant basis the total number of CH and CL flowers, as well as percentages of CH and CL capsules that aborted, were damaged, or successfully dispersed seeds. Only 62 plants that produced both flowers (Figure 2.1) were analyzed because of the paired nature of the data.

To prevent ballistic dispersal, developing CH and CL seed capsules were bagged with fine mesh tied around the peduncle of the flower two to three days before they opened. Dehisced capsules were then taken back to the laboratory where the number of seeds per capsule was counted. I used a one-way ANOVA with a fixed effect of flower type (CH and CL) to test for significant differences in number of seeds per capsule (using untransformed data). Because a difference in the number of seeds between CH and CL flowers may result from a difference in ovule number, I measured the number of ovules in 20 CH and 10 CL flowers. A t-test was used to test for a significant difference in ovule number between the two flower types.

![Figure 2.1. Fate of 86 flowering individuals of *V. pubescens* that were marked in 1996 and revisited in 1997.](image)
C. Differences in CH and CL Early Life History Stages

To test for fitness differences between CH and CL progeny in early life history stages, I measured seed mass and percent seedling emergence. I used seeds collected during the 1997 season (see above) and seeds gathered during 1996. All seeds were weighed individually to the nearest mg and a regression was used to determine if seed mass depended upon seed number. To test for a difference in seed emergence, I planted CH and CL seeds in two inch “community pots” filled with Promix potting medium on November 20, 1997. The pots were placed in a sandbox outside, and several layers of shade cloth and leaf litter were placed on top to simulate natural conditions. Seeding emergence began in March 1998 and was scored over a one and a half month period until no new seedlings appeared after several days. I used a two-way mixed model ANOVA with a random effect of year (1996 and 1997) and a fixed effect of flower type (CH and CL) to test for significant differences in seed mass and percent seedling emergence. To comply with ANOVA assumptions, seed mass data were log-transformed and percent emergence data were arc-sine-square root transformed.

D. Pollination of CH Flowers

I conducted a pollination experiment in 1999 to determine if CH flowers can autogamously self under field conditions and to verify that they can outcross under natural conditions following pollinator visitation. When grown inside a pollinator-free greenhouse, unmanipulated CH flowers frequently produced seeds while emasculated CH flowers failed to form fruit, indicating that autogamy, and not apomixis, occurs in V. pubescens (T. Culley, personal observation).
The pollination experiment consisted of four treatments with 40 randomly selected plants in each treatment (160 plants total). These were composed of the following: (1) Bagged – plants were covered with pollinator-exclusion cages (collapsible umbrellas used to cover plates of food); (2) Emasculated – CH buds on each plant were emasculated by carefully removing the stamens with fine forceps; (3) Hand-pollinated – CH buds on each plant were emasculated and then hand-pollinated two to three days later with pollen from another individual at least one meter away; (4) Control – plants left unmanipulated. In the first treatment, entire plants were bagged instead of individual flowers because the weight of a small mesh bag on a CH flower over several days could cause the peduncle to bend and break. Plants in all other treatments were left unbagged so they could be visited by pollinators. Thus, CH capsule development on emasculated plants in the field indicates pollinator activity, while seeds produced on bagged plants are evidence of CH autogamy. The hand-pollinated treatment was used to ensure that the emasculation technique did not adversely affect seed set.

I monitored flowers on all plants every two to three days over several weeks to determine if they developed into seed capsules and whether the capsules aborted, were damaged, or successfully dispersed seeds. A drought in late spring lasted approximately two to three weeks and caused the abortion of several CH capsules. Remaining capsules were bagged to prevent ballistic seed dispersal and were collected following dehiscence. The number of seeds per capsule was recorded, seeds were individually weighed, and mean seed mass was calculated for each capsule.

I used one-way ANOVAs to compare the effects of bagged, emasculated, hand-pollinated, and control treatments on percent fruit set (percentage of flowers forming
fruit), percent successful seed set (percentage of flowers forming seed), mean seed mass, number of seeds per capsule, and number of seeds per flower. The latter two parameters were chosen to measure seed number within flowers that successfully set seed (number of seeds per capsule) and within all flowers, regardless of whether or not they set seed (seed number per flower). Because percent seed set, percent fruit set, and seed number per flower data did not conform to ANOVA assumptions, they were rank-transformed; the number of seeds per capsule and mean seed mass data remained untransformed. A one-way ANOVA performed with ranked data is analogous to a Kruskal-Wallis non-parametric test (Conover and Iman 1981). For each ANOVA, least squares means (LSMEANS; SAS 1989) were used to determine significant differences among the four treatments.

Results

A. Flowering Phenology

CH and CL flowers were produced at different times during the season and this was consistent over a two-year period (Figure 2.2). As the plants emerged in early April, CH flowers appeared and peaked in frequency within 2-3 weeks. Production of CH flowers began to decline as the trees leafed out, and ended by the time the canopy was fully developed. At this point, CL flowers began to appear and were continuously produced until plants senesced in late autumn. This distinct switch-point between the production of CH and CL flowers was observed in 1996 and 1997 (Figure 2.2) as well as 1998 and 1999 (data not reported). Individual plants also varied in flower production.
of the 82 plants that emerged in 1997, three plants did not flower, 17 plants produced only CH or CL flowers, and 62 plants produced both CH and CL flowers.

Light availability differed between the times when CH and CL flowers were produced. The maximum amount of photosynthetically active radiation (PAR) was highest during CH flower production and declined to low levels before CL flowers appeared (Figure 2.2). As measured with individual sensors, the amount of PAR available to a plant on a 24-hour interval also differed before and after canopy formation (Figure 2.3). Because data were consistent from both quantum sensors, only data from one sensor is reported. Prior to tree leaf-out, the amount of photosynthetically active radiation (PAR) reached a higher maximum amount (1563 μmol m⁻¹ s⁻¹) with higher amounts available for longer periods, than after the forest canopy developed (maximum = 111 μmol m⁻¹ s⁻¹). The high amounts of PAR detected intermittently after tree leaf-out resulted from sunflecks on the forest floor.

B. Relative Seed Production in CH and CL Flowers

A total of 258 CH flowers and 296 CL flowers was produced by the 62 individuals in 1997. Most of these flowers or their developing fruits were damaged by herbivores (69% CH and 74% CL) or aborted naturally (9% CH and 16% CL). However, 22% of the CH flowers successfully dispersed seeds, compared with 10% of the CL flowers. In other words, of those flowers that successfully set seed, 64% were CH flowers and 36% were CL flowers.
Figure 2.2. The flowering phenology of *Viola pubescens* in (a) 1996 and (b) 1997. Closed circles represent the number of CH flowers and open circles signify the number of CL flowers in the group of marked individuals (see text). Forty plants were observed in 1996, and an additional 46 plants were added for the 1997 season. Squares signify photosynthetically active radiation (PAR) in 1997 (mean daily maximum for two sensors).
Figure 2.3. The amount of photosynthetically active radiation (PAR) reaching the forest floor during a 24 hour interval during (a) April 26 before tree leaf-out, and (b) May 21 after the canopy formed. Each point represents mean PAR for a five-minute interval.
On a per plant basis, there was no difference between cross types (CH and CL) in the number of flowers produced, the number of seeds per capsule, or the number of capsules that were aborted or damaged (Table 2.1). A mean of only 8-11 seeds formed in each capsule type, despite a large number of ovules per flower (24.3 in CH and 31.8 in CL flowers; t = 2.01, P = 0.07). CH capsules were nearly twice as likely to successfully form and disperse seeds as CL capsules (Table 2.1), indicating that progeny from CH flowers may be more common than those from CL flowers. Similar results were also obtained if data were analyzed on a per flower basis (all plants grouped together) rather than on a per plant basis.

<table>
<thead>
<tr>
<th>Per Plant:</th>
<th>CH</th>
<th>CL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td># Flowers</td>
<td>3.73 ± 0.30</td>
<td>4.60 ± 0.58</td>
<td>0.203</td>
</tr>
<tr>
<td># Successful capsules</td>
<td>0.82 ± 0.12</td>
<td>0.50 ± 0.16</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(21.9%)</td>
<td>(8.9%)</td>
<td></td>
</tr>
<tr>
<td># Aborted capsules and flowers</td>
<td>0.29 ± 0.07</td>
<td>0.73 ± 0.14</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td>(8.7%)</td>
<td>(14.4%)</td>
<td></td>
</tr>
<tr>
<td># Damaged capsules and flowers</td>
<td>2.61 ± 0.24</td>
<td>3.37 ± 0.44</td>
<td>0.111</td>
</tr>
<tr>
<td></td>
<td>(69.4%)</td>
<td>(76.7%)</td>
<td></td>
</tr>
<tr>
<td># Seeds per capsule</td>
<td>8.74 ± 0.90</td>
<td>10.91 ± 1.32</td>
<td>0.198</td>
</tr>
</tbody>
</table>

Table 2.1. A comparison of CH and CL parameters on a per plant basis for 62 individuals during the 1997 season. Successful capsules are those that disperse seeds. Standard errors are shown and P-values are results of sign rank tests on paired data. Of capsules that were successful, aborted, or damaged, the percentage of the total number of capsules is shown within parentheses.
C. Differences in CH and CL Early Life History Stages

There were no significant differences between CH and CL types in seed mass or percent seedling emergence (Tables 2.2, 2.3). Seed mass did not depend on seed number in either 1996 ($r^2 = 0.015$, $N = 52$, $P = 0.38$) or 1997 ($r^2 = 0.022$, $N = 46$, $P = 0.32$). Both CH and CL seeds weighed approximately 3 mg and emergence rates of both seedling types were only 16 - 21%. There were also no significant differences between years with regard to any of the parameters measured (Table 2.3).

<table>
<thead>
<tr>
<th>Character</th>
<th>1996</th>
<th>1997</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH</td>
<td>CL</td>
</tr>
<tr>
<td>Individual seed mass (mg)</td>
<td>3.08 (43.0.08)</td>
<td>2.93 (8.0.23)</td>
</tr>
<tr>
<td>Percent emergence</td>
<td>19.91 (18.5.30)</td>
<td>15.94 (13.4.51)</td>
</tr>
</tbody>
</table>

Table 2.2. Characteristics of CH and CL seeds in early life history stages. Numbers in parentheses are the sample size (number of capsules for seed mass, and number of pots for seedling emergence data) and the standard error for each mean. Percent seedling emergence for both years' seeds was measured in spring, 1998. None of the differences between cross types or years was significant (see Table 2.3).

D. Pollination of CH Flowers

Of the 40 plants in each treatment, some plants were removed from the analysis because they did not produce any CH flowers, and one bagged individual was removed because a moth was trapped in its cage. This left 38 plants in each of the four treatments.
Table 2.3. Results of mixed model ANOVAs with year (1996 and 1997) as a random factor and cross type (CH and CL) as a fixed factor. Dependent variables were individual seed mass and percent seedling emergence (as in Table 2.2).

<table>
<thead>
<tr>
<th>Character</th>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed mass</td>
<td>Year</td>
<td>1</td>
<td>0.0015</td>
<td>0.40</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Type</td>
<td>1</td>
<td>0.0232</td>
<td>3.51</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Year*Type</td>
<td>1</td>
<td>0.0066</td>
<td>1.72</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>93</td>
<td>0.0038</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent emergence</td>
<td>Year</td>
<td>1</td>
<td>200.5228</td>
<td>0.45</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Type</td>
<td>1</td>
<td>36.8457</td>
<td>0.15</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>Year*Type</td>
<td>1</td>
<td>252.8014</td>
<td>0.57</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>53</td>
<td>444.1844</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All plants produced similar numbers of CH flowers, and fruit set was in excess of 50% (Table 2.4). The majority of flowers that did not form fruit were produced at the end of the CH flowering season as leaf-out occurred; these CH flowers all aborted within a few days. Some capsules that did not survive (24.6%) were damaged by either deer, snails, or lepidopteran larvae. Other capsules aborted (13.3%) following a mid-season drought that began in mid May and lasted several weeks.

CH flowers were able to self-pollinate in the absence of pollinators, as indicated by the fruit set that occurred in bagged plants (Table 2.4). In terms of all flowers produced, seed number was significantly less in plants in the bagged treatment than those in the control group. Compared with control plants, bagged individuals had significantly fewer CH flowers that formed fruits (58.7% versus 88.3%), and a significantly smaller number of capsules surviving to successfully disperse seeds (5.0% versus 12.5%).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cross Type</th>
<th>N</th>
<th># Flowers</th>
<th>% Fruit set</th>
<th>% Successful</th>
<th># Seeds per capsule</th>
<th># Seeds per flower</th>
<th>Mean seed mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagged self</td>
<td>self</td>
<td>38</td>
<td>4.47a</td>
<td>58.66a</td>
<td>5.00a</td>
<td>11.75a</td>
<td>0.36a</td>
<td>3.00a</td>
</tr>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6.42)</td>
<td>(3.06)</td>
<td>(2.87)</td>
<td>(0.26)</td>
<td>(0.17)</td>
<td></td>
</tr>
<tr>
<td>Emasculated outcross</td>
<td>outcross</td>
<td>38</td>
<td>3.97a</td>
<td>58.46a</td>
<td>3.78a</td>
<td>5.17b</td>
<td>0.17ad</td>
<td>3.76b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(5.59)</td>
<td>(1.48)</td>
<td>(0.70)</td>
<td>(0.07)</td>
<td>(0.10)</td>
<td></td>
</tr>
<tr>
<td>Hand-pollinated</td>
<td>outcross</td>
<td>38</td>
<td>3.89a</td>
<td>68.68a</td>
<td>10.90b</td>
<td>6.50bc</td>
<td>0.64bc</td>
<td>3.08a</td>
</tr>
<tr>
<td>(Emasculated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4.76)</td>
<td>(2.64)</td>
<td>(0.81)</td>
<td>(0.18)</td>
<td>(0.12)</td>
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</tr>
<tr>
<td>Control</td>
<td>unknown</td>
<td>38</td>
<td>3.71a</td>
<td>88.33b</td>
<td>12.50b</td>
<td>8.93ac</td>
<td>1.01bcd</td>
<td>2.97a</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2.99)</td>
<td>(3.08)</td>
<td>(1.01)</td>
<td>(0.27)</td>
<td>(0.18)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4. Performance of CH flowers on plants in the four treatments on a per plant basis during 1999. Percent fruit set refers to flowers that formed fruit, and percent successful refers to capsules that successfully dispersed seeds. Number of seeds per capsule represents flowers that successfully set fruit, while number of seeds per flower was based on all flowers and takes into account those that did not set seed. Numbers in parentheses are the standard error of each mean. Superscripts of different letters denote significant differences among treatments (P < 0.05) using least squares means, and N refers to the number of individuals in each treatment.
However, plants in both the bagged and control treatments yielded capsules with similar numbers of seeds that had comparable seed mass (Table 2.4).

Pollinator visitation did occur, as shown by seed set in emasculated CH flowers (Table 2.4), and this was consistent with pollinator observations. During many hours of fieldwork during the 1996-1998 seasons, insects were not observed visiting CH flowers of *V. pubescens*. However in 1999, several potential pollinators were seen visiting the CH flowers. These included bee flies (*Bombylius major*, Bombyliidae, Diptera), skipper butterflies (*Erynnis juvénalis*, Hesperiidae, Lepidoptera), bumblebees (Apidae, Hymenoptera), carpenter bees (Anthophoridae, Hymenoptera), and four species of halictid bees (Halictidae, Hymenoptera). Although I did not verify that these floral visitors were effective pollinators, many of these insects have been documented as efficient pollinators of *V. pubescens* (referred to as *V. eriocarpa* in Beattie 1974).

Although outcross-pollinations naturally occurred in CH flowers, they were not always successful. Compared with individuals in the control treatment, emasculated plants had significantly lower fruit set, seed set, and number of seeds per capsule, but they produced seeds with a higher mean seed mass. The latter may reflect a negative correlation between the number of seeds per capsule and mean seed mass ($r^2 = -0.237, P = 0.002$). When seed set in all flowers was considered (regardless of whether a flower set seed or not), plants in the emasculated treatment had the lowest number of seeds per flower. This was due largely to low numbers of seeds in the few flowers that were successful (Table 2.4).

There appeared to be no adverse affect of the emasculation treatment, as emasculated flowers that were hand-pollinated had similar levels of fruit set as purely
emasculated flowers (Table 2.4). However, capsules that resulted from hand-pollinations were nearly three times as likely to set seed as capsules that originated from emasculated flowers that were left alone. Although plants with hand-pollinated flowers had lower fruit set than control plants, their capsules were just as likely to produce seeds (10.9% for pollinated plants and 12.5% for control plants), with the same number of seeds per capsule and mean seed mass as control plants (Table 2.4). For all flowers produced, the total number of seeds produced per plant was not significantly different for individuals within hand-pollinated and control treatments. Because hand-pollinated flowers were first emasculated, these results cannot be used to measure pollen limitation.

Although CL flowers were not the focus of this experiment, the performance of CH flowers could subsequently affect that of the CL flowers. if resources are limiting. However, there were no significant differences in the fruit set or seed set of CL flowers among the four treatments (data not shown), even though differences were detected with CH flowers.

Discussion

Both CH and CL flowers are important in the reproductive biology of Viola pubescens. During 1997, CH and CL seeds were produced within the population, but an individual plant did not always produce both seed types. In general, many (but not all) plants had at least one CH flower produce seeds (an average of 0.82 CH fruits were formed per plant), with even lower numbers of CL flowers forming seed (0.50 CL fruits were formed). These seed set levels were similar to those reported for other Viola species. Plants averaged less than one fruit per year in V. sororia (Solbrig et al. 1980), V.
pallens and V. blanda (Newell et al. 1981), and V. lanceolata (Solbrig et al. 1988), but 0.6 to 3.2 fruits per year were produced in V. fimbriatula (Solbrig et al. 1988). The two-fold reproductive advantage of CH flowers in V. pubescens occurred despite similar numbers of CH and CL flowers per plant. Of the CH and CL capsules that were produced, there was no difference in the number of seeds per capsule, mean seed mass, or emergence rates of CH and CL progeny. In Impatiens (Schemske 1978) and in other earlier studies of Viola (Darwin 1877; Bailey 1903; Clute 1907), CL flowers produced more seeds than CH flowers. However, seed set in the present study was only measured during one season and it is possible that it may vary seasonally depending upon pollinator availability, herbivory, and drought during seed formation (causing late fruit abortion).

There were no significant differences in early life history stages between CH and CL progeny, even though a CH fitness difference is usually expected because it would balance the higher cost of producing showy CH flowers (Schemske 1978; Waller 1979). Only a few studies have detected a significant CH fitness advantage in early life history stages. For example, CH fruits produced more seeds than CL fruits in Impatiens (Schemske 1978; but see Bennington and McGraw 1995), Viola canina (Darwin 1877), and Calathea micans (Le Corff 1993). CH seeds had higher seed mass in Impatiens (Schemske 1978; Mitchell-Olds and Waller 1985; Schmitt and Ehrhardt 1987; Schmitt and Gamble 1990; but see Schmitt et al. 1985), but not in Collomia grandiflora (Wilken 1982) and Calathea micans (Le Corff 1993; Le Corff and Horovitz 1995; Le Corff 1996). Emergence did not differ between CH and CL seedlings in I. capensis (Waller 1984; Antifinger 1986; Schmitt and Ehrhardt 1987; Schmitt and Ehrhardt 1990) and Calathea micans (Le Corff and Horovitz 1995; Le Corff 1996).
There are at least two explanations of why both CH and CL flowers are produced in *Viola pubescens*. First, light availability may be important. The discrete switch point between the production of CH and CL flowers corresponded with a reduction in photosynthetically active radiation (PAR) as the forest canopy formed (Figure 2.2). In the present study, light levels under the forest canopy decreased to 7% of the initial levels before the canopy formed, and in other *Viola* studies, PAR was 1-13% of that available above the canopy (Curtis and Kincaid 1984; Solbrig et al. 1980). Theoretically, high light availability would lead to increased photosynthetic rates, which would result in more resources available for production of energetically costly CH flowers (Schemske 1978; Waller 1979). CL flowers would subsequently appear under low light levels after tree leaf-out because they are less costly to produce. Flower production prior to leaf-out has also been observed in several spring ephemeral herbs (Schemske et al. 1978). In addition, light availability was important in *Impatiens* – in general, individuals near the forest edge produced CH flowers while plants in the shaded interior had predominately CL flowers, although flower production varied during the season as well (Schemske 1978; Schemske 1984). However, other factors not measured in this study, such as daylength, nutrient availability, moisture, and light climate the previous year, have been shown to be important in flower production of other understory herbs (Mayers and Lord 1983a; Dahlem and Boerner 1987; Gara and Muenchow 1990; Le Corff 1993).

A second reason why both CH and CL flowers are produced is that it may be an adaptation to changes in pollinator activity during the season. In elevated light environments during early spring, air temperatures are high enough (>12°C; Motten 1986) to allow for maximum pollinator activity and easy detection of flowers (Kerner
von Marilaun 1902; Schemske et al. 1978). This would favor production of showy CH flowers. For example, skipper butterflies and syrphid flies are more active in sunny locations or during non-cloudy days when they can elevate their temperatures by basking (Heinrich and Pantle 1975; Pivnick and McNeil 1987). In a study of seven spring ephemeral species (Schemske et al. 1978), flowering peaks occurred prior to tree leaf-out during the first period of weather consistently suitable for insect pollinators. Once the forest canopy has formed, temperatures may be too low for suitable insect thermoregulation, and insects may be restricted to sunflecks on the understory floor (Schultz 1998). In this situation, self-pollinated CL flowers would be favored in shaded environments because they do not depend on insect pollinators.

One advantage *Viola pubescens* has over most other spring wildflowers is the delayed selfing mechanism in CH flowers. When pollinators were excluded in bagged treatments, delayed selfing occurred in older CH flowers as the stigma slowly bent down and contacted pollen grains that had fallen out of the anthers and were resting on the anterior petal. This mechanism was previously unknown in *V. pubescens*, but has been detected in other *Viola* species (Kerner von Marilaun 1902; Culley, in review), with some exceptions (Knuth 1908; Beattie 1969; Banasinska and Kuta 1996). Delayed or induced selfing has also been found in other CH/CL species (Schoen and Brown 1991; Ruiz de Clavijo 1997), but not in *Impatiens* (Lloyd and Schoen 1992; Schmitt et al. 1985).

Delayed selfing is especially important in *V. pubescens* because flowering in the early spring is a high-risk option in terms of pollinator availability (Schemske et al. 1978; Motten 1986). In fact, delayed selfing should be selected if pollinators are unpredictable within or among flowering seasons (Kalisz et al. 1999). Thus, it is not surprising that this
mechanism was found in *V. pubescens* because I only observed pollinator activity during one of the four years (1999) I spent in the field. By enabling costly CH flowers to produce seeds in the absence of pollinators, delayed selfing is advantageous even if it has lower reproductive success than outcrossing (i.e., it is better to produce some inferior seeds, than none at all). In the present study, CH selfing resulted in lower seed set than if the emasculated CH flowers were hand-pollinated with outcross-pollen or left alone and available to pollinators (Table 2.4). This was true for those flowers that were successful and for all flowers that were produced. However, seed set on bagged individuals may have been lower than usual in 1999 because of a drought that occurred during fruit formation, causing early fruit abortion on plants in all treatments. In a preliminary experiment conducted in 1998 with only 10 bagged individuals, there was 100% fruit set compared with 59% seen in 1999. These field observations agree with a selfing rate study of *V. pubescens* in which intermediate selfing was detected in naturally-pollinated CH flowers (*s* > 0.50; T. Culley, unpublished data).

Although selfing was possible, outcrossing still occurred in CH flowers, as indicated by seed set in emasculated flowers (Table 2.4). Because most plants only produced 1-2 flowers at any one time, geitonogamous pollination was unlikely, although it cannot be completely ruled out. One unexpected outcome of the experiment was that seed set in emasculated flowers (~4%) was approximately one third of that in control flowers (12.5%). The emasculation technique was not directly responsible for this reduction because emasculated flowers that were hand-pollinated also had higher seed set (11%) than purely emasculated flowers. One possibility is that the emasculation technique indirectly affected seed set through its effect on pollinators. Because many
Viola pollinators, such as solitary bees, visit CH flowers for both nectar and pollen (Beattie 1972), pollinators may have been able to distinguish emasculated flowers (i.e., absence of pollen) prior to floral entry. Another reason for low seed set in emasculated flowers may have been that they were pollen limited. Unfortunately, pollen limitation could not be tested in this study because a unemasculated/hand-pollinated treatment was not included.

The reproductive biology of *V. pubescens* presented in this investigation differs from that of the widely studied *Impatiens* genus in three ways. First, there is a strict temporal separation of CH and CL flower production in *V. pubescens*, while both spatial and temporal separations exist in *Impatiens* (Schemske 1978; Schemske 1984). Second, CH flowers appear to have a greater role in seed production in *V. pubescens* than in *Impatiens*. Third, delayed selfing can occur in CH flowers of *V. pubescens*, but is structurally impossible in *Impatiens* (Schmitt et al. 1985; Lloyd and Schoen 1992; Stewart 1994), although both species may experience geitonogamy. The main similarity between *V. pubescens* and *Impatiens*, as in many CH/CL species, is that CH and CL fitness differences are either lacking or minimal during early life history stages (see above).

In *Viola pubescens*, both outcrossing through CH flowers and selfing through CL and CH flowers takes place. Thus, the species has a true mixed mating system that may account for its wide range. As Beattie (1971) and Waller (1984) suggest, outcrossing may serve to promote the formation of new genetic combinations that facilitate adaptation to new or changing habitats. On the other hand, selfing would result in progeny genetically similar to the parents, and would be beneficial in a locally-adapted
environment. Selfing may also promote the fixation of new, advantageous alleles introduced through outcrossing, which would be helpful in a newly colonizing population or in an older population faced with environmental change. The CH/CL mating system of *Viola* also provides a means of adapting to unpredictable pollinators in the early spring (through delayed selfing) and to pollinator absence during the rest of the season (through CL flowers). Beattie (1971) states that “this is a sexual system of great evolutionary versatility” and it may be one reason why *V. pubescens* is so common throughout eastern North America.
CHAPTER 3

SELFING RATES IN CHASMOGAMOUS FLOWERS OF THE CLEISTOGAMOUS HERB, Viola pubescens (VIOLACEAE)

Introduction

Many plant species have the ability to reproduce through self-pollination, a form of inbreeding. The rate of selfing is important in the evolution of plant mating systems (Lande and Schemske 1985; Charlesworth and Charlesworth 1987), especially if self-pollination produces inferior progeny compared to outcross-pollination (Darwin 1877). Selfing can also affect the genetic variation and structure of populations by promoting substructuring through the loss and/or fixation of certain alleles (Wright 1946, 1969).

Self-pollination can occur in several different ways (Lloyd and Schoen 1992). Selfing within an individual flower (autogamy) may take place before, during, or after opportunities for outcross-pollination occur (prior, competing, and delayed selfing; Lloyd and Schoen 1992) or when environmental conditions prevent flower opening (induced selfing; Schoen and Lloyd 1984). Selfing may also be facilitated by pollinator behavior within a flower, or as a pollinator visits a succession of flowers on the same plant (geitonogamy). A type of indirect selfing, known as biparental inbreeding, may also occur as a flower is fertilized with pollen from a related individual.
Another way that plants may self-pollinate is through cleistogamous (CL) flowers that never open and are automatically selfed. Species that have these flowers usually also produce showy chasmogamous (CH) flowers on the same plant (Darwin 1877; Lord 1981) - these species are known as cleistogamous or CH/CL species. CH flowers have often been assumed to be primarily outcross-pollinated rather than selfed (Waller 1979; Waller 1984; Mitchell-Olds and Waller 1985; Antifinger 1986; Schmitt and Ehrhardt 1987; McCall et al. 1989). These flowers typically have features that are considered attractive to pollinators (showy petals, nectar guides, nectar, fragrance, etc.), which may promote visitation rates and hence outcrossing. Another reason CH flowers are considered outcrossed is that many well-known studies of the CH/CL mating system have focused on the Impatiens genus, in which CH flowers are strongly protandrous and autogamy is mechanically impossible (Schmitt et al. 1985; Lloyd and Schoen 1992; Stewart 1994).

However, recent work has shown that selfing rates in CH flowers can be substantial, in addition to that already occurring in CL flowers (e.g., Stewart 1994). CH selfing rates in other unexplored CH/CL species also have the potential to be high because of mechanisms that promote autogamy within CH flowers. For example, delayed or induced selfing has been detected in CH flowers of Ajuga chamaepitys (Ruiz de Clavijo 1997), Glycine clandestina (Schoen and Brown 1991), Triodanis perfoliata (Bryan 1993), and Viola canadensis (Culley, in review). CH flowers are also able to self-pollinate in Centaurea melitensis (Porras and Álvarez 1999), Ceratocapnos heterocarpa (Ruiz de Clavijo and Jimenez 1993), Eurvale ferox (Kadono and Schneider 1989),
Medicago spp. (Novosyelova 1998), and Polygonum thunbergii (Momose and Inoue 1993).

Viola pubescens, the downy yellow violet, is a CH/CL species in which CH flowers are capable of delayed self-pollination, but selfing rates have never been measured. In this species, CH selfing occurs in older, unvisited flowers when the stigma bends down and contacts pollen grains that have fallen out of the anther cone and are resting on the anterior petal (T. Culley, personal observation). This mechanism has also been detected in Viola canadensis (Culley, in review) and in Viola spp. in the Melanium group (Kerner von Marilaun 1902), but not in V. odorata (Knuth 1908; Banasinska and Kuta 1996) or V. riviniana (Beattie 1969). Thus, delayed selfing may not be a widespread phenomenon in Viola. Geitonogamy is probably uncommon in V. pubescens because most individuals rarely have two or more compatible CH flowers open at the same time. A recent investigation of the population genetic structure of V. pubescens suggested that the species has an intermediate selfing rate (Culley and Wolfe, in review). The goal of this study was to measure the rate of selfing in CH flowers of V. pubescens over a two-year period. To my knowledge, this is the first study to measure selfing rates in a CH/CL species known to possess delayed selfing in CH flowers.

Materials and Methods

Viola pubescens (Violaceae) is a stemmed, nonclonal violet commonly found in mixed mesic forests across northeastern North America. In the early spring, this perennial species produces yellow chasmogamous (CH) flowers (mid-April to May) that are infrequently visited by a variety of solitary bees, bee flies, and lepidopterans (T.
Culley, unpublished data). Small, self-pollinated cleistogamous (CL) flowers appear after the canopy trees leaf out (May to September). CH capsules contain approximately nine seeds and are typically produced on longer peduncles than CL capsules (T. Culley, unpublished data). Seeds from both capsule types are ballistically dispersed up to 5.4 m with a mean distance of 1.2 m (Beattie and Lyons 1975; Culver and Beattie 1978). Both CH and CL seeds have elaisomes, indicating that secondary dispersal by ants may be common. The species is diploid with $2n = 12$ chromosomes (Clausen 1929; Canne 1987).

Selfing rates were measured in 1996 and 1997 in a population located at Ohio Wesleyan University's Bohannan Scientific Preserve, a 40.5 ha area in Delaware and Morrow Counties, Ohio (40°21' N and 82°55'W). Plants were randomly tagged throughout the population and when available, at least two CH seed capsules were collected from each individual. A total of 36 plants were sampled in 1996, and 13 individuals were sampled in 1997. Of these, nine plants were sampled in both years. To prevent ballistic dispersal of the seeds, the developing capsules were bagged two to three days before the capsules opened (about 20 days following the end of anthesis). Dehisced capsules were then collected and seeds were stored in glassine envelopes at 4°C for several weeks. Because of low seed germination in past studies (2 - 20%; T. Culley, unpublished data), seeds instead of seedling tissue were used for isozyme analysis.

Enzyme extraction began by first treating the seeds to break dormancy. Seeds were soaked in concentrated sulfuric acid for 30 minutes to scarify the seed coat, washed with 500 ml of cold water, and left to dry overnight on filter paper in petri dishes. The following morning, the seeds were transferred to new petri dishes filled with distilled
water, and stored at 4°C for three days. Seeds were ground individually in 1.5 ml eppendorf tubes, using a small amount of sand and approximately 1 drop of extraction buffer (Morden et al. 1987). The supernatant was absorbed onto 1 mm Whatmann 3M filter paper wicks, and stored at -80°C.

A morpholine-citrate gel buffer system at pH 6.1 (Clayton and Tretiak 1972) with a 1:19 dilution for the gel buffer was used to resolve the following three enzymes: aminopeptidase (AMP), isocitrate dehydrogenase (IDH), and glucose-6-phosphate isomerase (GPI). Several additional isozyme systems that worked well in a previous isozyme study using bud and leaf tissue (Culley and Wolfe, in review) were not sufficiently resolved using seed tissue, so they were not used in this investigation. Gels consisted of 11-12% potato starch (Starch Art. Smithville. Texas. USA) and markers of known genotypes were run on each gel to ensure consistent scoring. Gels were run at a constant voltage for 5 hours at 170-200 V. Staining recipes were as described in Culley and Wolfe (in review), and a single locus was resolved for all three isozyme systems. Five alleles were detected in the IDH locus, two alleles in the AMP locus, and three alleles were found in the PGI locus. Mendelian inheritance of these loci was confirmed using segregation patterns from CL seeds.

Selfing rate estimates were calculated for 1996 and 1997 using MLTR, a multilocus mating system computer program by K. Ritland (1990). Within each year, seeds from different capsules were grouped together to make up each maternal family. Only maternal families consisting of eight or more progeny were used in the analysis, although most families had more than 15 progeny. Family size was emphasized over family number to increase the accuracy of maternal genotype inference. The total
number of maternal families \(N = 13 - 36\) and progeny \(N = 124 - 654\) varied in the two years due to seed availability. Maternal genotypes were either known from a previous electrophoretic study (T. Culley, unpublished data) or were estimated using the most likely maternal genotype (Ritland 1990). All values were generated using the expectation-maximization method with pollen gene frequencies constrained to equal ovule frequencies, and standard deviations were based on 500 bootstraps. Multilocus \(t_m\) and single-locus \(t_s\) outcrossing rates were estimated, and selfing rates were calculated as \((1 - t_m)\). Also known as the effective selfing rate, \(t_s\) incorporates both effects of within-plant selfing and biparental inbreeding. Because \(t_m\) only measures the within-plant selfing rate, a positive difference between \(t_m\) and \(t_s\) indicates biparental inbreeding (Brown 1990).

The number of maternal families was sufficiently large enough in 1996 to allow for calculation of the inbreeding coefficient of maternal parents averaged over loci \((F \text{ or } F_b)\), as well as correlated mating parameters (Brown 1990; Ritland 1990). The latter were estimated using the sibling-pair model of Ritland (1989). The first parameter, \(r_s\), is the correlation of outcrossing rate between a randomly chosen pair of siblings of each maternal parent. If \(r_s\) is equal to 1, then siblings both result from either selfing or from outcrossing. The second parameter, \(r_p\), is the correlation of outcrossed paternity within families, or the probability that a randomly chosen pair of outcrossed progeny are full sibs (i.e., share the same paternal parent). Due to the limited number of capsules sampled per plant and software limitations of the number of alleles per locus, sib-pairs could not be analyzed within each capsule. Thus, I was unable to determine if progeny from one capsule were likely to be all selfed or all outcrossed. Instead, progeny from different
capsules had to be grouped together for each maternal family, which may underestimate \( r_t \) and \( r_p \).

**Results**

The rate of self-pollination in CH flowers was significantly higher in 1996 \((s = 0.60)\) than in 1997 \((s = 0.07\); \(Z\)-test, \(Z = 7.42, P < 0.001\); Table 3.1). Low to moderate levels of allelic variation were detected in the progeny (Table 3.2). The selfing rate estimate from 1996 was actually greater than the predicted estimate based on the inbreeding coefficient, assuming mating system equilibrium \((s = 1 - t, \text{ where } t = [1 - F] / [1 + F]; \text{ Jain 1979; Hedrick 1985})\). This was true if \( F \) was obtained from this study \((F = 0.28; s = 0.44)\), or from a previous, more extensive isozyme investigation of the population \((F = 0.26; s = 0.41; \text{ Culley and Wolfe, in review})\). These inbreeding coefficients suggest that moderately inbred plants exist in the population. However, biparental inbreeding was not substantial in either year, as the difference between \( t_m \) and \( t_s \) was close to zero (Table 3.1).

The correlated mating parameters calculated from the 1996 data indicated that siblings within a maternal family resulted from a mixture of selfing and outcrossing \((r_t = 0.56)\). Of those siblings that originated from outcross-pollinations, the majority were sired by only one paternal parent \((r_p = 0.98)\). This value was very high, despite the grouping of seeds from different capsules within a family.
Table 3.1. Selfing rate (s) results for 1996 and 1997. Shown for both years are the multi-locus outcrossing rate (tm), the single-locus outcrossing rate (ts), and the difference (tm - ts) indicating biparental inbreeding. The selfing rate (s) was calculated as (1 - tm). Family size was sufficiently large enough in 1996 for calculations of the parental inbreeding coefficient (F), the correlation of outcrossing rate within families (r_s), and the correlation of outcrossed paternity within families (r_p). Numbers shown in parentheses are standard deviations, based on 500 bootstraps. The number of progeny used in the analysis varied because not all progeny were scored for all three loci.

<table>
<thead>
<tr>
<th>Year</th>
<th># families</th>
<th># progeny</th>
<th>s</th>
<th>tm</th>
<th>ts</th>
<th>tm - ts</th>
<th>F</th>
<th>r_s</th>
<th>r_p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996</td>
<td>36</td>
<td>493-654</td>
<td>0.60</td>
<td>0.40</td>
<td>0.40</td>
<td>-0.003</td>
<td>0.28</td>
<td>0.56</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.09)</td>
<td>(0.07)</td>
<td>(0.02)</td>
<td>(0.10)</td>
<td>(0.18)</td>
<td>(0.02)</td>
</tr>
<tr>
<td>1997</td>
<td>13</td>
<td>124-190</td>
<td>0.07</td>
<td>0.94</td>
<td>0.86</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.05)</td>
<td>(0.06)</td>
<td>(0.03)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Estimated pollen allele frequencies for all plants sampled in 1996 and 1997 in the Bohannan population.

<table>
<thead>
<tr>
<th>Allele</th>
<th>1996</th>
<th>1997</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH-1</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>IDH-2</td>
<td>0.14</td>
<td>0.06</td>
</tr>
<tr>
<td>IDH-3</td>
<td>0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>IDH-4</td>
<td>0.75</td>
<td>0.86</td>
</tr>
<tr>
<td>IDH-5</td>
<td>&lt;0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>AMP-1</td>
<td>0.63</td>
<td>0.67</td>
</tr>
<tr>
<td>AMP-2</td>
<td>0.37</td>
<td>0.33</td>
</tr>
<tr>
<td>GPI-1</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>GPI-2</td>
<td>0.91</td>
<td>0.69</td>
</tr>
<tr>
<td>GPI-3</td>
<td>0.08</td>
<td>0.30</td>
</tr>
</tbody>
</table>

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Discussion

In contrast to the assumption that CH flowers of CH/CL species are primarily outcross-pollinated, CH selfing was detected in this central Ohio population of Viola pubescens, where rates ranged from low to substantial levels. Over half of the CH progeny in 1996 resulted from self-pollination, while less than a tenth were selfed the following year. Selfing was probably due to autogamy, rather than geitonogamy or biparental inbreeding, because most plants rarely had two or more compatible flowers open at the same time (T. Culley, personal observation) and levels of biparental inbreeding were very low. Autogamy was likely caused by delayed selfing in older CH flowers that were left unvisited by insect pollinators. Facilitated selfing by short-tongued pollinators, which has been detected in other Viola species (Knuth 1908; Beattie 1971), may also contribute to autogamy.

The large difference in selfing rates between years may have been caused by two factors. First, annual fluctuations in pollinator activity may explain some variation (see Kalisz et al. 1999), with higher pollinator visitation rates in 1997 leading to an increase in outcross-pollinations. In fact, progeny from six of the nine plants sampled in both years had a novel GPI allele present in 1997 (GPI^2), suggesting an outcrossed origin. Although I did not observe any pollinator activity while at the field site during 1996 and 1997, it is possible that visits the second year went undetected. Pollinators may have been more active in 1997 because of warmer temperatures that occurred in the beginning of the flowering season - average monthly temperatures were higher and skies were less cloudy than in 1996 (T. Culley, unpublished data). Pollinator activity may be reduced by low air temperatures and overcast skies because insects experience difficulties with
thermoregulation (Motten 1986). In addition, warmer temperatures in 1997 may have triggered plants to begin flowering approximately two weeks earlier than the previous year (T. Culley, unpublished data). Differences in flowering times may also explain variation in activity of certain pollinators; this has been suggested for _Penstemon penlandii_, in which different pollinator species were only present during one of two years (Tepedino et al. 1999).

Another possible reason for year-to-year variation in selfing is the number of maternal families used in the analysis. Due to low seed availability in 1997 because of herbivory (T. Culley, personal observation), only 13 families were analyzed, compared with 36 families in 1996. Although individual-based selfing rates were not measured because of insufficient family size, plants within a population may differ in their rates of selfing in a given year (A. Rankin, S. Weller, and A. Sakai, unpublished data). If only a small number of plants are sampled, the estimated selfing rate may vary by chance alone. For example, 25 random subsamples of 13 families from the 1996 dataset resulted in selfing rates ranging from 0.37 to 0.92. However, none of these values came close to approximating the 1997 selfing rate (s = 0.07), indicating that the differences between years may be more than just a sampling artifact. In addition, selfing rates calculated for the nine individuals sampled in both years were very different from one another (0.61 in 1996 and 0.02 in 1997). Nonetheless, because my sample sizes were low in 1997, only the 1996 selfing rate will be discussed further.

The CH selfing rate detected in the Ohio population of _V. pubescens_ in 1996 (s = 0.60) was well within the range of most values reported for other CH/CL species (Table 3.3). These studies along with the present investigation show that CH flowers of species
### Table 3.3

<table>
<thead>
<tr>
<th>Species</th>
<th># pop</th>
<th># families</th>
<th># loci</th>
<th>s</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danthonia spicata (Gramineae)</td>
<td>1</td>
<td>15</td>
<td>---</td>
<td>~0.92</td>
<td>Clay 1982</td>
</tr>
<tr>
<td>Impatiens capensis (Balsaminaceae)</td>
<td>2</td>
<td>60, 54</td>
<td>3,5</td>
<td>0.03, 0.46</td>
<td>Mitchell-Olds and Waller 1985</td>
</tr>
<tr>
<td>Impatiens capensis (Balsaminaceae)</td>
<td>6</td>
<td>16-71</td>
<td>1-3</td>
<td>0.50</td>
<td>Waller and Knight 1989</td>
</tr>
<tr>
<td>Impatiens pallida (Balsaminaceae)</td>
<td>1</td>
<td>352-360</td>
<td>5</td>
<td>0.50</td>
<td>Stewart 1994</td>
</tr>
<tr>
<td>Glycine argyrea (Fabaceae)</td>
<td>1</td>
<td>23</td>
<td>10</td>
<td>0.62</td>
<td>Brown et al. 1986</td>
</tr>
<tr>
<td>Lespedeza capitata (Fabaceae)</td>
<td>1</td>
<td>40</td>
<td>34</td>
<td>0.96</td>
<td>Cole and Biesboer 1992</td>
</tr>
<tr>
<td>Viola pubescens (Violaceae)</td>
<td>1</td>
<td>36, 13</td>
<td>3</td>
<td>0.60, 0.07</td>
<td>present study</td>
</tr>
</tbody>
</table>

1. estimated from genetic variation ($V_a$) and F
2. two populations
3. averaged over six populations in which selfing rates ranged from 0.30 to 0.68
4. averaged over three years in which selfing rates ranged from 0.34 to 0.51
5. two years

Table 3.3. Selfing rates ($s$) detected in chasmogamous (CH) flowers of CH/CL species. Shown are the number of populations, the number of maternal families, and the number of loci used in each analysis. In most cases, the selfing rate was calculated as ($1 - \text{the outcrossing rate}$). Unless noted, analyses involved isozyme electrophoresis of progeny arrays.
studied thus far are capable of substantial selfing rates in a given population or year.
Thus, it may be inaccurate to assume that CH flowers of CH/CL species are primarily
outcross-pollinated. Substantial selfing in CH flowers may also explain why large fitness
differences have not been found in CH and CL progeny of several species (e.g.,
Schemske 1978; Waller 1979; Waller 1984) because they share the same selfed cross
type (Antlfinger 1986; Stewart 1994).

It is interesting that V. pubescens with its delayed selfing mechanism has a lower
selfing rate than at least two of the CH/CL species lacking this ability (Table 3.3).
Theoretically, species with delayed selfing should have a higher selfing rate than other
species, because delayed selfing contributes directly to the selfing rate. However,
pollinator visits to V. pubescens may have been frequent enough so that delayed selfing
did not always occur, and as a result, CH flowers were not completely selfed (i.e., s ≈
1.0). In species without delayed selfing, CH selfing rates may be higher because of
substantial self-pollination by geitonogamy and/or biparental inbreeding (Waller and
Knight 1989; Stewart 1994), or pollinator-facilitated selfing (Cole and Biesboer 1992).
However, further studies are needed of CH/CL species with delayed selfing to further
explore the effects of this mechanism on CH selfing rates.

The selfing rates presented in this study should only be considered estimates of
the selfing rate level for V. pubescens in general. Only one population with a limited
number of maternal families was used (compared to the total population size), and selfing
rates may vary across populations (e.g., Mayer et al. 1996; Pascarella 1997). In addition,
Ritland and Jain (1981) recommend at least three to four unlinked loci with intermediate
gene frequencies to provide accurate multi-locus outcrossing rates in a selfing species. In
the present study, only three loci with low to moderate levels of allelic variation were used. Thus, the multilocus outcrossing rates ($t_m$) reported here should be considered minimum estimates, with the resulting selfing rates ($1 - t_m$) regarded as maximum estimates. Although many other selfing rate studies of CH/CL species have also focused on one population or used three or fewer loci (Table 3.3), future investigations should strive to increase the number of populations, families, and loci.

Mixed selfing was also detected within individual plants, in addition to that found on the population level. As indicated by the moderate level of correlated outcrossing ($r_t = 0.56$), siblings within a maternal family resulted from both self- and outcross-pollinations. Because seeds from different capsules were grouped together for each family, I could not determine if selfing and outcrossing were both occurring within one capsule or separately in different capsules. The latter could happen if one CH flower was visited by a pollinator carrying outcrossed pollen, while delayed selfing occurred in another flower. Delayed selfing should increase $r_t$ because there will be a higher probability that a randomly chosen pair of siblings are both selfed. Of those siblings that were outcross-pollinated, the majority was sired by only one father ($r_p = 0.98$), despite the grouping of seeds from different capsules. This could be due to repeated crossing with close neighbors, or low pollen carryover of pollinators. In addition, only a single visit may be required for full pollination in Viola (Beattie 1971), so it is possible that all seeds within a capsule result from one pollination event. However, more than one father could have also sired the outcrossed seeds if multiple fathers shared the same genotype - this would mask the number of potential fathers and overestimate $r_p$. This is possible because of the low to moderate levels of allelic variation detected in the study.
Stewart (1994) also estimated correlated mating parameters over three years in the CH/CL species, *Impatiens pallida*, for progeny sampled within the same capsule and between different capsules. Regardless of which capsules the progeny pairs originated from, Stewart (1994) found that at least some seeds in CH capsules resulted from selfing over a three-year period (average $r_t < 0.21$). After including the contribution of population substructure, Stewart concluded that most of the seeds in many CH capsules were selfed ($r_t$ averaged over three years was 0.69). This is similar to the value detected in *V. pubescens*. Of the progeny that were outcross-pollinated on a given plant, Stewart found that more than one paternal parent was involved, regardless of whether seeds were from the same capsule (average $r_p = 0.16$) or from different capsules (average $r_p = 0.14$). Thus, outcross progeny in *I. pallida* had a much lower probability of sharing the same paternal parent than in *V. pubescens* ($r_p = 0.98$).

Given the production of self-pollinated CL flowers in *Viola pubescens*, it is interesting that substantial CH selfing rates were detected. One explanation is that the delayed selfing mechanism in CH flowers has evolved in response to unpredictable pollinator availability (Kalisz et al. 1999). Pollinator unpredictability in the early spring has been observed in the Bohannan population over a four-year period (T. Culley, unpublished data). In cases such as this, it would be advantageous for a plant to produce some selfed seeds in the costly CH flowers (Schemske 1978; Waller 1979) after opportunities for outcrossing have passed, rather than have no seed set at all. As a result, selfing rates should be much higher when pollinators are scarce than when they are locally abundant. To further investigate this relationship between CH selfing rates and pollinator availability, more multi-year studies are needed in several populations. Such
investigations may help reveal why CH flowers of some CH/CL species are capable of high selfing rates.
CHAPTER 4

INBREEDING DEPRESSION AND FLORAL TYPE FITNESS DIFFERENCES IN Viola canadensis (VIOLACEAE), A SPECIES WITH CHASMOGAMOUS AND CLEISTOGAMOUS FLOWERS

Introduction

Inbreeding depression is thought to play a major role in the evolution of plant mating systems. Defined as the reduction in fitness of progeny from closely related individuals (relative to outcrossed progeny), inbreeding depression has been employed to explain the evolution and maintenance of mating systems that enhance cross-fertilization (Charlesworth and Charlesworth 1978, 1987; Lande and Schemske 1985). In recent years, there has been an increase in the number of studies of inbreeding depression in many different species (reviewed in Husband and Schemske 1996). However, only a few of these studies have focused on an unusual class of plant species - those that reproduce through both open, chasmogamous (CH) and closed, cleistogamous (CL) flowers.

Species with the CH/CL mating system usually have the ability to produce both floral types on the same plant, although CH and CL flowers may appear at different times or in different positions. Elaborate CH flowers are usually attractive to pollinators and are capable of outcross-pollination, while CL flowers resemble small buds that develop directly into fruit and automatically self-pollinate. Species with both floral types occur in at least 56 angiosperm families, comprising 287 species (Lord 1981). The few
dicotyledon CH/CL genera in which the reproductive biology has been studied include Impatiens (e.g., Schemske 1978; Waller 1984), Calathea (Le Corff and Horovitz 1995), Triodanis (Gara and Muenchow 1990), and Viola (Beattie 1971).

For this type of mixed mating system to be maintained, the overall level of inbreeding depression is expected to be low enough so that selfed (CL) progeny can survive (Schoen and Lloyd 1984). A difficulty in quantifying inbreeding depression in CH/CL species is that in many cases it cannot be accurately measured by simply comparing progeny from CH and CL flowers. One reason is that unless CH flowers are known to be fully outcross-pollinated, chasmogamy and cleistogamy are not equivalent to outcrossing and selfing (Clay and Antonovics 1985; Husband and Schemske 1996). In some species, CH flowers have been found to exhibit some degree of self-pollination, either through geitonogamy or biparental inbreeding (Mitchell-Olds and Waller 1985; Knight and Waller 1987; Waller and Knight 1989; Cole and Biesboer 1992; Stewart 1994). If self-pollination in CH flowers has been documented, it must be accounted for in a study measuring levels of inbreeding depression (as in Schmitt and Ehrhardt 1990).

A second and perhaps more important reason that a comparison of CH and CL progeny may be impractical in an inbreeding depression study is if fitness differences between the two progeny types are associated with flower type differences and not due to differences in mating type (Clay and Antonovics 1985, Husband and Schemske 1996). Thus, non-genetic differences could potentially confound genetic differences of the CH and CL progeny. For example, different numbers of ovules in CH and CL flowers of Impatiens pallida and I. biflora (Schemske 1978) may constrain the relative numbers of seeds produced in the two flower types and this would be independent of any genetic
effects associated with selfing. In some species, seed size may differ between floral types due to wind-dispersal of CH seeds (Clay 1983), and/or the subterranean production of CL seeds (Koller and Roth 1964; Weiss 1980; Cheplick and Quinn 1982). The effects of floral type differences have remained unexamined in studies of inbreeding depression and to the best of my knowledge, they have only been quantified once in a study of seed germination conditions and differences in adult biomass (Bryan and Muenchow 1991; Bryan 1993).

Both inbreeding depression and the effects of floral type differences can be measured in CH/CL species. Floral type differences can be quantified by comparing the progeny from self-pollinated CH flowers with those from CL flowers. This involves comparisons of progeny derived from different flower types (CH versus CL), but of the same mating type (self-pollinated). To accurately measure inbreeding depression without floral type effects, the fitness of self- and outcross-pollinated CH progeny can be compared (different mating types, but same floral type).

The first goal of this study was to measure the level of inbreeding depression in the CH/CL species, *Viola canadensis*. The reproductive biology of this species has remained unexplored in spite of its wide distribution across eastern North America. The second objective was to determine if progeny from CH and CL flowers exhibit fitness differences associated with flower type.

**Materials and Methods**

*Viola canadensis* is a herbaceous perennial found in the understory of beech-maple forests in eastern North America (Ballard 1994). This caulescent (stemmed)
species is nonclonal, making it easy to identify genetic individuals in the field. The CH flower consists of a white corolla with a yellow center, nectar guides, and a slightly sweet fragrance, suggesting insect pollination. Pollinator visitation appears to be very infrequent, but likely pollinators include bumblebees, skipper butterflies, solitary bees, and hoverflies that visit other violet species (Darwin 1877; Beattie 1971; T. Culley, personal observation). Prior to this study, it was unknown if CH flowers in *V. canadensis* were able to autogamously self-pollinate. CH flowers are produced primarily in the early spring before the forest trees leaf out and may also appear intermittently throughout the summer, whereas small, bud-like CL flowers subsequently appear after the canopy has fully developed (T. Culley, personal observation). CL flowers in this species are produced distally on the stem, in contrast to stemless *Viola* species that produce CL flowers near the base of the plant. CL seeds have elaisomes (absent on CH seeds), suggesting that secondary seed dispersal by ants may occur in this species (see Beattie and Lyons 1975). Both seed types are ballistically dispersed as the capsule valves dry and slowly squeeze shut, with seeds being dispersed up to four meters away from unobstructed parental plants (T. Culley, unpublished data). *Viola canadensis* is a tetraploid with $2n = 24$ chromosomes (Clausen 1929; Canne 1987), and belongs to section *Candenses* in the *Chamaemelanium* group, which typically has $x = 6$ chromosomes (Ballard et al. 1999).

A greenhouse crossing program was used to generate the selfed and outcrossed plants used in the study (Figure 4.1). To first obtain parental plants for use in the crosses, CH seeds were collected from a large number of individuals in a population in Emmet County, Michigan in summer, 1995. Unfortunately, seeds from only four field plants
germinated the following spring, thus limiting my ability to select genetically independent parental plants. From the seedlings that survived the following year, 15 individuals (representing all four field families, or grandmaternal parents) were randomly selected and these served as the parental plants in the crossing design.

Figure 4.1. Diagram of the crossing design for producing selfed and outcrossed progeny. Shown is only a sample of the actual number of plants used in the crossing program (see text). Outcross pollinations were conducted between individuals derived from different field plants (the grandmaternal generation; represented by dotted arrow).
The crossing program began in spring of 1997. To ensure that outcross pollinations were conducted between unrelated individuals, they were only made between pairs of individuals that had originated from different field plants. CH flowers were first emasculated and then pollinated three days later with pollen from either the same plant (self-CH treatment) or from an unrelated plant with a different field parent (outcross-CH treatment). Pollinations were achieved by brushing a set of newly dehisced anthers across the stigmatic surface, until it was covered with pollen. A subset of emasculated flowers was left unmanipulated and these failed to set seed, indicating that uncontrolled pollinations or apomixis had not occurred. To test for differences between artificial and natural pollinations, 67 unmanipulated CH flowers were allowed to set seed on their own (autonomously self-pollinated). Because there was no significant difference in fitness values between progeny from unmanipulated CH flowers and self-CH progeny (t-tests; P>0.06), the data from these two treatments were pooled to increase sample size. An additional pollination treatment consisted of CL flowers, which were unmanipulated (self-pollinated, by definition). Because the anthers dehisce early in the CL bud, it was not possible to conduct outcross pollinations on these flowers.

Three to four weeks after pollination, all developing fruit capsules were bagged to prevent ballistic seed dispersal. Following fruit collection, the number of seeds per capsule were recorded and all seeds were weighed individually. Seeds from a total of 126 CH capsules (self and outcross) and 59 CL capsules were stored at 4°C for approximately six to eight months until late autumn when they could be planted. Because a difference in seed number in CH and CL flowers could result from a disparity in ovule number, the average number of ovules produced in both floral types was also
measured. Buds were collected from 32 extra plants and the number of ovules was counted under magnification after dividing each ovary in half. The ovules were clearly visible and did not require any special clearing or staining procedures. Differences in ovule number between CH and CL flowers were analyzed with a Student's t-test.

In November 1997, a total of 2,139 seeds was planted in groups of ten (according to their maternal parent) in 5-cm diameter "community" pots filled with Promix® planting medium. These pots were then transported outside, immersed in a sandbox, and covered with several layers of shade cloth and leaf litter to simulate natural conditions. Germination and seedling emergence began early in February 1998, following an early warming period. Shortly after emergence began, outside temperatures fell dramatically, and because some seedlings began to suffer frost damage and a few (4%) died, the pots were moved into the greenhouse. Emergence and seedling survival were monitored every three to four days over a two month period. In April, all seedlings from the community pots were transplanted into individual 7.5-cm diameter pots. All pots were rotated every few days to minimize possible position effects. The plants began to produce CH flowers in mid-May and nearly all plants had flowered two months later. On July 17, 1998, the total number of CH flowers produced during the two month flowering interval were recorded after counting the number of flowers, seed capsules, or peduncles (for older, senesced flowers). None of the progeny had produced any CL flowers during this period, and because of space and time limitations, the study was terminated. The aboveground biomass of each plant was harvested, dried at 45°C for four days, and weighed to the nearest mg.
The cumulative relative fitness method was used to calculate the level of inbreeding depression and to measure floral type differences in *V. canadensis*. To determine the level of inbreeding depression, relative fitness was calculated for each life history stage as the following:

\[
\text{Relative Fitness} = \frac{W_{\text{S-CH}}}{W_{\text{O-CH}}} \tag{1}
\]

where \(W_{\text{S-CH}}\) is the fitness of the selfed-CH progeny and \(W_{\text{O-CH}}\) is the fitness of the outcross-CH progeny. The cumulative relative fitness was generated by multiplying the relative fitness values of the number of seeds per capsule, percent survival in community and individual pots, and vegetative biomass. The level of inbreeding depression in CH flowers was calculated as \((1 - \text{cumulative relative fitness})\). A value of zero indicates an absence of inbreeding depression, while negative values denote that selfed progeny outperformed outcrossed progeny. Positive values suggest that selfed progeny performed worse than outcrossed progeny.

To determine whether floral type differences exist, relative fitness was again calculated, but was adjusted accordingly:

\[
\text{Relative Fitness} = \frac{W_{\text{CL}}}{W_{\text{S-CH}}} \tag{2}
\]

where \(W_{\text{CL}}\) is the fitness of the CL progeny. The cumulative relative fitness was again calculated in the same way as previously, using the new relative fitness values. This statistic was then used as a measurement of floral type differences, where values close to one indicate that CL and self-CH progeny had similar fitness.

In calculating both cumulative relative fitness values, the life history stages mentioned above were chosen because of their importance in overall fitness and their presumed independence of one another. Some dependent life history stages were
excluded from the relative fitness calculations, although they were analyzed separately. Vegetative biomass was used instead of flower number because it may be a better indicator of fitness than just one period of flowering in this perennial species. Furthermore, flower number and vegetative biomass were significantly correlated \( r = 0.26, P < 0.0001 \). However, flower number was used in a separate analysis to see if it had any effect on the level of inbreeding depression. Seedling emergence was not included in the calculation of inbreeding depression because ungerminated seeds may still have been viable. Although little is known about the persistence of a seed bank in *V. canadensis*, over half (65%) of stored seeds have remained viable for three years (T. Culley, unpublished data), and seed banks have been found in other *Viola* species as well (Solbrig et al. 1988). Seed mass averaged per capsule was not used because it was negatively correlated with the number of seeds per capsule \( r = -0.28, P < 0.0001 \).

To investigate whether cross types differed significantly in the life history stages measured, ANOVA procedures were employed using SAS Release 6.12 (SAS Institute, Cary, NC). For seed mass, number of seeds per capsule, and percent seedling emergence data, a mixed model three-way ANOVA (see McIntosh 1983) was used to analyze the fixed effect of cross type, and random effects of grandmaternal parent, and maternal parent (nested within grandmaternal parent). Maternal parent was considered nested because parents were not entirely independent from one another, as they had originated from only four grandmaternal field plants. To test effects of grandmaternal parent and cross type, the denominator mean square for the F-tests was that of the maternal parent and the interaction term \((\text{grandmaternal parent} \times \text{cross})\), respectively. To test all other effects, the error mean square was used. Prior to running each analysis, the data were
examined for normality and homoscedasticity. Seed mass data did conform to these ANOVA assumptions, but the number of seeds per capsule had to be log-transformed and percent emergence was arcsine-square-root transformed.

For all remaining life history stages, a one-way ANOVA was used to examine the effect of cross type. This was necessary because emergence rates were not uniform across cross types in all maternal families, and so grandmaternal and maternal effects in later stages could not be accurately examined. To conform to ANOVA assumptions, percent survival in community pots was arcsine-square-root transformed, and the number of days to first CH flower was log-transformed. The number of CH flowers and vegetative biomass data were left untransformed. If cross type was found to be significant in any ANOVA, unorthogonal planned contrasts (CONTRAST; SAS 1989) were used to analyze differences between (1) self-CH and outcross-CH progeny, and (2) CL and self-CH progeny. Percent survival in individual pots was examined with a G-test of heterogeneity on untransformed data.

Results

Level of inbreeding depression

In the early life history stages, fitness components of selfed and outcrossed CH progeny were similar (Table 4.1). Both cross types had comparable numbers of seeds per capsule (approximately 9-10 seeds) with seeds of similar mass (Table 4.1), resulting in no significant differences between selfed and outcrossed CH progeny (Table 4.2). Seeds were produced in unemasculated CH flowers, indicating that autogamous selfing can
occur in this species. Seed mass varied among the different maternal parents within each
grandmaternal parent, as indicated by a significant maternal effect (Table 4.2).

Emergence of selfed and outcrossed CH seedlings was similar (Table 4.2). However, significant maternal effects were detected as percent emergence varied among the different maternal parents within each grandmaternal field parent (Table 4.2). A significant interaction between cross type and grandmaternal parent also occurred because selfed and outcrossed CH progeny were significantly different from one another for three of four grandmaternal field parents. The overall emergence rates measured during this study were low – only 17% (compared to previous emergence rates of over 50% following similar overwintering conditions). The observed reduction in seedling emergence may be caused by an extension of seed dormancy that could have been triggered by the abnormal warming period in early spring that was quickly followed by freezing temperatures (Baskin and Baskin 1998. p. 50).

Survival in both community and individual pots was high for selfed and outcrossed CH progeny, ranging from 81% to 100% (Table 4.1). Survival in community pots included persistence in early spring following frost damage. There was no significant difference in survival among cross types in community pots (Table 4.2) or in individual pots (G = 4.488. df = 2. P = 0.11).

Selfed and outcrossed plants from CH flowers began to flower at the same time, as measured by the number of days to first flower (Table 4.2). Over the two month period, progeny from the self-CH and outcross-CH treatments produced similar numbers of CH flowers (Table 4.1, Table 4.2). However, outcross-CH progeny had ~14% greater biomass than self-CH progeny (Table 4.1) and this difference was significant (Table 4.2).
Table 4.1. Performance of CL, self-CH, and outcross-CH progeny of *Viola canadensis* within each of the measured life history stages. Reported for each stage is the mean, followed by the standard error and sample size within parentheses. Sample sizes for the number of seeds per capsule and seed mass refer to the number of capsules; samples sizes for percent emergence and survival in community pots are based on the number of pots; samples sizes for the remaining characters refer to the number of individual plants sampled. Standard errors are not reported for survival in individual pots because data were based on counts.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Selfed CL</th>
<th>Self-CH</th>
<th>Outcrossed outcross-CH</th>
</tr>
</thead>
<tbody>
<tr>
<td># Seeds / capsule</td>
<td>8.97</td>
<td>9.72</td>
<td>9.14</td>
</tr>
<tr>
<td></td>
<td>(0.86, 59)</td>
<td>(0.79, 81)</td>
<td>(0.88, 48)</td>
</tr>
<tr>
<td>Seed mass</td>
<td>1.78</td>
<td>1.66</td>
<td>1.45</td>
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<td></td>
<td>(0.04, 59)</td>
<td>(0.03, 81)</td>
<td>(0.05, 48)</td>
</tr>
<tr>
<td>Percent emergence</td>
<td>27.9</td>
<td>16.5</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>(4.1, 77)</td>
<td>(2.2, 127)</td>
<td>(2.6, 58)</td>
</tr>
<tr>
<td>Survival (community)</td>
<td>88.5</td>
<td>85.0</td>
<td>80.6</td>
</tr>
<tr>
<td></td>
<td>(3.9, 42)</td>
<td>(4.2, 54)</td>
<td>(11.2, 12)</td>
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<tr>
<td>Survival (individual)</td>
<td>96.1</td>
<td>93.1</td>
<td>100.0</td>
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<tr>
<td></td>
<td>(----, 149)</td>
<td>(----, 149)</td>
<td>(----, 29)</td>
</tr>
<tr>
<td># Days to 1st flower</td>
<td>182.1</td>
<td>184.9</td>
<td>182.8</td>
</tr>
<tr>
<td></td>
<td>(0.6, 147)</td>
<td>(0.8, 127)</td>
<td>(1.4, 29)</td>
</tr>
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<td>CH flower number</td>
<td>91.9</td>
<td>79.4</td>
<td>75.6</td>
</tr>
<tr>
<td></td>
<td>(2.8, 147)</td>
<td>(2.7, 127)</td>
<td>(6.3, 29)</td>
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<td>5.10</td>
<td>5.93</td>
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<tr>
<td></td>
<td>(0.12, 147)</td>
<td>(0.13, 127)</td>
<td>(0.32, 29)</td>
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<td>Factor</td>
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<td>MS</td>
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<td>---------------------------</td>
<td>---------------------------</td>
<td>-----</td>
<td>---------</td>
</tr>
<tr>
<td># Seeds per capsule</td>
<td>Cross Type</td>
<td>2</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>Grandmother</td>
<td>3</td>
<td>0.168</td>
</tr>
<tr>
<td></td>
<td>Mom (Grandmother)</td>
<td>11</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Cross*Grandmother</td>
<td>6</td>
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</tr>
<tr>
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<td>Error</td>
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<td>Cross Type</td>
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<tr>
<td></td>
<td>Grandmother</td>
<td>3</td>
<td>11711.75</td>
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<tr>
<td></td>
<td>Mom (Grandmother)</td>
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<td>Cross*Grandmother</td>
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<td></td>
<td>Error</td>
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<td>3490.53</td>
</tr>
<tr>
<td>Survival</td>
<td>Cross Type</td>
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</tr>
<tr>
<td></td>
<td>Error</td>
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<td>738.56</td>
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<tr>
<td>Number of days to 1st</td>
<td>Cross Type</td>
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<td>0.001</td>
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<tr>
<td>flower</td>
<td>self-CH vs. outcross-CH</td>
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</tr>
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<td>1</td>
<td>0.003</td>
</tr>
<tr>
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<td>Error</td>
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<td>0.0003</td>
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<td>Total flower number</td>
<td>Cross Type</td>
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</tr>
<tr>
<td></td>
<td>self-CH vs. outcross-CH</td>
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<td>1078.95</td>
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<td>self-CH vs. CL</td>
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<td>7.28</td>
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<tr>
<td></td>
<td>Error</td>
<td>300</td>
<td>2.18</td>
</tr>
</tbody>
</table>

** P<0.05  ** P<0.01  *** P<0.001

Table 4.2. Effects of cross type and parental group on the number of seeds per capsule, seed mass, and percent seed germination using a mixed, nested ANOVA (see text). When cross type is significant, results of planned contrasts are shown between (1) selfed and outcrossed chasmogamous (CH) progeny, and (2) selfed CH and cleistogamous (CL) progeny. Because of low emergence rates, parental groups were combined for the remainder of the study. A one-way ANOVA was used to analyze the effect of cross type on percent seedling survival in community pots, number of days to first CH flower, total number of CH flowers, and vegetative biomass. Grandmother refers to the field parents and asterisks denote significance levels.
The cumulative level of inbreeding depression in *Viola canadensis* was low (\( \delta = 0.10 \); Table 4.3), as measured by the number of seeds per capsule, percent seedling survival in community and individual pots, and vegetative biomass. If flower number was substituted for vegetative biomass, the level of inbreeding depression was even lower (\( \delta = -0.14 \)). Inbreeding depression was not detected in early life history stages, but subsequently appeared during a later stage (vegetative biomass), when a significant difference between self and outcrossed CH progeny was observed.

<table>
<thead>
<tr>
<th>Comparison</th>
<th># Seeds / capsule</th>
<th>% Survival (community)</th>
<th>% Survival (individual)</th>
<th>Vegetative biomass</th>
<th>Cumulative fitness</th>
</tr>
</thead>
<tbody>
<tr>
<td>self CH</td>
<td>1.06</td>
<td>x</td>
<td>0.93</td>
<td>0.86 *</td>
<td>0.90</td>
</tr>
<tr>
<td>outcross CH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBD</td>
<td>-0.06</td>
<td>-0.06</td>
<td>0.07</td>
<td>0.14</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison</th>
<th># Seeds / capsule</th>
<th>% Survival (community)</th>
<th>% Survival (individual)</th>
<th>Vegetative biomass</th>
<th>Cumulative fitness</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>0.92</td>
<td>x</td>
<td>1.03</td>
<td>0.94</td>
<td>0.93</td>
</tr>
<tr>
<td>self CH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3. Relative fitness values in *Viola canadensis* progeny as measured in the number of seeds produced per capsule, percent seedling survival in community and individual pots, and vegetative biomass. Cumulative fitness is calculated as the product of all relative fitness values. The first comparison reveals the level of inbreeding depression (IBD) in chasmogamous (CH) flowers in each stage as (1 - relative fitness). Negative levels of inbreeding depression indicate that selfed progeny outperformed outcross progeny. The second comparison measures fitness differences attributed to flower type that may exist between CH and cleistogamous (CL) progeny. Asterisks indicate significant differences (\( P < 0.05 \)) between pairs of means in each comparison (see Table 4.1).
B. Floral Type Differences

Fitness differences associated with flower type (CH versus CL) were not detected until later stages. Both CL and self-CH pollinations resulted in similar numbers of seeds per capsule (9-10 seeds; Table 4.1), despite large differences in ovule number between CH (\(\bar{X} = 29.8 \pm 5.5\) SD, \(N = 16\)) and CL (\(\bar{X} = 17.4 \pm 3.7\) SD, \(N = 16\)) flowers (t-test, \(P < 0.0001\)). One initial concern was that the presence of an elaisome on CL seeds would interfere with seed mass comparisons, but CL seeds collected from the greenhouse-grown plants lacked elaisomes. Thus, the elaisome may be a plastic feature whose presence or absence is dictated by environmental influences. CL and self-CH seedlings also had similar survival rates in community and individual pots (Tables 4.1 and 4.2).

The only significant differences between CL and self-CH progeny were seen in the flowering stages (Table 4.2). CL progeny began to flower approximately 2 days earlier than self-CH progeny, and they produced more flowers on average than self-CH progeny (Table 4.1). However, both cross types produced similar amounts of vegetative biomass (Tables 4.1 and 4.2).

When relative fitness of CL and self-CH progeny was compared across several life history stages (Table 4.3), all values were close to one, indicating that both cross types had similar fitness. In addition, the cumulative relative fitness was very high (0.93), suggesting minimal fitness differences associated with flower type.

Discussion

The level of inbreeding depression in Viola canadensis low (\(\delta = 0.10\)), excluding effects of floral type differences. With the exception of later life history stages, self-CH
progeny performed as well as or better than outcross-CH progeny in all fitness measures, indicating there are few fitness disadvantages to selfing in this species. This level of inbreeding depression agrees with Schoen and Lloyd's (1984) model that predicted low levels of inbreeding depression if cleistogamy is to be maintained. Significant fitness differences due to floral type were also detected, but only in later stages involving the production of CH flowers. Overall, these fitness differences due to floral type were minimal.

There are several possible explanations for the low level of inbreeding depression observed in *V. canadensis*. First, increased ploidy level may affect the level of inbreeding depression in tetraploid *V. canadensis*. Recent theoretical work has shown that tetraploids, whether they be autopolyploids or allopolyploids, should express lower equilibrium levels of inbreeding depression than diploid species when mutations are lethal recessive or sublethal (Lande and Schemske 1985). For partially recessive mutations, Lande and Schemske predicted no differences in inbreeding depression levels for diploid and tetraploid species. However, Bennet (1976) argued that autopolyploid populations should accumulate higher frequencies of partially recessive mutations, resulting in higher levels of inbreeding depression than diploids. Ronfort (1999) disagreed and noted that for a given selfing rate, autotetraploids could have lower levels than diploids assuming identical mutation rates, selection, and dominance coefficients. In one empirical study of *Epilobium angustifolium*, lower levels of inbreeding depression were found in autotetraploid populations than in diploid populations (Husband and Schemske 1997), supporting the models of Lande and Schemske (1985) and Ronfort (1999). If differences in inbreeding depression levels are present between diploid and
polyploid populations, the magnitude may be affected by a variety of factors, including polyploid type (auto- versus allopolyploid) because of the associated mode of inheritance (tetrasomie versus disomic), age of the polyploid, degree of dominance (in the case of partial recessives), and selection regime (Lande and Schemske 1985; Husband and Schemske 1997; Ronfort 1999; but see Bennett 1976, and Bever and Felber 1992).

Polyplody may also affect inbreeding depression if genetic bottlenecks occurred during the formation of the species (Husband and Schemske 1997; T. Marcussen, personal communication). These bottlenecks could have resulted in increased rates of selfing and the purging of deleterious recessive alleles, thereby reducing levels of inbreeding depression in the species. Although increased ploidy level may make the formation of recessive homozygotes less likely (Stebbins 1950; Busbice and Wilsie 1966; Clay and Antonovics 1985), purging can still occur and may actually be more efficient in tetraploids than in diploids (Ronfort 1999).

Another factor that may contribute to low levels of inbreeding depression is if a purging of the deleterious alleles responsible for inbreeding depression occurred during the initial evolution of cleistogamy in the species (Schemske 1983; Clay and Antonovics 1985; Antlfinger 1986). Deleterious alleles would then enter the gene pool only via mutation or by distant gene flow (assuming local adaptation of the populations). These recessive alleles could then be removed from the gene pool following their expression in selfed progeny through CL and/or CH flowers. This idea is supported by the observation that the level of inbreeding depression in _V. canadensis_ is more similar to the average level detected in predominately selfing species (0.23) than in outcrossing species (0.53; Husband and Schemske 1996). Furthermore, the timing of inbreeding depression in _V.
**canadensis** follows the same pattern as for selfing species— inbreeding depression was expressed late in the life cycle, at the stage of vegetative growth and reproduction (*V. canadensis* δ = 0.14, Table 4.2; selfing species δ = 0.17, Husband and Schemske 1996). Inbreeding has likely purged the population of recessive lethal alleles causing early-acting inbreeding depression and the remaining low levels of inbreeding depression in later stages are probably due to weakly deleterious mutations that are difficult to purge (Husband and Schemske 1996).

One way selfing may occur in populations of *V. canadensis* is if reproduction is mainly through self-pollinated CL flowers. A primary role of CL flowers in reproduction in *Viola* was supported both by early botanists (see Darwin 1877; Bailey 1903; Clute 1907), who reported that CH flowers appeared to produce either few or no seeds, and by Beattie (1971), who documented a higher ratio of CL to CH seeds despite substantial CH seed production. Because I have readily collected both seed types in a natural population of *V. canadensis*, it is likely that both CH and CL flowers have the potential to play important roles in reproduction. Furthermore, CH flowers in an Ohio population of a related species, *V. pubescens*, were twice as likely to set seed as CL flowers and were responsible for 64% of fruits produced (T. Culley, unpublished data).

Selfing might also take place in populations of *V. canadensis* if it occurs in CH flowers that are responsible for a large amount of a population's seed production. If true, this would violate one of the assumptions for the direct comparison of CH and CL progeny in inbreeding depression studies (i.e., chasmogamy and cleistogamy are equivalent to outcrossing and selfing). In this study, CH flowers were capable of delayed self-pollination as unemasculated CH flowers set fruit automatically in the absence of
pollination. Delayed selfing occurred in older flowers as the stigma bent down and contacted pollen grains that had fallen out of the anther cone and were resting on the anterior petal (T. Culley, personal observation). Although delayed selfing has also been detected in Viola pubescens (T. Culley, unpublished data), it has not been found in V. odorata (Banasinska and Kuta 1996) or in V. riviniana (Beattie 1969), indicating that it may not be a widespread phenomenon in Viola. Beattie (1971) also suggested that pollinator-mediated selfing may occur in many Viola species as pollen deposited on the anterior petal is transferred to the stigma of the same flower by short-tongued pollinators. In addition, inbreeding may occur in CH flowers through geitonogamy and biparental inbreeding (Lloyd and Schoen 1992). Thus, CH flowers of V. canadensis may not be completely outcross-pollinated under field conditions, especially with low rates of pollinator visitation. In fact, a substantial CH selfing rate (> 0.50) has already been detected in a preliminary study of V. pubescens (T. Culley, unpublished data).

Self-fertilization in CH flowers has been found in other CH/CL species as well. Delayed or induced selfing has been detected in Glycine argyrea and G. clandestina (Schoen and Brown 1991), Triodanis perfoliata (Bryan 1993), and in Ajuga chamaepitys (Ruiz de Clavijo 1997). Other forms of CH autogamy have been found in Euryale ferox (Kadono and Schneider 1989), Ceratocapnos heterocarpa (Ruiz de Clavijo and Jimenez 1993), Polygonum thunbergii (Momose and Inoue 1993), and Medicago spp. (Novosyelova 1998). Significant selfing rates have been measured in CH flowers of Impatiens capensis (Waller and Knight 1989), Impatiens pallida (Stewart 1994), Collomia grandiflora (Wilken 1982), and Lespedeza capitata (Cole and Biesboer 1992). Because CH flowers in these species may not possess autonomous pollination or delayed
selfing (Lloyd and Schoen 1992), significant selfing rates may result from geitonogamy and/or biparental inbreeding (Schoen and Lloyd 1992). In these species, it would only be correct to assume that CH flowers are completely outcrossed if all methods of inbreeding are found to be insignificant.

Past studies of CH/CL species are difficult to compare with the present results because many only involve comparisons between CH and CL progeny (Koller and Roth 1964; McNamara and Quinn 1977; Weiss 1980; Cheplick and Quinn 1982; Wilken 1982; Clay 1983; Gara and Muenchow 1990), with some of these studies assuming that chasmogamy and cleistogamy are equivalent to outcrossing and selfing - mainly because of low selfing rates in CH flowers (Waller 1979; Waller 1984; Mitchell-Olds and Waller 1985; Antlfinger 1986; Schmitt and Ehrhardt 1987; McCall et al. 1989). However, a few investigations of CH/CL species are comparable to the present study because they utilized selfed and outcrossed CH progeny, or incorporated the outcrossing rate in some way. Schmitt and Ehrhardt (1990) found that the level of inbreeding depression in Impatiens capensis was 0.14 for vegetative biomass; this is identical to the level of inbreeding depression found in V. canadensis. However, a second harvest later revealed that the vegetative biomass of CL progeny had increased substantially as compared to CH progeny, resulting in a low level of inbreeding depression (-0.09; Schmitt and Ehrhardt 1990). Although Wilken (1982) did not specifically measure inbreeding depression in Collomia grandiflora, I calculated levels from his reported means for selfed (CL and selfed-CH) and outcrossed CH progeny (Wilken’s Table 6). For vegetative biomass, levels of inbreeding depression in C. grandiflora ranged from -0.07 to 0.08 in five populations. As with V. canadensis, there was no significant difference in CH flower
number between selfed and outcrossed progeny (in most populations; Wilken 1982).

There also were no significant differences in CL flower number of selfed and outcrossed progeny in most populations. Schemske (1978) did not detect any significant differences in fruit and seed set among selfed or outcrossed CH flowers in Impatiens biflora and I. pallida. Finally, Bryan (1993) found that following pollinations of CH flowers in Triodanis perfoliata, selfed seeds took significantly longer to germinate than outcrossed seeds, and that self-CH progeny had lower adult biomass than outcross-CH progeny. Levels of inbreeding depression calculated from the adult biomass of self- and outcross-CH progeny ranged from 0.52 to 0.12 with increasing density treatments (Bryan 1993).

With the exception of the latter investigation, these studies are largely consistent with the low level of inbreeding depression detected in V. canadensis. In this species, the role of CH flowers may be to introduce new alleles into the population through occasional outcrossing, followed by the fixation of favorable alleles following selfing in the CL flowers.

Even though the level of inbreeding depression was determined to be low in Viola canadensis, it may have been underestimated because only early life history stages were studied in this perennial species, and not all fitness comparisons were measured under competitive conditions (Schmitt and Ehrhardt 1990) because of low sample sizes. Levels may also be underestimated because the experiment was not performed under field conditions (Dudash 1990; Jarne and Charlesworth 1993; Eckert and Barrett 1994; Ramsey and Vaughton 1998). It was necessary to continue the experiments in the greenhouse because of early seeding emergence caused by temperature fluctuations due to El Niño.
One concern in measuring levels of inbreeding depression in CH/CL species is that floral type differences (CH versus CL) may confound fitness differences due to mating type (outcross versus self). This was true in the present study if chasmogamy and cleistogamy were considered equivalent to outcrossing and selfing. In this case, fitness comparisons based on CL and outcrossed-CH progeny would have slightly over-estimated the level of inbreeding depression ($\delta = 0.17$). In addition, significant effects of cross type in two life history stages (number of days to first flower and total number of CH flowers) would have been erroneously attributed to mating type differences, when in fact they were due to floral type differences. These results demonstrate the importance of separating fitness differences due to mating type and floral type in CH/CL species, as suggested by Clay and Antonovics (1985).

It is likely that under field conditions, fitness differences due to floral type may occur in *V. canadensis* because of changes in natural conditions during the time of flowering. CH and CL seeds in many *Viola* species are produced at different times during the season – CH seeds mature in early spring and CL seeds are produced during the summer and early fall. The energy available to the plant may change during this time, especially as the forest canopy forms and shades the understory plants in the early spring, thereby reducing their photosynthetic rates. Although untested, this could translate into lower resource availability for CL seed production. A comparison of field-collected CH and CL seed capsules in *V. canadensis* revealed no difference in the number of CH and CL seeds, but the latter were significantly larger (T. Culley, unpublished data).

In other CH/CL species, floral type differences may be even more prominent and thus may have a larger impact on the measurement of levels of inbreeding depression. In
**Tridanis perfoliata**, Bryan and Muenchow (1991) and Bryan (1993) found that self-CH seeds germinated significantly more slowly than CL seeds, although there was no difference in final adult biomass of the two progeny types. Floral differences may be especially important in species that possess dimorphic seeds, such as *Gymnarrhena micrantha* (Koller and Roth 1964), *Emex spinosa* (Weiss 1980), *Amphicarpum purshii* (McNamera and Quinn 1977; Cheplick and Quinn 1982), and *Danthonia spicata* (Clay 1983). In *Amphicarpa bracteata*, which produces both aerial and subterranean CL flowers, there were no floral type differences detected between self-CH and aerial CL progeny; however, significant differences were apparent when self-CH progeny were compared to progeny from subterranean CL flowers (Trapp and Hendrix 1988). In other species, such as *Impatiens biflora* and *I. pallida*, floral type differences may be less obvious but may exist nevertheless (Schemske 1978). Studies in these types of species should be especially careful to account for differences of floral type so that levels of inbreeding depression will only reflect genetic effects due to mating type differences.

**Summary**

A low level of inbreeding depression was detected in *Viola canadensis*, a species that reproduces through both open, CH flowers and closed, self-pollinated CL flowers. Evidence from other CH/CL species also suggests low levels, but additional studies are needed to quantify levels of inbreeding depression over several populations in these types of species. These comparisons should involve known selfed and outcrossed CH progeny under field conditions, if possible. In addition, future investigations of CH/CL species
should examine and control for the possibility of floral-type differences, especially in species with dimorphic seed types.
CHAPTER 5

POPULATION GENETIC STRUCTURE OF THE CLEISTOGAMOUS PLANT SPECIES, *Viola pubescens* AITON (VIOLACEAE), AS INDICATED BY ISOZYME AND ISSR MOLECULAR MARKERS

Introduction

The amount and distribution of genetic variation among populations can be influenced by the mating system of a species (Hamrick and Godt 1990; Schoen et al. 1996). Wright (1946, 1969) demonstrated that selfing and inbreeding reduce gene flow and promote population substructuring through the loss and/or fixation of certain alleles, resulting in lower overall genetic diversity. In contrast, outcrossing species may have significant gene flow via pollen, resulting in higher levels of genetic variation, with populations sharing a number of alleles. However, some species have a mixed mating system in which both outcrossing and selfing occur within an individual. This could result in a range of genetic diversity levels that reflects the frequency of outcrossing and selfing in a population. In these species, the same plant produces both open, chasmogamous (CH) flowers and closed, cleistogamous (CL) flowers. The elaborate CH flowers are often attractive to pollinators and are thought to facilitate outcrossing, while the less conspicuous CL flowers are automatically self-pollinated. These species, known as cleistogamous or CH/CL species, are widely distributed throughout the world and are present in at least 56 angiosperm families, comprising 287 species (Lord 1981).
The CH/CL system is of interest because it is a stable mixed mating system, in contrast to past theory predicting that mating systems consisting of both selfing and outcrossing should not be evolutionary stable (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). The CH/CL mating system may be an adaptation that allows exploitation of different environmental conditions. For example, the less costly, self-pollinated CL flowers (Schemske 1978; Waller 1979) may be a means of reproductive assurance when pollinators are scarce (Kerner von Marilaun 1902; Redbo-Torstensson and Berg 1995) or when environmental conditions are not optimal (e.g., low light, water, or nutrient availability; Le Corff 1993). In addition, the overall contribution of CH and CL flowers to the next generation may also be influenced by other factors, such as inbreeding depression, selection of outcrossed or selfed progeny, and differential seedling recruitment.

To date, the population genetic structure of CH/CL species has been studied in only six species (Schoen 1984; Knight and Waller 1987; Lesica et al. 1988; Cole and Biesboer 1992; Sun 1999). Overall, these studies showed that there is usually high inbreeding, little or no genetic variability within populations, and large genetic differences among populations of CH/CL species – as would be expected with a highly selfing species. In many of these species, selfed CL flowers are responsible for the majority of seed production (Sun 1999; Schoen 1984), but rates of self-pollination in CH flowers may also be higher than expected. For example, CH selfing rates greater than or equal to 0.50 have been found in Impatiens pallida (Stewart 1994), Lespedeza capitata (Cole and Biesboer 1992), and Danthonia spicata (Clay 1982). Selfing in CH flowers could occur through geitonogamy (pollination between flowers on the same plant),
pollinator-mediated selfing within the same flower, or autogamy (self-fertilization within an individual flower) (Lloyd and Schoen 1992). Sun (1999) suggested that additional studies of more CH/CL species are needed to determine if other species share this pattern of genetic structure.

We chose to study the population genetic structure of a CH/CL violet species, Viola pubescens Aiton, because little is known of its genetic variation and CH flowers appear to be more important in reproduction than in other CH/CL species. CH flowers were responsible for most of the seeds that were successfully dispersed in an Ohio population of V. pubescens in 1997 (T. Culley, unpublished data). Although individuals produced similar numbers of CH and CL flowers, CH flowers were more than twice as likely to produce viable fruits than their counterparts (22% of all CH flowers successfully set seed, compared to 9% of CL flowers). The selfing rate in CH flowers appears to be intermediate (>0.50: T. Culley, unpublished data), and CH flowers are capable of delayed selfing if left unvisited by insect pollinators (T. Culley, unpublished data). Gene flow via seed dispersal is not likely to be very significant because populations are generally isolated from one another and seeds are only dispersed a few meters by ballistic and ant dispersal mechanisms (see below).

Little is known about the population genetic structure of Viola species, many of which possess both CH and CL flowers. Viola has a worldwide distribution of approximately 500 - 600 species (Ballard 1996; Judd et al. 1999), with about 84 species located in North America (Britton 1913). The few studies that have measured genetic variation in Viola have used it as a method to delineate Nordic and Korean taxa (Kim et al. 1991; Nordal and Jonsell 1998), or to determine the origin of a Nordic species or
group of species (Marcussen and Nordal 1998; Marcussen and Borgen, in press). These studies have generally found low variation within or among Viola populations, with some exceptions (T. Marcussen, personal communication).

Most of the published studies of population structure involving CH/CL species were conducted using isozymes (Sun [1999] also analyzed RAPD data). It is possible that isozyme studies will underestimate diversity, because additional variation has been seen when other molecular markers have been employed (Esselman et al. 1999 and references therein). We were therefore interested in measuring population genetic structure using isozymes and an additional technique. We used a relatively new method with molecular markers known as intersimple sequence repeats (ISSR). The ISSR technique is similar to that for RAPDs (Wolfe and Liston 1998), except that the ISSR primer consists of a di- or trinucleotide repeat with a 5' or 3' anchoring sequence of 1-3 nucleotides. The primer sequence is usually larger than the 10'mer used for the RAPD primer. This allows for a higher primer annealing temperature, resulting in greater band reproducibility than RAPD markers (see the ISSR Resource Website at: http://www.biosci.ohio-state.edu/~awolfe/ISSR/ISSR.html). ISSRs have been used in cultivated species since 1994, but they have only recently been employed to study intrapopulation variation in vascular plants (see Wolfe and Liston 1998; Wolfe et al. 1998a,b). Only one other study has directly compared genetic diversity estimates based on isozyme and ISSR data (Esselman et al. 1999).

ISSR markers have several advantages over isozymes (Wolfe and Liston 1998). ISSR markers are usually hypervariable enough (Wolfe et al. 1998a,b) to be of use in within-population studies when isozymes show little or no variation. Fresh or large
quantities of material are not required for ISSR markers, but are necessary for isozyme analysis. In addition, material for ISSR analysis can be dried and stored at room temperature, while isozyme material must be kept at cold temperatures to prevent enzyme degradation. Limitations of the ISSR technique are that bands are scored as dominant markers, genetic diversity estimates are based on diallelic characters, and ISSRs do not yet have a solid foundation in the literature for comparative purposes.

Here we report the findings of a population genetic study of the CH/CL species, *Viola pubescens*. The purpose of this investigation was twofold. Our first objective was to determine the population genetic structure of *V. pubescens*, using six populations distributed over a geographical range. Our second objective was to compare results for isozymes with those for ISSRs, a new molecular method.

**Materials and Methods**

*Viola pubescens* Aiton (Violaceae) is a herbaceous perennial commonly found in the understory of mixed mesic forests in northeastern North America. This stemmed species produces yellow chasmogamous (CH) flowers in the early spring (mid-April to May) and small, self-pollinated cleistogamous (CL) flowers after the forest trees leaf out (May to September). The species is nonclonal, making it easy to identify genetic individuals in the field. Foliage may be either densely pubescent (var. *pubescens* Hort., previously *V. pubescens* Aiton or *V. pensylvanica* Michx.) or glabrous (var. *scabriuscula* Schwein., previously *V. eriocarpa* Schwein.), although seed capsules of both varieties may be hairy or glabrous (Lévesque and Dansereau 1966; Cain 1967; Ballard 1994). The species is diploid with $2n=12$ chromosomes (Clausen 1929; Canne 1987).
Pollinators of *V. pubescens* are infrequent and include carpenter bees, halictid bees, bee flies, and skipper butterflies (T. Culley, personal observation). A single pollinator visit may be sufficient for full CH seed set in *Viola* species (Beattie 1971), and in the absence of pollinators, CH flowers of *V. pubescens* can self-pollinate (T. Culley, unpublished data). CH capsules are typically produced on longer peduncles than CL capsules, but seeds from both capsule types are ballistically dispersed as the valves dry and slowly squeeze shut. Seeds can be dispersed up to 2.0 m from an unobstructed maternal plant (T. Culley, unpublished data). Both CH and CL seeds have elaisomes, indicating that secondary dispersal by ants may be common.

Genetic variation was analyzed in five populations of *V. pubescens* var. *scabriuscula* and one population of *V. pubescens* var. *pubescens* located within a two-state area (Figure 5.1). On a local scale, three isolated populations of var. *scabriuscula* (Etter, Hill, and Stump) were located in agricultural woodlots in Crawford County, Ohio (OH) and were chosen because of their similar size (> 21 ha). These were compared to a population of var. *scabriuscula* at Ohio Wesleyan University’s Bohannan Scientific Preserve (40.5 ha) in nearby Delaware and Morrow Counties, OH. Finally, a distant population in Emmet County, Michigan (>50 ha; on land owned by the University of Michigan’s Biological Station) served as an outgroup comparison to measure long-distance genetic relatedness. Initially, all plants collected from the Michigan (MI) site were identified as var. *scabriuscula*, but upon closer examination, approximately half of them were actually var. *pubescens*. Because the genetic relationship of the two varieties was unknown, they were analyzed separately as two different populations. For
Figure 5.1. Five sites of *Viola pubescens* sampled in the study. The Michigan site (▼) is found in Emmet County, Michigan, and the Bohannan site (★) is located in Delaware and Morrow Counties, Ohio. Etter (●), Hill (■), and Stump (▲) sites are all found within Crawford County, Ohio.
simplicity, we will refer to the var. *scabriuscula* population as the “Michigan” population, and the var. *pubescens* population found at the same site as the “var. *pubescens*” population.

Leaf and CL bud tissue was collected from 36 to 46 plants located at least two meters apart in each of the OH populations in spring, 1997, and in the MI populations in 1998. A total of 191 plants were sampled in the study. The tissue samples were stored on ice or dry ice for transport back to the laboratory, where they were then placed in eppendorf tubes, frozen with liquid nitrogen, and stored at -80°C.

A. Isozyme Analysis

Small portions of samples from all populations were ground using a Sorghum extraction buffer (Morden et al. 1987). The supernatant was absorbed onto 1 mm Whatmann 3M filter paper wicks that were then stored at -80°C until electrophoresis was carried out several weeks later.

Two buffer systems were used to resolve seven enzymes, resulting in a total of nine loci and 31 alleles. Isocitrate dehydrogenase (IDH), phosphogluconate dehydrogenase (PGD), and shikimate dehydrogenase (SKD) were resolved using a morpholine-citrate gel buffer system at pH 6.1 (Clayton and Tretiak 1972) with a 1:19 dilution for the gel buffer. Aminopeptidase (AMP), malate dehydrogenase (MDH), glucose-6-phosphate isomerase (GPI), and phosphoglucomutase (PGM) were resolved using a histidine-citrate buffer of pH 5.7 (Stuber et al. 1977) with a 1:6 dilution for the gel buffer. Gels were made of 11-12% potato starch (Starch Art, Smithville, Texas, USA). Samples from all five populations were run alongside markers of known
genotypes to ensure consistent scoring. Gels were run at a constant voltage for 5 hours at 170-180 V (morpholine-citrate buffer) or 190-210 V (histidine-citrate buffer).

Staining recipes for AMP were from Morden et al. (1987), and all others were those of Wendel and Weeden (1989) with some slight modifications: GPI was modified as an agar overlay, and IDH was best resolved as a stain bath (Tris-HCl buffer increased to 50ml). IDH, MDH, GPI, and PGD were scored as dimers and AMP, PGM, and SKD were scored as monomers. Alleles were designated as numbers representing band migration distance, with the lowest number assigned to the most anodal isozyme. Two loci of MDH and AMP were used: for each of the remaining isozymes, a single locus was resolved. Mendelian inheritance of these loci was confirmed using segregation patterns from CL seeds. Enzymes that were not included because of resolution difficulties were aconitase (ACO), adenylate kinase (ADK), hexokinase (HEX), and lactate dehydrogenase (LDH).

Measures of genetic variation within and among the populations were calculated using the GDA software package (Lewis and Zaykin 1999). For each population, the following measurements were calculated: allele frequencies per locus, number of alleles per locus (A) and per polymorphic loci (A_p), and the percentage of polymorphic loci (P; 0.95 level). We also calculated Nei’s (1978) unbiased estimate of the expected proportion of heterozygous loci per individual (H_e), and observed heterozygosity (H_o). Both Hardy-Weinberg equilibrium and linkage disequilibrium between pairs of loci were tested in all populations, using Bonferroni corrections of the rejection level (Rice 1989).

To compare the amount of total genetic variation partitioned within and among populations, we calculated both F-statistics and genetic distances using isozyme data.
F-statistics were computed using the method of Weir and Cockerham (1984), which is similar to Wright’s (1951) procedure, except that it incorporates effects of small and uneven sample numbers. Weir and Cockerham’s $f$ is analogous to $F_{IS}$, and measures the correlation of genes within individuals in populations. $F$ (similar to $F_{IT}$) measures the correlation of genes within individuals in the pooled population. Finally, $\theta$ is analogous to $F_{ST}$ and measures the amount of differentiation among populations relative to the total diversity. We also calculated Nei’s (1978) unbiased genetic distance for each pair of populations using GDA. and generated a neighbor-joining tree in NTSYSpc (Rohlf 1998) to visualize population relationships.

Although it may be considered inappropriate to use both F-statistics and genetic distance measures on the same data set due to different assumptions in their underlying models (Reynolds et al. 1983; Weir 1996, p. 197), we calculated both for two reasons. First, we estimated Nei’s (1978) genetic distance because both drift and mutation could be important in differentiation of the populations used in this study (divergence due to mutation might be important between geographically distant populations, while drift might be more significant in neighboring, but isolated populations within Crawford County). Second, we calculated F-statistics because we wanted to compare our results with other studies of CH/CL species in which these measures are usually presented.

B. ISSR Analysis

DNA was extracted with a modified mini-prep technique of Doyle and Doyle (1987), using the same tissue samples that were analyzed with isozyme electrophoresis. DNA samples were then stored at $-20^\circ$C until further analysis.
We used three simple sequence repeats (SSR) as primers to generate a total of 83 bands in single-primer reactions. The trinucleotide primer, Mao [(CTC)\textsubscript{4}R], yielded 23 bands, and two nucleotide primers, 17898 [(CA)\textsubscript{6}RY] and 844 [(CT)\textsubscript{8}RG] generated 32 and 28 bands, respectively (R represents either of the purine bases - A or G). All single-tube reactions were optimized by adjusting the amounts of MgCl\textsubscript{2}, Taq (GIBCO/BRL), and the primer annealing temperature. Mao was best optimized using 3 mM MgCl\textsubscript{2}, 0.25 U Taq, and a 45°C annealing temperature. Reactions with primer 17898 contained 3 mM MgCl\textsubscript{2} and 0.50 U Taq at 45°C, while primer 844 was best resolved with 2 mM MgCl\textsubscript{2} and 0.25 U Taq at 46°C. The remaining cocktail ingredients per reaction (25μL total in each) were the same for each of the three primers: 0.5 μL DNA, 0.2 mM dNTPs, 0.4 mM primer, and 1X Taq DNA polymerase buffer. The polymerase chain reaction (PCR) was conducted in a Stratagene RoboCycler\textsuperscript{®} 40 and the program was 1.5 min at 94°C; 35 X 45 s at 94°C, 45 s at 45°C or 46°C, 1.5 min at 72°C; 45 s at 94°C, 45 s at 45°C or 46°C, 5 min at 76°C; 6°C soak.

Following PCR, 1.5 μL bromophenol blue marker dye was added to each reaction and the samples were loaded onto a 1.2% agarose gel in 1 X TAE buffer. Additionally, 1 kb ladders (GIBCO/BRL), and negative and positive controls were loaded onto each gel. The gels were run at constant voltage (~ 144V - 150V) until the marker dye migrated 10 cm (~ 2 h). Each gel was stained with ethidium bromide and digitized under UV light using the Alpha Innotech imaging system (Alpha Innotech, San Leandro, California, USA). The images were saved in TIFF format and analyzed using the Kodak 1D software package (Eastman Kodak), which assigns a fragment size to each band using an
algorithm based on the 1 kb ladder. These fragment sizes were used to assign loci for each primer, and bands for each assigned locus were scored as diallelic (1 = band present; 0 = band absent). To ensure repeatability of the results, a replicate of each gel was also run and only bands that were common to both gels were used in the final analysis. Thirteen individuals were removed from the final analysis because of non-repeatability of bands with one or more primers. This resulted in a total of 178 individuals sampled in the ISSR study.

Our analysis of the ISSR data included the following assumptions. First, we assumed that marker alleles (bands) from different loci did not comigrate to the same position on the gel, and that bands shared by two individuals descend from a common ancestor. This is likely, as a preliminary study indicates that ISSR bands are largely homologous (S. Datwyler and A. Wolfe, unpublished data). Second, we assumed that each locus consisted of only two alleles that segregate in a Mendelian fashion.

Analysis of ISSR data is not as straightforward as for isozymes because of the dominant nature of the ISSR markers. This makes it difficult to calculate allele frequencies that are used to generate many traditional statistics (e.g., $H_e$, $F_{st}$, $\theta$, $G_{st}$). As with RAPD or AFLP data, the presence of a band can denote either a dominant homozygote (band present/present) or a heterozygote (band present/absent); it is generally not possible to distinguish between genotypes, although not entirely impossible (Lynch and Milligan 1994). To determine allele frequencies at a locus for dominant data, traditional methods assume Hardy-Weinberg equilibrium and calculate the frequency of the absent or null allele ($q$) from the observed number of individuals without a band ($q^2$). The frequency of the dominant allele ($p$) is then calculated as $(1-q)$. One problem with
this approach is that the assumption of Hardy-Weinberg equilibrium could easily be violated with dominant markers, as sometimes occurs with co-dominant isozyme markers (see below). Another problem is that for dominant data such as ISSRs, the absence of a band may not always indicate a homozygous recessive genotype, as band absence may be caused by loss of a primer annealing site (because of nucleotide sequence differences), insertions or deletions in the fragment between the two primer sites, or experimental error (which can be reduced by running replicate gels). Consequently, it may be unlikely that the absence of the same band in two individuals arose from identical ancestral mutations. The use of shared band absences may overestimate relatedness among individuals and should be used with caution in population analyses, but never should be used above the species level (Black 1995).

We addressed this problem by employing genetic statistics that used the bands themselves and did not involve assumptions of Hardy-Weinberg equilibrium to create allele frequencies (e.g., Lynch and Milligan 1994). To characterize the variation present in the ISSR data, we calculated the number of shared and unique bands, along with the percentage of polymorphic and fixed loci in the populations. To measure population differentiation, we used the Nei and Li (1979) similarity coefficient (also known as the Dice [1945] or Sørensen [1948] coefficient; see Sneath and Sokal 1973) to compare the number of bands that were shared between individuals or populations (excluding shared absences). The Nei and Li coefficient was calculated for each pair of populations using !WAVSIML (V. Ford, unpublished; available via anonymous FTP at 140.254.12.151 in incoming/ISSR; see Crawford et al. 1998 for formulae) and genetic distances were then computed for each pair of populations as (1-similarity). These distances were then used
to construct a population-level neighbor-joining tree in NTSYSpc (Rohlf 1998). To
determine how individuals from all populations clustered together, we performed
Principle Coordinates Analysis (PCoA) in NTSYSpc using Nei and Li genetic distances
generated among all pairs of individuals across the populations. This matrix was
generated in WXDNL (V. Ford, unpublished; available via FTP as described above).

Results

A. Isozyme Variation

The isozyme analysis revealed a high amount of genetic variation in populations
of Viola dubescens. In each population, five to eight of the nine loci examined were
polymorphic, and only one locus (MDH-2) was monomorphic throughout all populations
(Table 5.1). Heterozygous individuals were detected in all populations at five
polymorphic loci (IDH, MDH-1, SKD-1, PGD-1, and PGM-1). Because MDH-1 was
fixed in the heterozygous condition (or was a duplicated locus) and did not behave in a
Mendelian fashion, it was removed from all subsequent analyses. With the remaining
eight loci, the mean value of observed heterozygosity was 0.35, which was slightly larger
than the value expected under Hardy-Weinberg conditions ($H_e = 0.32$; Table 5.2).

The mean number of alleles per locus ($A$) ranged from 2.1 within the Crawford
County populations (Etter, Hill, and Stump), to 3.4 in the Bohannan populations,
respectively (Table 5.2). The Crawford County and var. pubescens populations had a
lower percentage of polymorphic loci (50% - 62%) than either the Bohannan or Michigan
populations (both 88%). Observed and expected heterozygosity were consistently high in
all populations ($>0.28$), with the greatest difference occurring in the Bohannan
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<tr>
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<th>Stump</th>
<th>Bohannan</th>
<th>Michigan</th>
<th>var. pub</th>
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<td>0.01</td>
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Table 5.1. Allele frequencies for nine isozyme loci in five populations of Viola pubescens var. scabriuscula and one population of V. pubescens var. pubescens (var. pub). N refers to the number of individuals sampled in each population for each locus.
population (Table 5.2). The Crawford County populations and the var. *pubescens* population had a slightly greater number of observed heterozygotes than expected, but none of these differences were significant (paired t-test; \( t = 0.783, \text{df} = 5, \ P = 0.469 \)).

Population structuring was evident in the isozyme analysis. The Crawford County populations (Etter, Hill, and Stump) contained only a subset of the genetic variation present in the other populations, and two alleles (IDH-1\(^0\) and GPI-1\(^1\)) were completely missing from the Crawford County group (Table 5.1). In addition, unique alleles were found in the Bohannan population (SKD-1\(^1\), AMP-1\(^1\), GPI-1\(^1\)), and in the Michigan population (PGD-1\(^1\)). The Michigan and var. *pubescens* populations also shared one unique allele (IDH-1\(^1\)). In most cases, these unique alleles were at very low frequencies and it is possible that increased sampling may reveal their existence in other populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>A</th>
<th>( A_p )</th>
<th>P</th>
<th>( H_e )</th>
<th>( H_o )</th>
</tr>
</thead>
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<td>Etter</td>
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<td>3.0</td>
<td>50.0</td>
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<td>0.39</td>
</tr>
<tr>
<td>Hill</td>
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<td>3.2</td>
<td>50.0</td>
<td>0.28</td>
<td>0.37</td>
</tr>
<tr>
<td>Stump</td>
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<td>2.4</td>
<td>3.0</td>
<td>62.5</td>
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<td>0.32</td>
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<tr>
<td>Bohannan</td>
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<td>3.7</td>
<td>87.5</td>
<td>0.41</td>
<td>0.30</td>
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<tr>
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<td>3.1</td>
<td>87.5</td>
<td>0.37</td>
<td>0.35</td>
</tr>
<tr>
<td>var. pub</td>
<td>22.2</td>
<td>2.5</td>
<td>3.0</td>
<td>62.5</td>
<td>0.29</td>
<td>0.35</td>
</tr>
<tr>
<td>Total</td>
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<td>2.6</td>
<td>3.2</td>
<td>66.7</td>
<td>0.32</td>
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</table>

Table 5.2. Genetic variability at eight isozyme loci in five populations of *Viola pubescens* var. *scabriuscula* and one population of *V. pubescens* var. *pubescens* (var. *pub*). MDH-1 was excluded because it was either a duplicated locus or exhibited fixed heterozygosity. Shown are the mean sample sizes per locus (N), mean number of alleles per locus (A) and per polymorphic loci (\( A_p \)), percentage of polymorphic loci (P), Nei's (1978) unbiased estimate of the expected proportion of heterozygous loci per individual (\( H_e \)), and observed heterozygosity (\( H_o \)). A locus was considered polymorphic if the frequency of the most common allele was less than 0.95.
Significant deviations from Hardy-Weinberg equilibrium occurred in only one locus (PGD-1) across all populations. The remaining loci were found to be in Hardy-Weinberg equilibrium in at least one population. The direction of deviations was not consistent across comparisons – it was influenced both by heterozygote excess and deficiency. In addition, significant linkage disequilibrium was not detected within most populations, except in the Bohannan and Michigan populations, in which 3% and 14% of the 28 pairwise loci comparisons were significant.

B. ISSR Variation

High genetic variation was also observed using ISSR markers. Within each population, over 70% of the 83 loci were polymorphic, with the Bohannan population exhibiting the highest number of polymorphic loci (Table 5.3). At the species level, 100% of the loci were polymorphic.

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<th>% fixed</th>
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<tr>
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<tr>
<td>var. pub</td>
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<td>21</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>77.1</strong></td>
<td><strong>1.2</strong></td>
<td><strong>178</strong></td>
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</table>

Table 5.3. Proportions of the 83 ISSR loci that were polymorphic and fixed in each population. A polymorphic locus was one in which a band was both present and absent in a population, and locus was considered fixed if a band was always present. Sample size (N) refers to the number of individuals sampled in each group.
Population structuring was evident with the ISSR data. A unique band was detected in each of the Etter and Hill populations while the Bohannan and var. pubescens populations had two and three unique bands, respectively (data available upon request). Of the 83 loci, 36 (43%) contained bands that were found in all six populations in at least some individuals. One band was fixed in the Crawford County populations and was near fixation (average frequency of 82.4%) in the remaining populations. A second band was also fixed throughout the Etter, Hill, and var. pubescens populations.

C. F-statistics

The inbreeding coefficient within populations (f) calculated from the isozyme data was not significantly different from zero (Table 5.4; bootstrapping over loci, 1000 replicates, 95% CI = 0.21 - 0.31). The negative value indicated an excess of heterozygotes, which was largely due to high heterozygote frequencies in the Crawford County and var. pubescens populations. The range of f values across loci were larger than normal (Table 5.4), suggesting that evolutionary forces other than nonrandom mating may be acting differently on individual loci. However, this range of f values is not unique among CH/CL species (Cole and Biesboer 1992).

The value of θ derived from the isozyme data was 0.29 and was significantly different from zero (bootstrapping over loci, 1000 replicates, 95% CI = 0.48 - 0.16), indicating that genetic differentiation has occurred among the sampled populations. Mating may not be completely random within populations because F was lower than θ, even though f was not different from zero (see Weir and Cockerham 1984).
<table>
<thead>
<tr>
<th>Locus</th>
<th>f</th>
<th>F</th>
<th>θ</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH-1</td>
<td>0.08</td>
<td>0.24</td>
<td>0.18</td>
</tr>
<tr>
<td>PGD-1</td>
<td>-0.25</td>
<td>-0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>SKD-1</td>
<td>-0.44</td>
<td>-0.23</td>
<td>0.15</td>
</tr>
<tr>
<td>PGM-1</td>
<td>-0.47</td>
<td>-0.23</td>
<td>0.16</td>
</tr>
<tr>
<td>MDH-2</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>AMP-1</td>
<td>0.66</td>
<td>0.84</td>
<td>0.52</td>
</tr>
<tr>
<td>AMP-2</td>
<td>0.12</td>
<td>0.68</td>
<td>0.63</td>
</tr>
<tr>
<td>GPI-1</td>
<td>0.80</td>
<td>0.92</td>
<td>0.59</td>
</tr>
<tr>
<td>Mean</td>
<td>-0.09</td>
<td>0.22</td>
<td>0.29</td>
</tr>
<tr>
<td>Upper CI</td>
<td>0.21</td>
<td>0.57</td>
<td>0.48</td>
</tr>
<tr>
<td>Lower CI</td>
<td>-0.31</td>
<td>-0.05</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 5.4. Summary of F-statistics (Weir and Cockerham 1984) at eight isozyme loci analyzed in six populations of Viola pubescens. Asterisks indicate a monomorphic locus and confidence intervals (CI) are derived from bootstrapping across loci (1000 replicates).

D. Genetic Distance

Nei's (1978) genetic distance based on isozyme allele frequencies was estimated for all 15 pairwise comparisons among the populations (Table 5.5). The mean distance for all comparisons was 0.268, ranging from 0.010 to 0.760. This range is very large, but is most similar to that reported for highly selfing species (Crawford 1983). The lowest genetic distances were found within the Crawford County populations (Etter, Hill, and Stump), while the var. pubescens population was the most distantly related to all other populations (Table 5.5).
Table 5.5. Genetic distances between pairs of populations using ISSR and isozyme data. Above the diagonal are Nei and Li distances using 83 ISSR loci, and below the diagonal are Nei's (1978) unbiased genetic distances based on eight isozyme loci (excluding MDH-1).

Genetic distances for all pairwise comparisons were also calculated from the ISSR data using the Nei and Li (1979) distance coefficient. Overall, the ISSR pairwise distances were higher than those generated with isozymes (Table 5.5). For each population, the average distance among populations derived from the ISSR data were always more than within populations (Table 5.6) – thus there are more differences among populations than within them. Again, the Crawford County populations were most closely related to one another. The var. pubescens population was most distantly related to many of the other populations, but the Michigan population also expressed large genetic distances in certain comparisons (Table 5.5). The neighbor-joining phenogram derived from isozyme data (Figure 5.2a) shared the same topology as the ISSR-based phenogram (Figure 5.2b), although there were some slight differences in branch lengths. Overall, the genetic relationships among populations of V. pubescens var. scabriuscula corresponded with their geographical distances (Figure 5.2).
<table>
<thead>
<tr>
<th>Population</th>
<th>Within Populations</th>
<th>Mean Distance</th>
<th>N_i</th>
<th>Among Populations</th>
<th>Mean Distance</th>
<th>N_p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etter</td>
<td>0.317</td>
<td>37</td>
<td></td>
<td>0.499</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Hill</td>
<td>0.323</td>
<td>35</td>
<td></td>
<td>0.493</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Stump</td>
<td>0.415</td>
<td>34</td>
<td></td>
<td>0.511</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Bohannan</td>
<td>0.567</td>
<td>33</td>
<td></td>
<td>0.578</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Michigan</td>
<td>0.554</td>
<td>18</td>
<td></td>
<td>0.616</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>var. pub</td>
<td>0.524</td>
<td>21</td>
<td></td>
<td>0.640</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.450</td>
<td></td>
<td></td>
<td>0.556</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.6. The average genetic distances within and among populations, as calculated from the ISSR data using Nei and Li’s (1979) coefficient. The among population measure is the mean distance calculated from pairwise comparisons of the population of interest to all other populations (see Table 5.5). N_i refers to the number of individuals sampled within each population and N_p is the number of pairwise comparisons used to calculate mean distances among populations.

We also examined how individuals were related to one another within the total population, using Principal Coordinates Analysis (PCoA) (Figure 5.3). Samples from the Crawford County populations mostly grouped together, and samples from the Michigan and var. pubescens populations (located at the same site) sorted into a cluster. Most samples from the Bohannan population clustered largely with the Crawford County group, but several samples overlapped with the MI populations. As expected, var. pubescens samples clustered furthest from the other populations, all of which consisted of a different species variety. This plot indicates that the differentiation between the two varieties in MI is about as extreme as the differentiation among all Ohio populations.
Figure 5.2. Neighbor-joining phenograms of six populations of *Viola pubescens* based on (a) isozyme data and Nei's (1978) unbiased genetic distance; (b) ISSR data and the Nei and Li (1979) distance coefficient. All populations consisted of var. *scabriuscula* except for one population of var. *pubescens*. Numbers above each branch represent the length of the branch.
Figure 5.3. Principal Coordinates Analysis (PCoA) using the Nei and Li (1979) distance coefficient computed from ISSR data from all 178 individuals. All populations consisted of var. scabriuscula except for one population of var. pubescens.
Discussion

A. Population Genetic Structure

Our study provides evidence that the genetic diversity of Viola pubescens is very different from other CH/CL species studied so far, and is typical of an outcrossing species (Hamrick and Godt 1990). Viola pubescens displayed a surprisingly large amount of genetic variation, with isozyme-based measures well outside of the range of values reported for other CH/CL species (Table 5.7). This large amount of genetic diversity was also found using ISSR markers. In general, V. pubescens also possesses more genetic variation than most Viola species studied thus far. There was no isozyme variation within the Asian V. albida complex (Kim et al. 1991), and in V. collina (Marcussen and Borgen, in press). Most populations of V. suavis and associated species were generally monomorphic (Marcussen and Nordal 1998), and low levels of variation were found in Viola rupestris (Nordall and Jonsell 1998) and in nine Viola taxa (Marcussen and Borgen, in press). However, these low measures of genetic variability in the latter study may reflect past glaciation events because variability declined in a northward direction from Mediterranean populations to those in Norway (T. Marcussen, personal communication). In a large Mediterranean population, three out of five isozyme systems were polymorphic (T. Marcussen, personal communication), which is comparable to the variation found in some populations of V. pubescens.

The distribution of the total genetic variation among populations in V. pubescens was lower than other CH/CL species (Fst or θ; Table 5.7). One reason the population genetic structure of V. pubescens is so different may be that CH flowers are responsible for more of the overall reproduction in V. pubescens than in other CH/CL species.
<table>
<thead>
<tr>
<th>Species (Family)</th>
<th># pop</th>
<th># loci</th>
<th>A</th>
<th>A_P</th>
<th>P</th>
<th>H_E</th>
<th>H_O</th>
<th>F_S (f)</th>
<th>F_T (F)</th>
<th>F_ST (0)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Howellia aquatilis</em></td>
<td>4</td>
<td>18</td>
<td>1.00</td>
<td>------</td>
<td>0.0</td>
<td>0.00</td>
<td>0.00</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>Lesica et al. 1988</td>
</tr>
<tr>
<td>(Campanulaceae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Impatiens capensis</em></td>
<td>11</td>
<td>31</td>
<td>------</td>
<td>2.02</td>
<td>10.0*</td>
<td>------</td>
<td>------</td>
<td>0.57*</td>
<td>0.77*</td>
<td>0.46*</td>
<td>Knight and Waller 1987</td>
</tr>
<tr>
<td>(Balsaminaceae)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Lespedeza capitata</em></td>
<td>12</td>
<td>34</td>
<td>1.08</td>
<td>------</td>
<td>7.8</td>
<td>0.02</td>
<td>0.01</td>
<td>0.56</td>
<td>0.78</td>
<td>0.51</td>
<td>Cole and Biesboer 1992</td>
</tr>
<tr>
<td>(Fabaceae)</td>
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</tr>
<tr>
<td><em>Lespedeza leptostachya</em></td>
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<td>32</td>
<td>1.00</td>
<td>------</td>
<td>0.0</td>
<td>0.00</td>
<td>0.00</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>Cole and Biesboer 1992</td>
</tr>
<tr>
<td>(Fabaceae)</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td><em>Microlaena polynoda</em></td>
<td>1</td>
<td>9</td>
<td>1.00</td>
<td>------</td>
<td>0.0</td>
<td>0.00</td>
<td>0.00</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>Schoen 1984</td>
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<tr>
<td>(Gramineae)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Scutellaria indica</em></td>
<td>20</td>
<td>30</td>
<td>1.02</td>
<td>2.03</td>
<td>2.4</td>
<td>0.01</td>
<td>0.001</td>
<td>0.74*</td>
<td>0.98*</td>
<td>0.92*</td>
<td>Sun 1999</td>
</tr>
<tr>
<td>(Labiatae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Viola pubescens</em></td>
<td>6</td>
<td>8</td>
<td>2.63</td>
<td>3.18</td>
<td>66.7</td>
<td>0.32</td>
<td>0.35</td>
<td>-0.09</td>
<td>0.22</td>
<td>0.29</td>
<td>Present study</td>
</tr>
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<td>(Violaceae)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* measured for polymorphic loci only

Table 5.7. Genetic variability measures derived from isozymes for species with both chasmogamous and cleistogamous flowers. In most cases, the percentage of polymorphic loci (P) was not specified as 95% or 99%. See text for details of notation.
Outcrossing through CH flowers would introduce more genetic variation into populations and prevent population differentiation. Outcrossing is also suggested by the $F_{is}$ in the populations. However, some selfing does occur in CH flowers, because they have an intermediate selfing rate, and can self-pollinate in the absence of pollinators via a delayed-selfing mechanism (T. Culley, unpublished data).

In other CH/CL species, it appears that either CL flowers are primarily responsible for reproduction, or that selfing rates in CH flowers are very high. Most reproduction is thought to occur through selfed CL flowers in *Scutellaria indica* (Sun 1999) and *Microlaena polynoda* (Schoen 1984). In *Howellia aquatilis*, CH flowers appear to be self-pollinated prior to flower opening, although the possibility of an occasional outcrossing event cannot be discounted (Lesica et al. 1988). CH flowers in some populations of *Impatiens capensis* may be self-pollinated via geitonogamy or undergo biparental inbreeding (Waller and Knight 1989). The selfing rate in CH flowers of *Lespedeza capitata* is also very high – at least 96.6% (Cole and Biesboer 1992). In some species, selfing rates are thought to be so high that there is no genetic variation in the populations (Schoen 1984; Lesica et al. 1988; Cole and Biesboer 1992).

Several other reasons could explain the difference in population structure between *V. pubescens* and other CH/CL species. Gene flow via seeds may occur more frequently in *V. pubescens* than in other CH/CL species. Although seed dispersal can be up to two meters in *V. pubescens* because of ballistic dispersal and the potential for secondary ant dispersal, it is probably not enough to transverse the wide distances of agricultural monocultures separating populations in Crawford County. The distribution of a species could also affect population structure because common, widespread species typically
have higher genetic variation than rare species (Barrett and Kohn 1991). However, this
do not appear to apply in this case because both Impatiens capensis and V. pubescens
are widespread CH/CL taxa that frequently co-occur and still differ in their population
遗传结构。In addition, an increased mutation rate in V. pubescens (compared to
other species) could introduce new variation into the populations. This seems unlikely
because the mutation rate would have to be quite substantial to explain the magnitude of
the differences in population structure, and the same new mutations would have to arise
concurrently in all populations (unless gene flow via CH flowers is very strong).

Although the genetic structure of V. pubescens may be strongly affected by the
mixed mating system, genetic drift may play a minor role. With the exception of
Bohannan, most sampled populations consisted of less than 100 individuals (T. Culley,
personal observation). In addition, many Viola populations in Ohio may have gone
through genetic bottlenecks as their habitat decreased following conversion of Ohio
forests to agricultural fields during the nineteenth and early twentieth centuries. Today,
many populations in Ohio (e.g., Etter, Hill, and Stump) can only be found in small,
isolated woodlots subjected to many disturbances (e.g., invasion of exotic species,
selective logging, etc.). Drift, combined with selfing in CL flowers, could have caused
the loss or fixation of unique alleles in some of these populations.

One unexpected result of this study was the genetic differences detected between
the two varieties of V. pubescens at the MI site. In most cases, the two varieties were
nearly fixed for different alleles at several isozyme loci. It was almost possible to assign
individuals to the correct variety solely based on isozyme genotypes. This genetic
difference between varieties was also seen in other analyses. The neighbor-joining
phenograms (Figure 5.2) constructed from isozyme and ISSR data show that the var. pubescens population clusters away from the Michigan population (and all other var. scabriuscula populations). The same was seen in the principal coordinates analysis (Figure 5.3), although there was slight overlap of the two varieties from the MI site. To verify these observations, we recollected samples from the same populations in spring 1999 and repeated the isozyme analysis, obtaining the same results. These two varieties are morphologically similar, but do differ in their degree of pubescence, number of stems and basal leaves, and teeth number on leaf margins (Lévesque and Dansereau 1966; Cain 1967; Ballard 1994). These were initially considered separate species, but were grouped together because of several morphologically intermediate herbarium specimens (Ballard 1994). It would be interesting to see if genetic differences are found in additional populations, and if the two varieties are cross compatible. This may indicate whether a revision of the two varieties is warranted.

B. Comparison of Isozymes and ISSRs

Overall, the isozyme and ISSR techniques gave similar results in this study. Both detected a high amount of genetic variation (e.g., $P_{\text{isozyme}} = 66.7$ and $P_{\text{ISSR}} = 77.1$). Even though genetic distances were computed differently for isozyme and ISSR data, the relationships among pairwise genetic distances were similar for both techniques, except that the isozyme method detected larger distances on average. For both methods, the lowest pairwise differences were always found among the Crawford County populations. In addition, the topology of neighbor-joining phenograms constructed from both data sets was identical, although the branch lengths differed slightly.
Presently, only one other study (Esselman et al. 1999) has compared isozymes with ISSR markers. They found that in the clonal plant species, *Calamagrostis porteri*, very little variation was detected with isozymes, whereas over 10% of ISSR bands were variable. In fact, many studies of natural populations that have compared isozymes with other DNA techniques, such as RAPDs, have generally found that isozymes express lower amounts of genetic variation (Wang et al. 1996; Huang et al. 1998; Sun 1999; Sun et al. 1999).

The similarity of overall conclusions based on isozymes and ISSR markers in this investigation shows the potential of ISSR markers for population genetic studies. Although dominant markers do not provide the same accuracy of estimation as with codominant markers (Lynch and Milligan 1994), we believe that ISSR markers provide a suitable alternative to isozymes, especially in cases involving rare or clonal species that typically express low levels of isozyme variation. Compared with widely used RAPD markers, ISSRs are advantageous because they have higher band repeatability. As this is only the second population genetic study to compare isozyme and ISSR markers, additional investigations are needed in other species to create a benchmark with which to compare future results.

One caveat for future population genetics studies involving ISSRs is that the method of genetic analysis must be selected carefully because of the dominant nature of the markers. We suggest that traditional methods not be used because these estimate allele frequencies based on Hardy-Weinberg equilibrium and band absences. There are several alternatives, but each has different assumptions and limitations. For example, Lynch and Milligan (1994) incorporated the sampling variance of the frequency of null
homozygotes into calculations of allele frequencies, providing an estimate of Wright’s (1951) \( F_{st} \), but this method assumes Hardy-Weinberg equilibrium and random mating. An analysis of molecular variance (AMOVA) can also be used to partition observed variation into within and among population (\( \phi_{st} \), analogous to \( \theta \)) components. However, data can presently only be treated as haplotypes (data made up of completely linked sites with no recombination) rather than as RAPD, ISSR, or AFLP data (made up of independent sites with no linkage disequilibrium). The resulting \( \phi_{st} \) is not yet directly comparable with \( \theta \) generated from codominant markers (see Stewart and Excoffier 1996), but software is currently under development to adequately handle dominant data. The Shannon-Weaver diversity statistic has also been used to describe population differentiation (see Whitkus et al. 1998) by using band phenotypes and thus avoiding Hardy-Weinberg assumptions, but this method uses shared band absences. Both the Jaccard (1908) coefficient and the method we used, the Nei and Li coefficient, also use band phenotypes without Hardy-Weinberg assumptions, but these exclude use of shared band absences. Whichever method is chosen, we suggest that results must be carefully interpreted in light of the assumptions and limitations of each technique.

Conclusions

*Viola pubescens* has high genetic variation within populations and moderate levels of differentiation among populations, for both isozyme and ISSR markers. This genetic structure is very different from other CH/CL species studied thus far, probably because outcross-pollinated CH flowers play a more important role in *V. pubescens* than in other species. Additional studies measuring genetic variation in other species would
be especially helpful to determine if the high levels of genetic variation detected in *V. pubescens* are unique among other CH/CL species.
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