THE EFFECTS OF FLORAL AND PLOIDY VARIATION ON REPRODUCTIVE ISOLATION IN *COLLINSIA PARVIFLORA* AND *C. GRANDIFLORA* (VERONICACEAE).

by

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ABSTRACT

Understanding how new species are formed through the development of reproductive barriers is a major goal of evolutionary research. To determine how floral and genomic variation contribute to reproductive isolation between populations of *Collinsia parviflora* and *C. grandiflora* from British Columbia, Washington, Oregon and California, I surveyed flower size and genomic content in populations throughout that area. I found continuous variation in flower size and four distinct ploidy levels among the populations surveyed. Reciprocal crossing experiments between populations representing three flower size classes (small, intermediate and large) and four ploidy levels were performed. Total reproductive isolation was calculated based on fitness differences in seed production, F1 germination, F1 survival to flowering and F1 fertility and revealed that isolation among populations is due primarily to a combination of ploidy and flower size differences. The results of this study indicate that a reclassification of this group of Collinsia is in order.

Keywords: *Collinsia parviflora*; *Collinsia grandiflora*; Floral Variation; Polyploidy; Reproductive Isolation; Speciation.

DEDICATION

I dedicate this thesis to my parents who have made untold sacrifices so that I could succeed. Thank-you, Mom and Dad.

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1: CHAPTER ONE: GENERAL INTRODUCTION

The study of speciation has been a topic of growing interest over the past century and many ideas regarding what actually defines a species have arisen. Early species concepts include the Biological Species Concept, which defines a species as a group of organisms that are able to interbreed with each other but not with organisms outside of their group (Mayr 1942), and the Morphological Species Concept, by which species are classified based on differences among groups in morphological characters (Coyne and Orr 2004). Although morphological traits are of great utility for the expedient identification of individuals in the field, because there are many cryptic species in nature morphology can be misleading. Convergent evolution can result in superficially similar organisms that are neither closely related to nor able to interbreed with each other. In recent decades, numerous additional species concepts have been proposed including those based on between-group differences in genetic content, ecological range limits or evolutionary history (Coyne and Orr 2004). Although there has been much debate among evolutionary biologists regarding which species concept is the most appropriate, and even whether species are real entities at all, one thing that does seem to be agreed upon is the fact that none of the existing concepts can be unilaterally applied (Coyne and Orr 2004).

The difficulty in defining species arises from the fact that there are groups from throughout the tree of life that are difficult to categorize using existing

species concepts. Plants can display extensive within- and among-species variation in floral, leaf or overall structure that can lead to misclassification of multiple species where only one, or few, actually exist. Polyploidy, an increase in the number of copies of an individual's genome above that of the diploid state, can result in instantaneous reproductive isolation (Stebbins 1950) and is thought to be particularly common in plant lineages (Otto and Whitton 2000). Changes in ploidy are known to result in reproductive barriers between polyploids and their diploid progenitors, but taxonomists tend not to classify autopolyploids (those that arise from a single parental species) as separate species (Husband and Sabara 2003). Although this may be due to the fact that polyploid phenotypes often fall within the normal range of phenotypic variation exhibited by the parental species (Otto &Whitton 2000), allopolyploids (polyploids that arise following hybridization) are more often classified as separate species despite the dearth of research on reproductive isolation in that group (Husband and Sabara 2003).

Hybridization, another process that complicates the identification of species, is also very common in plants. Ellstrand et al. (2006) estimated hybridization rates in five floras from varied geographic regions in Europe, North America and the tropics and found that, across all five floras, natural hybrids occurred at an average frequency of 11%. If hybrids are able to successfully interbreed with each other and evolve reproductive barriers to prevent interbreeding with the parental species (polyploidy, for example), then they can form new species (Grant 1981, Rieseberg 1997). Hybridization is a clear

violation of the Biological Species Concept, yet it commonly occurs among groups that are still considered independent species.

Due to the myriad problems which arise in trying to unilaterally apply any single species concept, biologists tend favour the one most relevant to their field of research. In the field of speciation, the Biological Species Concept has been adopted by many as their working species definition and, thus, research has focused on understanding how groups become reproductively isolated from each other.

In studies of speciation in flowering plants, floral variation is of particular interest to evolutionary biologists because flowers contain the reproductive organs of the plant and, therefore, their morphology is of primary importance in the movement of male gametes (pollen) between individuals. The size, structure, colour, and fragrance of flowers can all be important traits for promoting the movement and capture of pollen via wind, water or animal carriers. Groups of pollinators such as bees, hummingbirds and butterflies are known to prefer specific floral traits and, due to their role in facilitating reproduction, they can act as strong agents of selection for those preferred traits (Kiester et al. 1984). Differences in floral morphology among groups can, in turn, result in differential pollinator visitation and therefore reproductive isolation (Hodges and Arnold 1994, Schemske and Bradshaw 1999, Ramsey et al. 2003).

In addition to the selection pressures imposed by biotic agents, the abiotic conditions of the environment can also have a strong effect on floral form. Edaphic and atmospheric conditions such as salinity, chemical composition and

moisture availability can all act as selective agents on floral morphology and development (Galen et al. 1999, Elle 2004, Lambrecht and Dawson 2007). If differential adaptation in floral traits occurs among populations within a species, then reproductive barriers can arise that may, ultimately, result in speciation.

The primary focus of recent research in the field of speciation has been on determining what specific barriers are involved in the evolution of reproductive isolation and how important each barrier is in maintaining that isolation. Extensive research in this area has been conducted in plants, including the genera *Mimulus* (Ramsey et al. 2003, Martin and Willis 2007, Lowry et al. 2008), *Costus* (Kay 2006), *Penstemon* (Chari and Wilson 2001), *Chamaecrista* (Costa et al. 2007), and *Chamerion* (Husband and Sabara 2003). This work has examined the effect of various pre- and post-zygotic barriers on total reproductive isolation between groups.

Collinsia parviflora and *C. grandiflora* are small and large-flowered sister species (Armbruster et al. 2002) in the family Veronicaceae which occur from British Columbia (BC), Canada in the north to California (CA), United States in the south (Douglas et al. 2000). The range of *C. grandiflora* extends east from the Pacific Ocean to the Cascade and Sierra Nevada mountains while *C. parviflora* extends past the mountains to States as far east as Pennsylvania (Douglas et al. 2000). Although the different floral forms in the United States have been described as two distinct diploid species in the literature (Garber 1956, 1958), recent research indicates that populations in BC are tetraploids with

continuously variable flower size (Ganders and Krause 1986). The discrepancies in the literature have left the taxonomic status of the BC populations ambiguous.

To determine what constitutes a species among these populations I conducted an extensive survey of Western populations from BC to CA, and sampled a single population from Michigan in the eastern part of the *C. parviflora* range. In Chapter 2, I quantify the floral and ploidy variation among these populations and propose a reclassification of the group. In Chapter 3, I report the results of a reciprocal crossing experiment among populations of varying flower size and ploidy from throughout the study area to determine the validity of my suggested reclassification scheme. The purpose of this research was to determine how flower size, ploidy and geographic location affect species boundaries within this group of *Collinsia*.

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2: CHAPTER TWO: VARIATION IN FLORAL MORPHOLOGY AND PLOIDY LEVEL AMONG POPULATIONS OF *COLLINSIA PARVIFLORA* AND *C. GRANDIFLORA*.

2.1 Introduction

Floral traits, such as size, shape and colour, are directly linked to plant reproduction and, therefore, among population variation in these traits can be particularly important in driving the evolution of reproductive isolation. Factors that impose selection on heritable traits associated with greater relative fitness will cause a shift in the population mean favoring such traits. As long as a specific trait serves to increase plant fitness it will persist in the population. Pollinators have long been recognized as important agents of selection for flowering plants because they facilitate reproduction by moving gametes between individuals. Due to the important role of pollinators in plant reproduction, strong selective pressure can be exerted for floral adaptations that increase the attractiveness of the flower to its most common visitors and the efficiency by which pollen is moved between conspecific individuals (Stebbins 1974).

There are many examples of the important role of pollinators in the evolution of floral variation. Nilsson (1988) showed the importance of the fit between plant and pollinator morphology by experimentally reducing the nectar spur length of orchids from the genus *Plantanthera.* Manipulated orchids experienced a reduction in successful pollination by natural pollinators which, in

turn, resulted in a significant reduction in reproductive fitness compared to controls. Another experiment by (Schemske and Bradshaw 1999) demonstrated that pollinator preferences for specific floral traits can affect visitation rates. Observations of pollinator behavior in *Mimulus cardinalis* and *M. lewisii* showed that hummingbird versus bee visits were strongly dependent on variation in flower colour (Schemske and Bradshaw 1999). Pollinator preference was later shown by Ramsey et al. (2003) to be the most important barrier contributing to reproductive isolation between these two species. Evidence of the ability of pollinator preferences to affect floral trait evolution has also been demonstrated in a study of *Polemonium viscosum* by Galen (1996). *P. viscosum* populations that occur at low altitudes are serviced by generalist pollinators and have mean corolla widths significantly smaller than that of higher altitude populations, which are pollinated mainly by large bodied bumblebees (Galen 1996). When plants from low altitude populations were isolated from their natural pollinators and exposed exclusively to bumblebees, mean corolla width increased by 9% after only one generation of selection (Galen 1996).

Abiotic features of the plant habitat, such as edaphic and climatic conditions, can also impose strong selection resulting in intra-specific variation in floral traits (Galen et al. 1999, Elle 2004, Lambrecht and Dawson 2007). Moisture poor soils can drive selection for reduced flower size to avoid excessive moisture loss due to transpiration (Elle 2004, Lambrecht and Dawson 2007), growth in serpentine soils can result in changes in phenology, which can affect reproductive isolation (Wright et al. 2006) and variation among populations in

altitude or latitude can result in changes in flower size or phenology (Jonas and Geber 1999).

Floral variation may also be affected by variation in chromosome complement between species. The transition to polyploidy is often accompanied by increased cell size which is predicted to result in increased size of either individual flowers or whole plants (*gigas* effects); however, the effects on organ and plant size are not consistent across taxa (Stebbins 1950, Grant 1981, Otto and Whitton 2000). In many cases polyploid phenotypes are not outside the normal range of the parent species (Otto and Whitton 2000). Self-fertilization is predicted to promote the establishment of new polyploid lineages because it allows polyploids to avoid cross pollination with their diploid progenitors which would result in the production of less fertile triploid offspring (Grant 1981). Contrary to the prediction that polyploids should have larger flowers, selection for reduced flower size may accompany the selection for increased selfing (Vamosi et al. 2007).

Collinsia parviflora and *C. grandiflora* are sister species (Armbruster et al. 2002) which are differentiated primarily by flower size (Douglas et al. 2000). Although they have been classically described as distinct small and large-flowered species with diploid chromosome numbers ($2n=2x=14$; Garber 1956, 1958), recent research has indicated that, in British Columbia (BC), Canada, flower size is continuously variable among populations (Ganders and Krause 1986, Elle and Carney 2003) and that all populations, regardless of flower size are tetraploid ($2n=4x=28$; Ganders and Krause 1986). Currently, research

presenting quantitative differences in flower size is limited to populations in BC (Ganders and Krause 1986, Elle and Carney 2003) and chromosome counts have only been done for a few populations in BC (Ganders and Krause 1986) and the US (Garber 1956, 1958).

Given the continuous variation in flower size among populations in BC and the differences in ploidy between BC populations and those previously examined in CA, it is unclear how BC populations fit into the current taxonomy. Because flower size is the primary trait used to differentiate *C. parviflora* and *C. grandiflora* in taxonomic keys (Hitchcock and Cronquist 1973, Douglas et al. 2000), continuous variation in size makes species identification somewhat dubious. Ganders and Krause (1986) suggested that all BC populations be included in *C. parviflora* with the subspecies *grandiflora* and *parviflora* used to distinguish between small and large-flowered populations, however, their reason for choosing *C. parviflora* as the species name is unclear, and the subspecies descriptions are also hampered by reliance on the continuously varying flower size of the group. Due to these ambiguities in the current taxonomy, I will refer to the populations included in this study as "BC *Collinsia*", "US small-flowered", or "US large-flowered" rather than attempting to assign them a species identity. Note, however, that small vs. large flower size is both a relative and somewhat arbitrary way to define populations for ease of description in the current research.

The purpose of this study was to expand the dataset describing flower size and ploidy variation to include populations from throughout Washington (WA), Oregon (OR), and California (CA). Through documentation of geographic

variation in floral morphology and ploidy I endeavored to clarify the species boundaries among these morphologically variable populations of *Collinsia*.

2.2 Methods

2.2.1 Study Species

Collinsia parviflora and *C. grandiflora* (Veronicaceae) are described as small and large-flowered winter annuals, which occur throughout the Pacific Coast from northern BC to southern CA (Figure 2-1; Table 2-1; Douglas et al. 2000). Large-flowered populations are restricted to Vancouver Island in BC and west of the Cascade and Sierra Nevada mountain ranges in WA, OR and CA, however, the range of the small-flowered populations extends as far east as Pennsylvania (Douglas et al. 2000). Flower morphology is highly variable among (but not within) the populations in BC (Ganders and Krause 1986, Elle and Carney 2003), such that there are "intermediate" flower sizes, but there has been no quantification of floral variation among populations in the US.

To locate potential study populations from BC to CA, I examined the reference collections at the University of British Columbia Herbarium, the Marion Ownbey Herbarium at Washington State University, the Oregon State University Herbarium and the Jepson Herbarium at the University of California, Berkeley. Collection locations identified from herbarium specimens were visited as were areas with appropriate habitat during the likely bloom time of the species. Flower morphology was measured at all locations where plants were located (below). Populations were re-visited later and seeds were collected haphazardly from 40

plants per population spaced at least 1m apart. The seeds were stored in paper envelopes for 2-4 years before being grown for ploidy analysis.

2.2.2 Chromosome Counts

I planted seeds for ploidy analysis, as needed, from 2005 through 2008 from the collections described above. Seeds were germinated in 48 cell flats in a common environment at 8 hr 20ºC light/16hr 10ºC dark and then switched to 16 hours daylight after eight weeks to stimulate flowering. I determined experimentally that flower buds fixed approximately 3-4 hours after the environmental chamber switched to daylight were more likely to be in late metaphase or anaphase. As chromosomes were easiest to count at these stages, I made all collections around this time of day.

I collected young buds approximately 1-2mm in length and added 175μL of Carnoy's II solution (Singh 2003). Buds were removed from Carnoy's solution after 3 hours and placed in 70% ethanol for at least 1 hour at room temperature. This step was repeated twice to ensure the removal of all fixative. Samples were stored in 70% ethanol at 4ºC until they could be processed. I stained the buds in 2% Aceto-Orcein (Singh 2003) in the dark at room temperature for a minimum of 4 hours before preparing slides. Some samples were in the stain longer (up to a maximum of 24 hours) as samples stayed in the stain until they could be further processed and visualized, which depended on the time needed to process other samples prepared that day. I dissected individual buds to isolate pollen mother cells (PMC), added 1% Aceto-Orcein and allowed the sample to sit under a cover slip for 5 minutes before squashing and setting the stain over an alcohol flame.

Because finding cells with clearly separated chromosomes was extremely difficult, I counted chromosomes from cells at several stages including metaphase, anaphase and telophase. In many cases, clearly separated chromosomes could only be obtained for one plant per population (Table 2-1).

2.2.3 Estimation of DNA Content Using Flow Cytometry

Flow cytometry is a fast and effective means to estimate the relative 2C-DNA content of tissue samples from organisms of unknown ploidy. The flow cytometer operates by focussing a laser beam into a high-speed stream of fluorochrome labelled DNA particles in solution. Detectors in the flow cytometer measure the amount of fluorescence emitted from the labelled DNA and this value is used to estimate the genomic content of the unknown sample relative to that of a sample of known genomic content (the internal standard). Ploidy of the unknown sample can be inferred if the 2C-DNA content matches that of a sample of known ploidy, e.g. where chromosomes have been counted (Section 2.2.2).

In 2008, I planted seeds from collections made throughout the Pacific Coast in 2004 and 2005 and from a single population from Michigan collected in 2006. I planted the seeds in 48 cell flats and germinated at 8 hr 20ºC light/16hr 10ºC dark. Daylight was increased to 16 hours after 8 weeks if plants had not yet been analyzed. Methods used in preparing material for analysis were adapted from Suda and Travnicek (2006) by Chris Sears (personal communication 2008). Where possible, leaf material was collected from young plants either prior to flowering or in the early stages of flowering. One to two small leaves were collected from the plants immediately prior to use. The leaves were chopped to a

fine consistency in 800 μl ice cold Otto I buffer (Otto 1990, Dolezel and Gohde 1995) and 1 μl ml^{-1} ß-mercaptoethanol using a fresh stainless steel razor blade. An additional 800 μl of Otto I buffer was added and samples were left in solution for at least 4 minutes before filtering through a 50-μm Partec column. After centrifuging at 500 g's for 4 minutes the supernatant was removed and the pellet re-suspended in 200μl Otto I buffer. Samples were placed on ice for a minimum of 30 minutes prior to analysis.

The stain solution was prepared by adding 50μl propidium iodide (1mg ml^{-1}) and 100μl RNase (1mg ml^{-1}) to 850μl Otto II buffer (Otto 1990, Dolezel and Gohde 1995) and then added to samples at a rate of 4 parts solution to 1 part nuclei suspension. Samples were stained for 2 minutes prior to analysis in the flow cytometer (Becton Dickinson FacScan bench top analyzer equipped with a 488 nm laser). The internal standard (*Zea mays* Ce-777) was run individually at the beginning of each session and the BD CellQuest program (BD, 1 Becton Drive, Franklin Lakes, New Jersey, USA 07417) was used to determine fluorescence of the internal standard and *Collinsia* samples. Files created in BD CellQuest were processed in FlowJo 8.7 (Tree Star Inc., 101-340 A Street, Ashland, Oregon, USA 97520). Unknown 2C-DNA values were determined for all *Collinsia* samples by comparison to the internal standard *Zea mays*, which has a known 2C-DNA value of 5.43pg (Dolezel et al. 2007).

2.2.4 Morphological Measurements

Population sampling occurred in 2004 in the US and in 2005 in the BC part of the range. Although it is possible that some phenotypic variation was due

to plastic responses to year-to-year variation in climate, flower size has been shown to have a strong genetic basis in many of the populations included in this survey (Elle 2004, Chapter 3: Figure 3-3). Thus, the environment is unlikely to have significantly altered the *relative* among-population variation that was the focus of this study. Two transects were placed haphazardly in areas where plants were present; the patchy distribution of plants precluded random placement of transects. One flower was measured from each of 20 individuals per transect at 0.5m intervals. For each flower, I measured: width of a single banner petal, total corolla width across both banner petals at the widest point, the length of the floral tube from the join of the upper and lower petals to the saccate bend in the corolla tube, and total corolla length from the tip of the keel to the base of the ovary (Figure 2-2). Measured flowers were collected and preserved in 95% ethanol for later measurement of the declined angle of the corolla tube to the calyx. On the same plants, I also collected buds for counts of pollen and ovules (below). To measure declined angle the flower was removed from ethanol, flattened laterally under clear plastic, and photographed from above. I then measured the inside angle of the corolla tube to the calyx on each photograph using the angle tool in Adobe Photoshop 6.0 (Adobe Systems Inc., 601 Townsend St., San Francisco, California, USA 94103).

2.2.5 Reproductive Allocation

To determine pollen to ovule ratios for US and small and large-flowered plants, I dissected buds collected from 6 large-flowered (FRg, HUMg, TC, TRg, CP, CAM) and 6 small-flowered (AW, HUMp, TRp, MV, EL, RL) populations

included in the morphological survey (described above) and stored in 95% ethanol. I counted the number of ovules in one bud from each measured plant. To determine pollen number I used methods outlined in Parachnowitsch and Elle (2004). Anthers were removed from 95% ethanol; air dried and added to a vial containing 25ul of 3:1 lactic acid and glycerin solution. The anthers were ground with a pestle to release the pollen grains, and vortexed 3 minutes to suspend the pollen in solution. I added 5ul of solution to each of two hemocytometer grids and counted the number of pollen grains in three randomly selected 1 X 1mm squares in each grid. As the total solution added to each hemocytometer grid was 10^{-4}ml, each count was multiplied by 250 to estimate the total number of pollen grains per plant in 25μl solution. I then selected the 3 pollen grains closest to three predetermined points on the two grids of the hemocytometer and measured mean pollen diameter. Pollen:ovule ratios and pollen grain sizes for BC populations were not included in this study as they have already been reported for small, intermediate and large flower sizes by Parachnowitsch and Elle (2004).

2.2.6 Statistical Analysis

To determine if floral traits were correlated, I performed a Pearson's correlation analysis using the CORR procedure in SAS 9.1.3 (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina, USA 27513). To assess whether populations differed for the five floral traits measured, I performed a multivariate analysis of variance (MANOVA) with population and transect nested within population as main effects, followed by univariate ANOVAs and Ryan-Einot-

Gabriel-Welsch Multiple Range tests in SAS. I square root transformed tube length, corolla length, and corolla width, log transformed banner width and squared angle data to eliminate heteroscedasticity (Sokal and Rolph 1995). I used a principal components analysis to provide a visual representation of floral size and shape variation in two-dimensional space. Univariate ANOVA with population and transect within population as main effects was used to determine variation in pollen to ovule ratios and pollen grain diameter among the twelve populations measured. Pollen to ovule ratio was log transformed to eliminate heteroscedasticity.

2.3 Results

2.3.1 Ploidy Variation

Through the observation of chromosomes in the rapidly dividing PMCs I found both diploid (2n = 14) and tetraploid (2n = 28) plants among the populations studied (Table 2-1). Unfortunately, due to methodological difficulties in obtaining PMCs with chromosomes adequately separated for counting, I was able to obtain chromosome counts for only 13 out of the 29 populations for which plant material was available. However, because I was able to identify both diploid and tetraploid plants it was possible to calibrate the relative estimates of genomic content from the flow cytometry analysis to actual ploidy levels.

Flow cytometric analysis revealed four independent 2C-DNA content levels within the populations sampled (Table 2-1). I found that all BC populations had 2C-DNA content corresponding to the tetraploid samples (Mean = 4.43pg; Table 2-1). Ploidy levels varied among the US populations. Large-flowered

populations that were sympatric with small-flowered tetraploid populations generally had 2C-DNA levels consistent with diploidy (Mean = 2.38pg) with the exception of FRg where one of the five plants measured was tetraploid. The allopatric large-flowered populations were all consistent with tetraploidy. Three ploidy levels were found among the small-flowered populations in the US; most populations were tetraploid (mean 2C-DNA content = 4.43pg), three populations had 2C-DNA contents greater than 6.0pg and one population had a 2C-DNA content less than 2.0pg (Table 2-1). Unfortunately, chromosome counts could only be obtained for diploid and tetraploid plants leaving it unclear what factors contributed to these differences in genomic DNA content among small-flowered populations.

2.3.2 Morphological Variation

The results of the correlation analysis indicated that corolla length, corolla tube length, banner petal width, and corolla width were all positively correlated with each other, and all were negatively correlated with the angle of declination of the corolla tube to the calyx (Table 2-2). Floral traits were found to differ significantly among populations using MANOVA (Wilks' Lambda F = 15.75, $P<0.0001$). Subsequent univariate tests demonstrated significant differences among populations for all measured traits (angle, F = 77.80, $P<0.0001$; corolla tube, F = 5.60, $P<0.0001$; corolla length, F = 7.72, $P<0.0001$; banner petal width, F = 7.98, $P<0.0001$ and total corolla width F = 9.96, $P<0.0001$), as well as significant effects of transect nested within population (angle, F = 1.80, $P<0.01$;

corolla tube, $F = 2.61$, $P<0.0001$; corolla length, $F = 2.30$, $P<0.001$; banner petal width, $F = 1.88$, $P<0.01$ and total corolla width, $F = 3.45$, $P<0.0001$).

Populations tended to sort according to a combination of flower shape and size in the principal components analysis (Figure 2-3). The first component explained 88.6% of the variation in floral traits and distinguished between populations with small flowers and larger declined angles and populations with larger flowers and smaller declined angles (Table 2-3). The US large-flowered populations were tightly grouped on the positive end of the first principal component axis indicating larger flower size and smaller declined angle (Figure 2-3). Although the small-flowered populations from the US were much more loosely grouped along the negative end of the first component, indicating smaller flower size and larger declined angle, they were clearly separate from the large-flowered US populations. Populations from BC were distributed from the negative to positive ends of the first component demonstrating the wide variation in flower sizes found in that region.

The second principal component explained only 6.3% of the variation and distinguished between populations with wider corollas, shorter corolla tubes and larger angles and those with narrower corollas, longer tubes, and smaller angles (Table 2-3). The US populations of both flower sizes tended to be distributed along the negative end of the second component, indicating longer, narrower flowers whereas the BC populations were generally distributed along the positive end of the axis, indicating shorter, wider flowers.

2.3.3 Reproductive Allocation

Pollen:ovule ratios differed among populations with plants from large-flowered populations all producing significantly more pollen per ovule than plants from the small-flowered populations ($F=99.58$, $P<0.0001$; Figure 2-4). There was no effect of transect nested within population on pollen:ovule ratio ($F=1.08$, $P>0.05$). Although small-flowered plants produced significantly less pollen ($F=105.72$, $P<0.0001$), the pollen grains were significantly larger than those produced by the large-flowered plants ($F=30.42$, $P<0.0001$; Figure 2-5). There was no effect of transect within population on pollen production ($F=1.41$, $P>0.05$), however, pollen size differed among transects ($F=2.18$, $P<0.05$).

2.4 Discussion

2.4.1 Ploidy Variation

Previous work in the genus *Collinsia* indicated that populations of *C. grandiflora* and *C. parviflora* in the US were diploid (Garber 1956, 1958) while populations in BC were tetraploid (Ganders and Krause 1986). I found that all BC populations and, in contrast to Garber (1956, 1958), most US populations had 2C-DNA contents consistent with a tetraploid genome regardless of flower size (Table 2-1). Disparities between this study and those of Garber (1956, 1958) are likely due to differences in sample size—the Garber studies included only two pollen mother cells for the determination of ploidy in *C. parviflora* (Garber 1956). Although 30 pollen mother cells were analyzed for ploidy determination in *C. grandiflora*, the number of individual plants comprised in that sample was not reported (Garber 1958). The extremely small sample size for *C.*

parviflora is especially concerning as it was small-flowered US populations that exhibited the most ploidy variation. Variation in corolla length was continuous within the tetraploid group demonstrating that flower size is not associated with variation in ploidy level (Figure 2-6A). Tetraploid populations were broadly distributed from BC to southern CA and across the Cascade and Sierra Nevada mountain ranges whereas diploids occurred only in select locations from central WA to southern CA west of the mountains.

In this study, the large-flowered diploids were only found at sites where small and large-flowered populations occurred in sympatry (FR and TR). In all locations included in this study where large and small-flowered populations co-occurred I found that the small-flowered plants were tetraploid and the large-flowered plants were diploid (except HUM where no data could be obtained regarding the ploidy of large-flowered plants; Table 2-1). In another site, not included in this study, (Butte Falls Road, OR, 42 32.681'N, 122 44.360'W) where small and large-flowered populations co-occur (April Randle, personal communication) mean 2C-DNA contents were 2.39pg (consistent with diploid) for the large-flowered plants and 4.45pg (consistent with tetraploid) for the small-flowered plants. In contrast, large-flowered populations growing in allopatry were invariably tetraploid. The observed pattern of ploidy variation demonstrates that it may be typical for there to be ploidy differences between large and small-flowered populations when they co-occur. Flower size variation may have evolved in sympatry as a result of selection to avoid mating between plants of different ploidy. Crosses between diploid and tetraploid plants result in triploid

hybrid offspring that often experience reduced fertility compared to the parental species (Ramsey and Schemske 1998).

In addition to the variation among large-flowered populations, I also found ploidy variation among the small-flowered populations in the US. Mean 2C-DNA content was greater than 6.0pg in three of the populations included in this study (ELTH, MM53 and MV) and less than 2.0pg in the eastern population (MI) (Table 2-1). These differences in 2C-DNA content are not consistent with multiplication of the full haploid genome and may represent aneuploid chromosome numbers. Aneuploidy is common in many plant genera and occurs as a result of either the increase or decrease in basic chromosome number, generally by one chromosome per event (Stebbins 1950, Grant 1981). It can occur in either diploids or polyploids by several mechanisms generally related to irregularities in chromosome pairing at meiosis (Grant 1981). However, it is also possible that these abnormal genomic contents are due to molecular variations in the DNA that do not lead to change in chromosome number. Leitch and Bennet (2005) show that the total amount of DNA contained in the genome can be increased by transposon amplification or decreased by a number of other recombinational mechanisms. Further cytological work will be required to determine haploid chromosome number for these irregular populations

2.4.2 Morphological Variation

In this study, flower size measured in the field varied continuously among tetraploid populations, but tended to be either large or small for other ploidy levels (Figure 2-6A). When grown in a common environment, *Collinsia* exhibits

the same pattern of among-population variation as in the field, indicating that flower size variation is due to genetic differences rather than a plastic response to local conditions (Krause 1978, Elle 2004, Chapter 3–Figure 3-1). The potential selective agents driving the genetic variation in flower size may include biotic sources such as insect pollinators or abiotic factors such as edaphic or climatic conditions.

Pollinator preferences can impose strong selective pressure for increased flower size in plant populations (Galen et al. 1999, Herrera 2005, Lambrecht and Dawson 2007). In BC, the pollinator environment is known to differ among large and small-flowered populations (Elle 2004). Pollinators tend to prefer large-flowered over small-flowered plants in experimental arrays (Elle and Carney, 2003) and insect visit rates are higher for large compared to small-flowered populations (Elle and Carney 2003, Kennedy and Elle 2008). Differences in flower size among populations could be the result of selection for larger flower size in pollinator rich environments and selection for reduced flower size to promote autonomous selfing in pollen-limited conditions (Elle and Carney 2003, Kennedy and Elle 2008). However, this hypothesis predicts discontinuous variation in flower size, rather than the continuous variation observed in this study; an explanation for intermediate flower sizes is therefore needed.

In addition to selection imposed by the pollinator community, abiotic factors including soil or atmospheric moisture content (Galen et al. 1999, Elle 2004, Lambrecht and Dawson 2007) serpentine soil conditions (Wright et al. 2006) and latitudinal or elevation gradients (Jonas and Geber 1999, Herrera

2005) can also affect variation in flower size and development. Flower size is positively correlated with annual precipitation for *Collinsia* populations in BC (Elle 2004) and research across plant species and families has shown that significant reductions in individual flower and total display size are associated with moisture limitation (Galen et al. 1999, Jonas and Geber 1999, Herrera 2005, Lambrecht and Dawson 2007).

There are two potential explanations for reduced flower size under drought conditions. Because flowers generally lack stomata, transpiration rates tend to be very high in floral tissues; reduction in flower size may aid plants in their ability to control water loss in xeric habitats (Lambrecht and Dawson 2007). Galen et al. (1999) found that drought stress tended to result in reduced carbon assimilation in large, compared to small-flowered, *Polemonium viscosum*, indicating that large-flowered plants were at a disadvantage due to the necessity of closing stomata—therefore reducing photosynthesis—to control water loss. Alternatively, plants which grow in ephemerally moist environments may be subject to selection for more rapid development which indirectly results in reduced flower size if development time and flower size are genetically correlated (Elle 2004).

The continuous variation in flower size found among populations in this study may be due to differences in the relative strength of the competing biotic and abiotic selection pressures among sites. Pollinators tend to select for larger flower sizes while drought conditions impose strong selection for reduced flower size (Elle 2004). Large flower sizes may be restricted to mesic sites because,

with excess water availability, the plants are released from the selection for reduced flower size associated with water limitation and respond instead to the stronger selective force of pollinator preference for larger flower size (Elle 2004). Populations of intermediate flower size in BC have been shown to experience intermediate levels of precipitation (Elle 2004). The strength of abiotic selection due to moisture availability at these sites may be strong enough to temper, but not eliminate, the effects of pollinator selection, resulting in the intermediate flower sizes observed in this study.

Although it is difficult to single out a group of populations as intermediate when flower size varies continuously, it is interesting to note that four of the populations (JP, KB, AW, RB) which group near the center of the distribution of corolla lengths (Figure 2-6B) are located within 100m of the ocean. Both plant and pollinator populations in close proximity to the ocean may face different selection regimes due to abiotic conditions that differ between coastal and inland areas such as soil salinity, salt spray, atmospheric moisture, and temperature (Lowry et al. 2008). The effects of abiotic and biotic characters of the habitat on flower size should be studied throughout the geographic range to determine their combined effects on flower size.

2.4.3 Reproductive Allocation

The differences in pollen to ovule ratios between small and large-flowered populations in the US corresponded to results found by Parachnowitsch and Elle (2004) in a study of six BC populations. I found that for large-flowered populations the number of pollen grains produced per ovule greatly exceeded

that of small-flowered populations, with pollen production up to 90 times greater in some large-flowered populations. BC populations of intermediate flower size produced intermediate numbers of pollen grains per ovule when compared to large and small-flowered populations (Parachnowitsch and Elle 2004). In the present study, I found that pollen diameter was significantly smaller among large-flowered compared to small-flowered populations from the US, as in the BC populations studied by Parachnowitsch and Elle (2004).

Although it is possible that smaller pollen grains take less energy to produce, it seems unlikely that this difference alone could explain the dramatic disparity in pollen production between the two flower sizes. Although outcrossing rates are currently unavailable, based on differences observed among BC populations in autonomous selfing rates (Elle 2004), pollinator visit rates (Elle and Carney 2003, Kennedy and Elle 2007), and allocation to male and female primary and secondary reproductive traits (Parachnowitsch and Elle 2004) it appears likely that large-flowered plants rely more on outcrossing than small-flowered plants. Although we do not have data regarding autonomous selfing or pollinator visitation for the US populations surveyed in this study, the extreme difference in the amount of pollen produced per ovule between the large and small-flowered populations could be indicative of higher outcrossing rates in the large-flowered plants. It is widely recognized that higher allocation to male traits is associated with outcrossing, and lower allocation with selfing, in hermaphroditic flowers (Cruden and Lyon 1985).

2.4.4 Evolution of Variation

Without more detailed cytological data indicating chromosome number for the populations with 2C-DNA contents that fall outside the typical values for known diploid and tetraploid groups, or further knowledge of the relationships among populations in this group of *Collinsia*, it is not possible to say, with any certainty, how morphological and ploidy variation evolved. It is clear from the data that variation in flower size is not linked to ploidy level. However, the patterns I observed in this study do suggest a potential hypothesis. For all sites where small and large-flowered plants grow in sympatry I have observed that the large-flowered plants are diploid whereas the small-flowered plants are tetraploid. When polyploids arise in a population, they can be out-competed by the majority cytotype at that site due to reduced fertility in the offspring of between-cytotype mating (Levin 2002, Ramsey and Schemske 1998, Husband and Sabara 2004). The polyploid lineages that are most likely to persist in such mixed ploidy groups are those that are able to escape minority cytotype exclusion. One means of overcoming minority cytotype disadvantage is via increased self-fertilization (Grant 1981, Husband and Sabara 2004). In BC, small-flowered plants have higher autonomous selfing rates than large-flowered plants due to reduction in anther stigma separation and synchronization of male and female reproductive maturity (Elle 2004). If this autonomous selfing ability also occurs in small-flowered *Collinsia* populations in the US, selection for increased selfing in polyploids may have driven the disparity in flower sizes between tetraploids and diploids growing in sympatry.

Polyploids often have wider geographic distributions than diploids. This is thought to be due to the increased heterozygosity in neopolyploids, which may make them more adaptable to marginal habitats (Grant 1981, Levin 2002). If tetraploid populations of *Collinsia* were successful in colonizing habitats outside the range of their diploid progenitors then they may have been released from the selection pressure for selfing. If this were the case then allopatric populations would have been free to evolve larger or smaller flower sizes depending on the biotic and abiotic agents of selection present in their new habitat. This may be why we see such broad variation in flower size among the allopatric tetraploids.

2.4.5 Implications for Collinsia Taxonomy

Plant taxonomy has historically been based on morphological variation among groups (Stebbins 1950, Grant 1981). From the taxonomist's perspective, species or sub-species groupings must be based on morphological discontinuities between whole populations; if continuous variation is observed among populations then there is no way to clearly identify intermediate forms (Stebbins 1950). Traits related to flower size are the primary features considered in the literature for distinguishing between *C. parviflora* and *C. grandiflora* (Hitchcock and Cronquist 1973, Douglas et al. 2000). Variation in the floral traits measured in this study was continuous throughout the range of populations (Figure 2-3, 2-6B). To address the problem of assigning populations with continuous floral variation to the separate species described above, Ganders and Krause (1986) proposed that all populations be reclassified as the single species, *C. parviflora*, with two subspecies *parviflora* and *grandiflora* to reflect the

variation in flower size. However, given that there is no clear discontinuity between small- and large-flowered populations, division into two distinct subspecies does not really address the problem of classification in this group.

From an evolutionary perspective, species are identified based on the ability of individuals within a group to produce viable and fertile offspring (Mayr 1942). Barriers to reproduction may result from morphological, mechanical or genomic incompatibilities between groups (Coyne and Orr 2004). Crosses made between plants of different ploidy frequently result in either the failure to form seeds or reduction in offspring fertility due to meiotic irregularities (Ramsey and Schemske 1998). In Chapter 3, I present results showing reproductive isolation among a subset of the populations described in this chapter including all four ploidy levels identified. The primary cause of reproductive isolation among these populations was reduced fertility in the offspring of crosses between populations that varied in 2C-DNA content or ploidy level.

The existing taxonomic classification of this group of *Collinsia*, which describes two species based on flower size differences, appears to be insufficient when the continuous variation in flower size and the variation in genomic content found among the populations in this study are taken into account. In place of the classification of two species or subspecies based on flower size, I propose that species be designated based on differences in genomic content. I suggest that the continuously variable group of tetraploids compose a single species that occurs from BC to Southern CA. I recommend that this group be designated *Collinsia variabilis* (Corolla Length 4-15mm) to

reflect the widespread variation in flower size observed among populations. Although an argument could be made for recognizing two ecological races (small-flowered, drought tolerant and large-flowered, mesic) within *C. variabilis*, due to the continuous variation observed, identification of such races would still be problematic. I find that the large-flowered diploids (found only to occur in sympatry with small-flowered tetraploids in this study) fit into the current taxonomic classification of *C. grandiflora*, and occur west of the Cascade and Sierra Nevada mountain ranges from WA to CA.

I did not find any populations that fit the classification of diploid, *C. parviflora*. It is possible that this species does exist, but was simply missed in this survey. However, because Garber (1956) identified this species as diploid based on the results of chromosome counts made using only two pollen mother cells (he does not mention if these cells were obtained from a single individual or two separate plants) it is possible that this result was an aberration from the norm.

Based on the differences in genomic content and the resultant reproductive isolation incurred (see Chapter 3) it is possible that the two small-flowered populations with odd 2C-DNA contents are distinct species. However, without chromosome counts, assigning these groups full species status is a somewhat tenuous proposal. Evidence revealing the exact chromosome complement and the genetic relationships between these populations and the described diploid and tetraploid groups will be necessary before a complete taxonomic reassessment of this group can be performed.

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2.6 Tables

Table 2-1: Population locations, ploidy, and mean values (±SE) for five floral traits of *Collinsia* measured in Canada and the United States. ND = no data. Populations are arranged alphabetically within Canadian Province or US State of origin. Number of unrelated plants used for chromosome counts or flow cytometry in brackets. Flow cytometry results are followed by range of values below. Floral trait means followed by the same letter within a column do not differ significantly (ANOVA followed by Ryan's Q; see Methods). Floral traits for MI are from a separate, growth chamber experiment (Chapter 3) and provided for comparison only (not included in analysis; N = 5).

British Columbia Populations

Population	Pop Code	Location	2n No.	2C-DNA pg	Corolla Width	Banner Width	Corolla Length	Tube Length	Declined Angle
Cowichan River	CR	48 46'39.0"N 123 56'30.2"W	28 (1)	4.58 (2) 4.49-4.67	8.15 ± 0.16^{CDE}	4.35 ± 0.10^{BCD}	12.21 ± 0.19^{DE}	6.48 ± 0.13^{BC}	133.03 ± 1.40^{H}
Elk Falls	EF	50 02'34.9"N 125 19'32.0"W	28 (2)	4.62 (2) 4.57-4.66	8.35 ± 0.16^{CD}	4.46 ± 0.09^{BCD}	12.76 ± 0.17^{CD}	6.86 ± 0.13^{AB}	121.46 ± 1.24^{J}
Garry Oak Preserve	GO	48 48'30.8"N 123 37'52.5"W	28 (1)	4.47 (3) 4.43-4.56	4.15 ± 0.13^{IJ}	2.18 ± 0.06^{G}	7.15 ± 0.15^{J}	3.06 ± 0.09^{IJ}	157.96 ± 1.14^{BC}
Jack Point	JP	49 09'29.9"N 123 53'35.0"W	28 (2)	4.32 (2) 4.20-4.44	7.05 ± 0.18^{G}	3.69 ± 0.09^{E}	9.02 ± 0.19^{G}	4.14 ± 0.14^{EF}	141.49 ± 1.12^{G}
Kin Beach	KB	49 43'44.8"N 124 53'51.8"W	ND	4.39 (4) 4.36-4.43	7.28 ± 0.16^{FG}	3.97 ± 0.11^{DE}	8.64 ± 0.17^{GH}	4.21 ± 0.13^{EF}	150.07 ± 1.18^{DE}
Nanoose Hill	NH	49 16'29.0"N 124 09'52.2"W	ND	4.40 (2) 4.35-4.45	4.28 ± 0.10^{I}	2.12 ± 0.05^{G}	7.95 ± 0.12^{I}	3.66 ± 0.08^{GH}	148.73 ± 0.95^{EF}
Rathtrevor Beach	RB	49 19'21.2"N 124 15'50.5"W	ND	4.43 (2) 4.31-4.55	5.62 ± 0.15^{H}	2.89 ± 0.07^{F}	8.22 ± 0.14^{HI}	3.67 ± 0.12^{G}	158.04 ± 1.23^{BC}
Stoltz Meadows	SM	48 46'54.0"N 123 53'06.9"W	28 (1)	4.44 (2) 4.39-4.49	7.79 ± 0.15^{DEF}	4.23 ± 0.09^{CDE}	11.80 ± 0.26^{DE}	6.32 ± 0.14^{BC}	134.00 ± 1.53^{H}

Population	Pop Code	Location	2n No.	2C-DNA pg	Corolla Width	Banner Width	Corolla Length	Tube Length	Declined Angle
Sooke Potholes	SP	48 25'44.3"N 123 42'40.9"W	28 (2)	4.59 (2) 4.57-4.60	7.51 ± 0.14^{EFG}	3.95 ± 0.09^{DE}	10.24 ± 0.14^{F}	5.56 ± 0.10^{D}	143.54 ± 1.08^{FG}
Thetis Lake	TL	48 28'00.9"N 123 27'58.6"W	28 (1)	4.29 (3) 4.27-4.31	3.89 ± 0.10^{IJ}	2.08 ± 0.04^{G}	6.58 ± 0.11^{JK}	3.12 ± 0.07^{I}	151.92 ± 0.95^{DE}

Washington Populations

Population	Pop Code	Location	2n No.	2C-DNA pg	Corolla Width	Banner Width	Corolla Length	Tube Length	Declined Angle
Anacortes Washington	AW	48 29'41.9"N 122 42'06.1"W	ND	4.40 (4) 4.20-4.69	5.85 ± 0.12^{H}	3.08 ± 0.07^{F}	8.36 ± 0.12^{HI}	4.37 ± 0.07^{E}	148.20 ± 1.38^{FE}
Ellensburg-Thorpe	ELTH	47 06'56.4"N 120 42'47.4"W	ND	6.06 (1)	2.94 ± 0.12^{KL}	1.60 ± 0.03^{H}	5.10 ± 0.10^{KL}	2.64 ± 0.07^{IJK}	164.91 ± 0.93^{A}
Fossil Rock grandiflora	FRg	46 49'34.3"N 122 27'57.2"W	ND	a) 2.39 (4) 2.26-2.51 b) 4.53 (1)	7.54 ± 0.12^{EFG}	4.04 ± 0.13^{E}	13.79 ± 0.14^{AB}	7.47 ± 0.11^{A}	125.43 ± 1.31^{IJ}
Fossil Rock parviflora	FRp	46 49'34.3"N 122 27'57.2"W	ND	4.49 (4) 4.33-4.71	ND	ND	ND	ND	ND
Rowland Lake	RL	45 42'45.6"N 121 22'52.7"W	28 (1)	4.44 (3) 4.40-4.53	3.86 ± 0.15^{J}	2.03 ± 0.06^{G}	7.99 ± 0.15^{I}	4.34 ± 0.08^{E}	142.33 ± 1.60^{G}

Oregon Populations

Population	Pop Code	Location	2n No.	2C-DNA pg	Corolla Width	Banner Width	Corolla Length	Tube Length	Declined Angle
Camassia Natural Area	CAM	45 21’40.1”N 122 37’09.0”W	28 (2)	4.53 (4) 4.38-4.63	8.56 ± 0.14^{BCD}	4.86 ± 0.07^{B}	12.27 ± 0.13^{DE}	7.19 ± 0.14^{A}	129.25 ± 1.75^{HI}
Crown Point	CP	45 32’12.9”N 122 14’21.5”W	ND	4.58 (2) 4.50-4.66	7.47 ± 0.12^{EFG}	4.15 ± 0.07^{DE}	12.80 ± 0.13^{CD}	6.17 ± 0.11^{C}	126.85 ± 0.10^{IJ}
Mile Marker 53	MM53	45 02’18.2”N 120 42’36.3”W	ND	6.09 (3) 5.83-6.22	2.82 ± 0.12^{KLM}	1.69 ± 0.06^{H}	6.51 ± 0.11^{JK}	3.13 ± 0.10^{I}	159.08 ± 1.06^{BC}
Saddle Mountain	SAD	45 57’46.8”N 123 41’01.3”W	28 (1)	4.37 (2) 4.24-4.47	3.62 ± 0.07^{J}	1.90 ± 0.04^{G}	6.85 ± 0.08^{J}	3.12 ± 0.07^{HI}	157.69 ± 1.15^{BC}
Spencer Butte	SB	43 56’54.0”N 123 02’27.4”W	ND	4.26 (2) 4.23-4.29	2.97 ± 0.07^{KL}	1.52 ± 0.03^{HI}	6.60 ± 0.09^{JK}	3.85 ± 0.07^{FG}	148.10 ± 0.93^{EF}
Trout Creek	TC	44º24’02.2”N 122º 21’13.9”W	28 (1)	4.36 (3) 4.20-4.57	8.74 ± 0.13^{BC}	4.74 ± 0.09^{BC}	14.62 ± 0.15^{A}	7.36 ± 0.10^{A}	129.67 ± 1.42^{HI}
Table Rock grandiflora	TRg	42º27’15.7”N 122º 57’01.0”W	14 (2)	2.39 (3) 2.35-2.43	9.96 ± 0.14^{A}	5.71 ± 0.11^{A}	13.18 ± 0.13^{BC}	7.43 ± 0.11^{A}	126.08 ± 1.32^{IJ}
Table Rock parviflora	TRP	42º27’15.7”N 122º 57’01.0”W	28 (1)	4.42 (2) 4.27-4.52	3.11 ± 0.09^{K}	1.59 ± 0.04^{H}	6.35 ± 0.12^{KL}	3.21 ± 0.05^{HI}	153.87 ± 1.05^{CD}

California Populations

Population	Pop Code	Location	2n No.	2C-DNA pg	Corolla Width	Banner Width	Corolla Length	Tube Length	Declined Angle
Gazelle-Callahan	GC	41 25'48.1"N 122 38'18.1"W	ND	ND	2.63 ± 0.08^{LM}	1.40 ± 0.04^{I}	6.21 ± 0.12^{KL}	2.57 ± 0.08^{K}	159.06 ± 0.88^{BC}
Humboldt grandiflora	HUMg	40 46'54.6"N 123 40'48.6"W	ND	ND	9.34 ± 0.26^{B}	5.08 ± 0.13^{B}	11.65 ± 0.15^{E}	6.20 ± 0.12^{C}	124.56 ± 0.92^{IJ}
Humboldt parviflora	HUMp	40 46'54.6"N 123 40'48.6"W	ND	4.29 (1)	2.30 ± 0.06^{M}	1.38 ± 0.04^{I}	5.71 ± 0.09^{L}	2.53 ± 0.07^{K}	159.26 ± 1.03^{B}
Markleeville	MV	38° 41'36.8"N 119º 46'04.3"W	ND	6.15 (2) 6.13-6.18	2.46 ± 0.08^{M}	1.37 ± 0.05^{I}	6.06 ± 0.10^{KL}	2.85 ± 0.10^{IJK}	157.75 ± 0.79^{BC}
Susanville	SV	40 37'05.6"N 120 38'02.1"W	ND	4.61 (2) 4.57-4.65	1.90 ± 0.04^{N}	1.14 ± 0.03^{J}	4.85 ± 0.10^{M}	2.07 ± 0.06^{L}	162.32 ± 0.93^{AB}

Michigan Population

Population	Pop Code	Location	2n No.	2C-DNA pg	Corolla Width	Banner Width	Corolla Length	Tube Length	Declined Angle
Michigan	MI	46º 52'24"N 87º 55'00"W	ND	1.95 (2) 1.94-1.97 2.45(1)	1.82 ± 0.04	ND	2.92 ± 0.16	ND	ND

Table 2-2 Pearson's correlation between measured floral traits across 27 populations of *Collinsia* from the United States and Canada. (*Correlation significant with $P<0.0001$).

Variable	Tube Length	Corolla Length	Banner Width	Corolla Width
Angle	-0.830*	-0.815*	-0.751*	-0.759*
Tube Length		0.939*	0.855*	0.870*
Corolla Length			0.887*	0.898*
Banner Width				0.967*

Table 2-3 Eigenvectors and Pearson's correlation coefficients associated with the principal components analysis of floral traits measured in 27 populations of *Collinsia*. The first principal component explained 88.6% of the variation and the second principal component explained 6.3%. (Correlation significant with p-value ** <0.0001, * <0.05)

Variable	Eigenvector PC1	Correlation PC1	Eigenvector PC2	Correlation PC2
Angle	-0.418	-0.880**	0.776	0.436**
Tube Length	0.454	0.956**	-0.145	-0.082*
Corolla Length	0.459	0.965**	-0.004	-0.002
Banner Width	0.450	0.948**	0.449	0.253**
Corolla Width	0.454	0.956**	0.418	0.235**

2.7 Figures

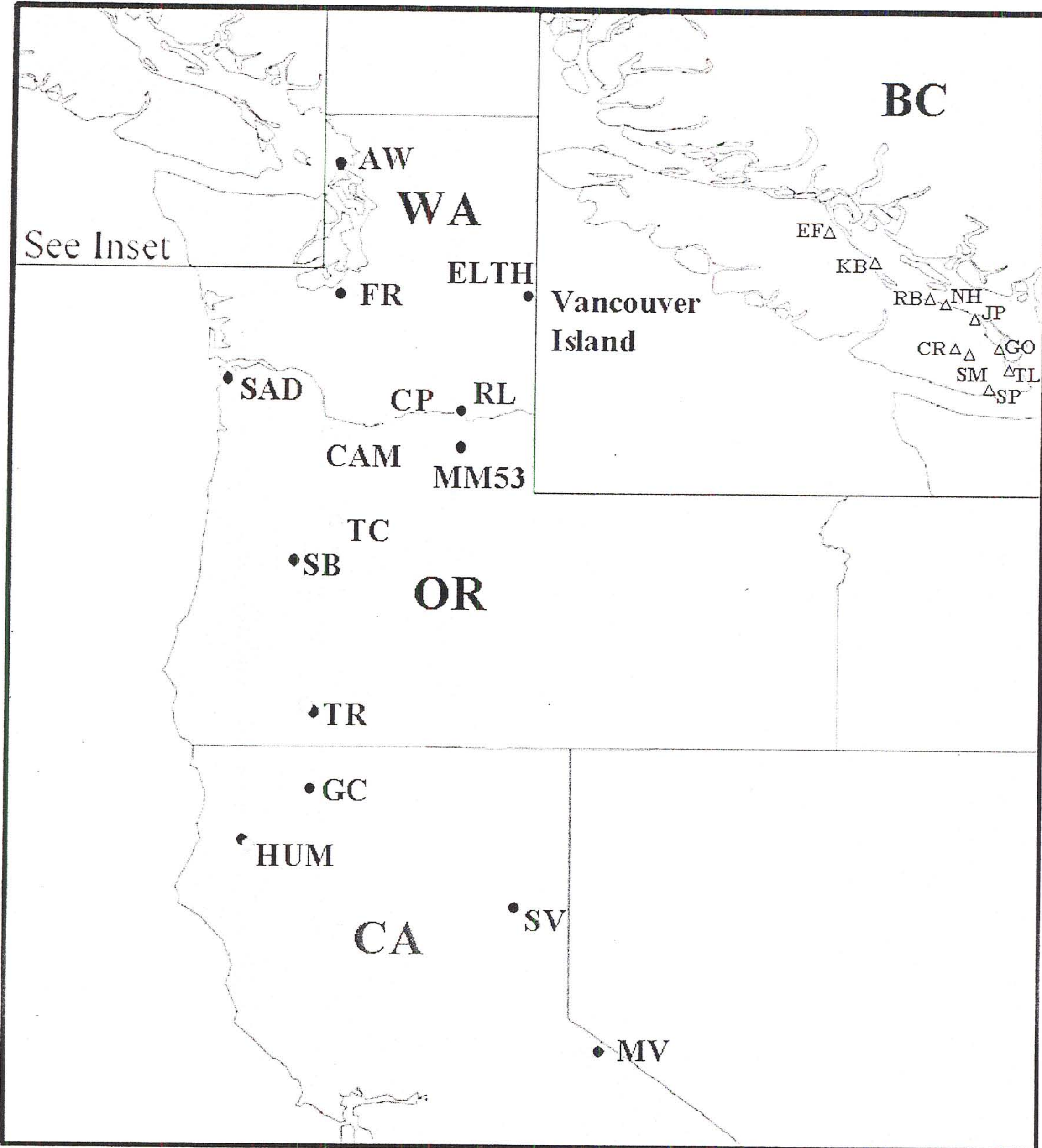

Figure 2-1 Locations of *Collinsia* populations used in this research, located along the Pacific Coast of North America. US small-flowered populations, black circles; US large-flowered populations, white circles; BC populations all flower sizes, grey triangles. The MI population, not shown, occurs in the mid-western state of Michigan.

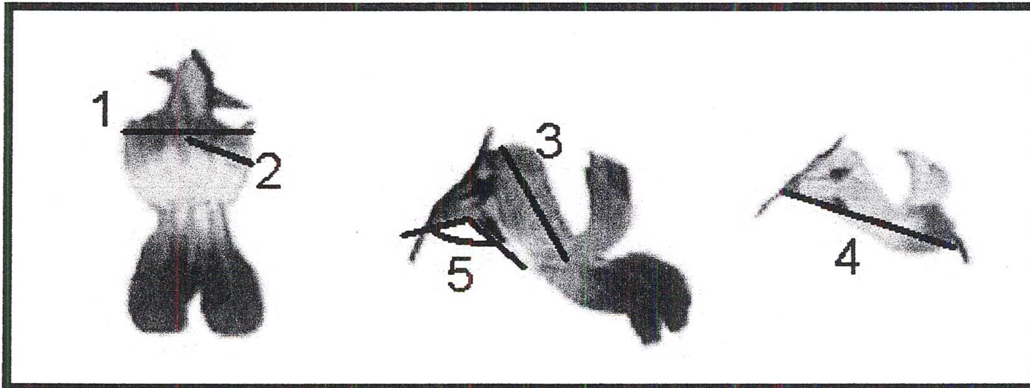

Figure 2-2 Measurement of floral traits in *Collinsia* populations from BC, WA, OR, and CA. 1, corolla width; 2, banner petal; 3, corolla tube; 4, corolla length; 5, declined angle.

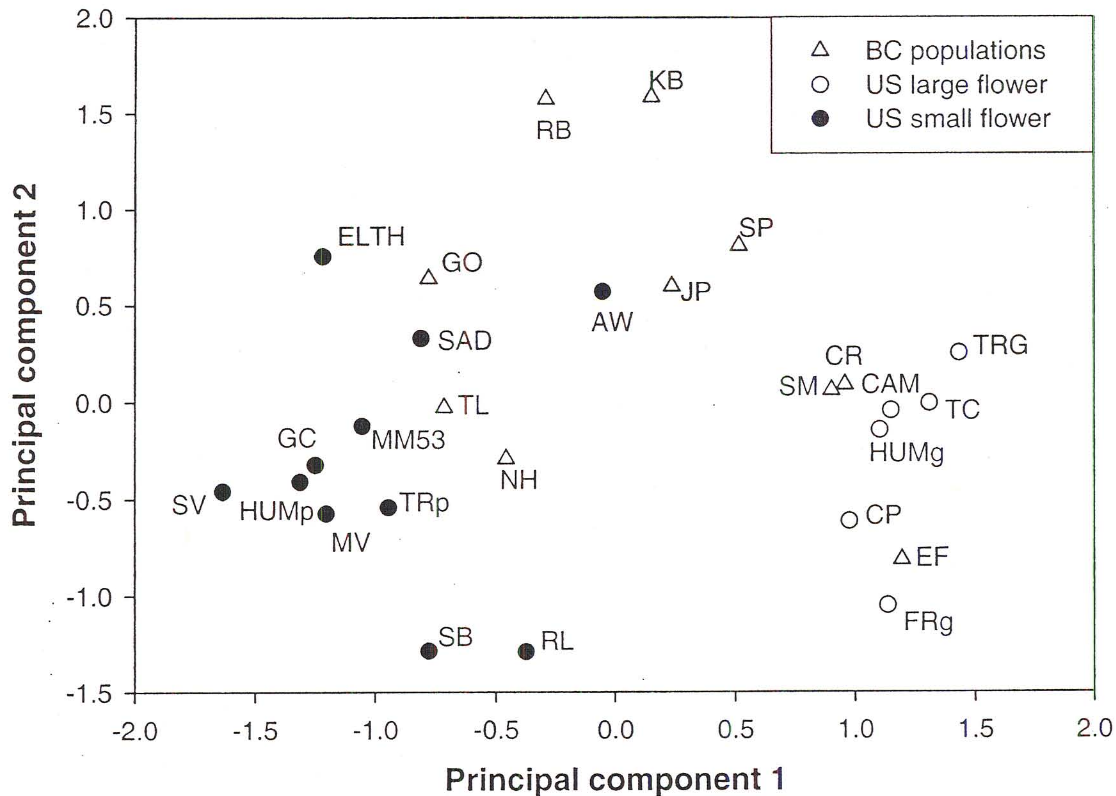

Figure 2-3 Principal component analysis of floral traits measured in *Collinsia* populations from BC, WA, OR, and CA. PC1 can be interpreted as distinguishing among populations with large flowers and small declined angles (positive loading), and populations with the alternate traits. PC2 can be interpreted as distinguishing among populations with large corolla and banner petal widths, short corolla and tube lengths, and large declined angles (positive loading), and populations with alternate traits. The following data transformations were done to correct for heteroscedasticity: angle data was raised to the power of 2, tube length and corolla length and width were square root transformed, and the banner width was log transformed.

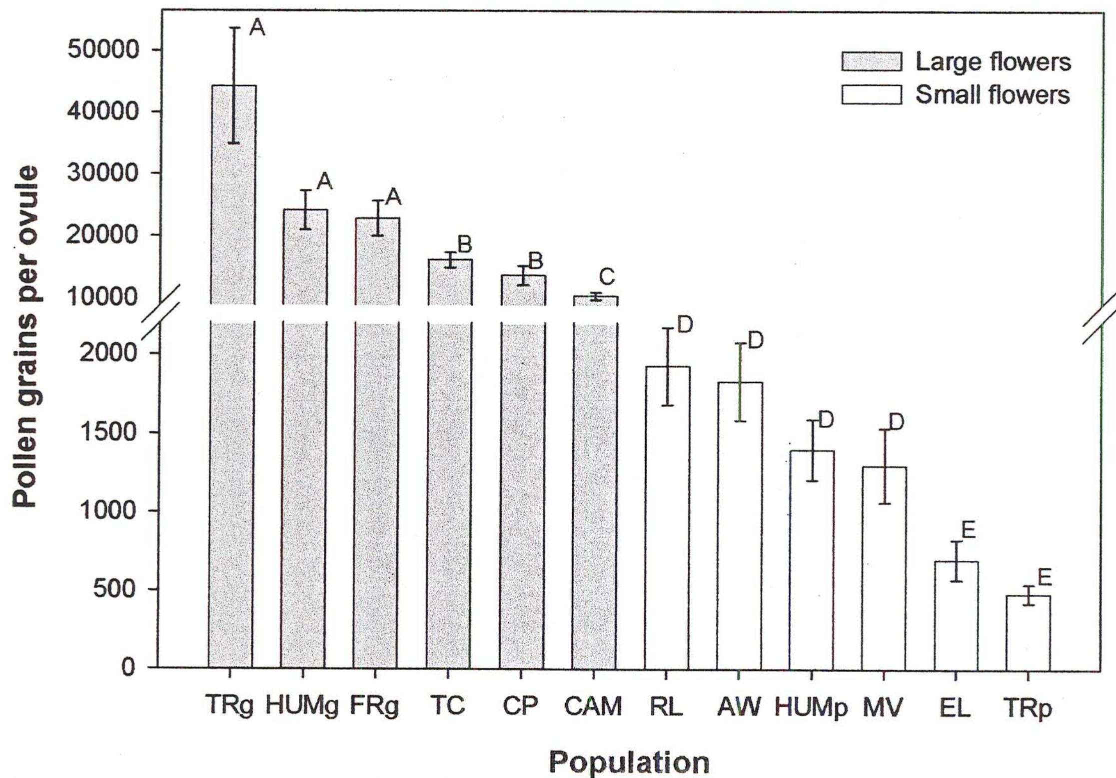

Figure 2-4 Mean pollen grains per ovule (PGO) ± SE for *Collinsia* from 12 small- and large-flowered US populations. Note the break in y-axis scale (2200-9000 PGO), included to clarify differences among small-flowered populations. Data were log transformed to correct for heteroscedasticity; untransformed data are shown here.

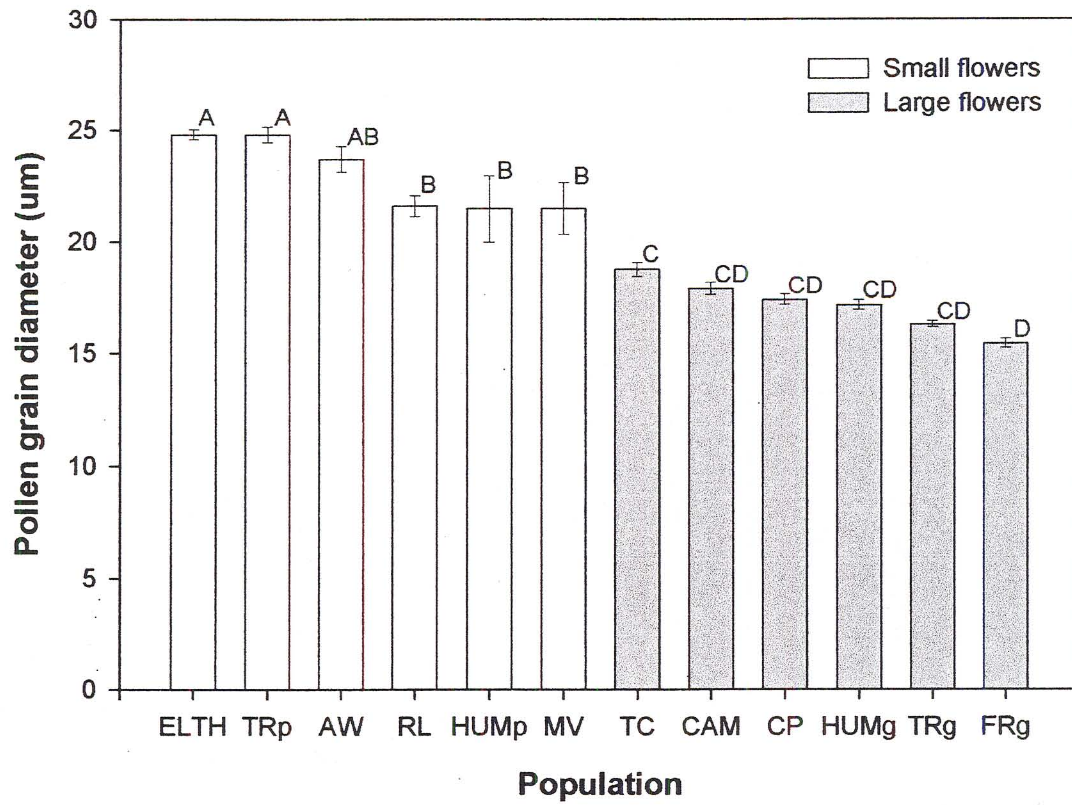

Figure 2-5 Mean pollen grain diameter ± SE for *Collinsia* from 12 small- and large-flowered US populations.

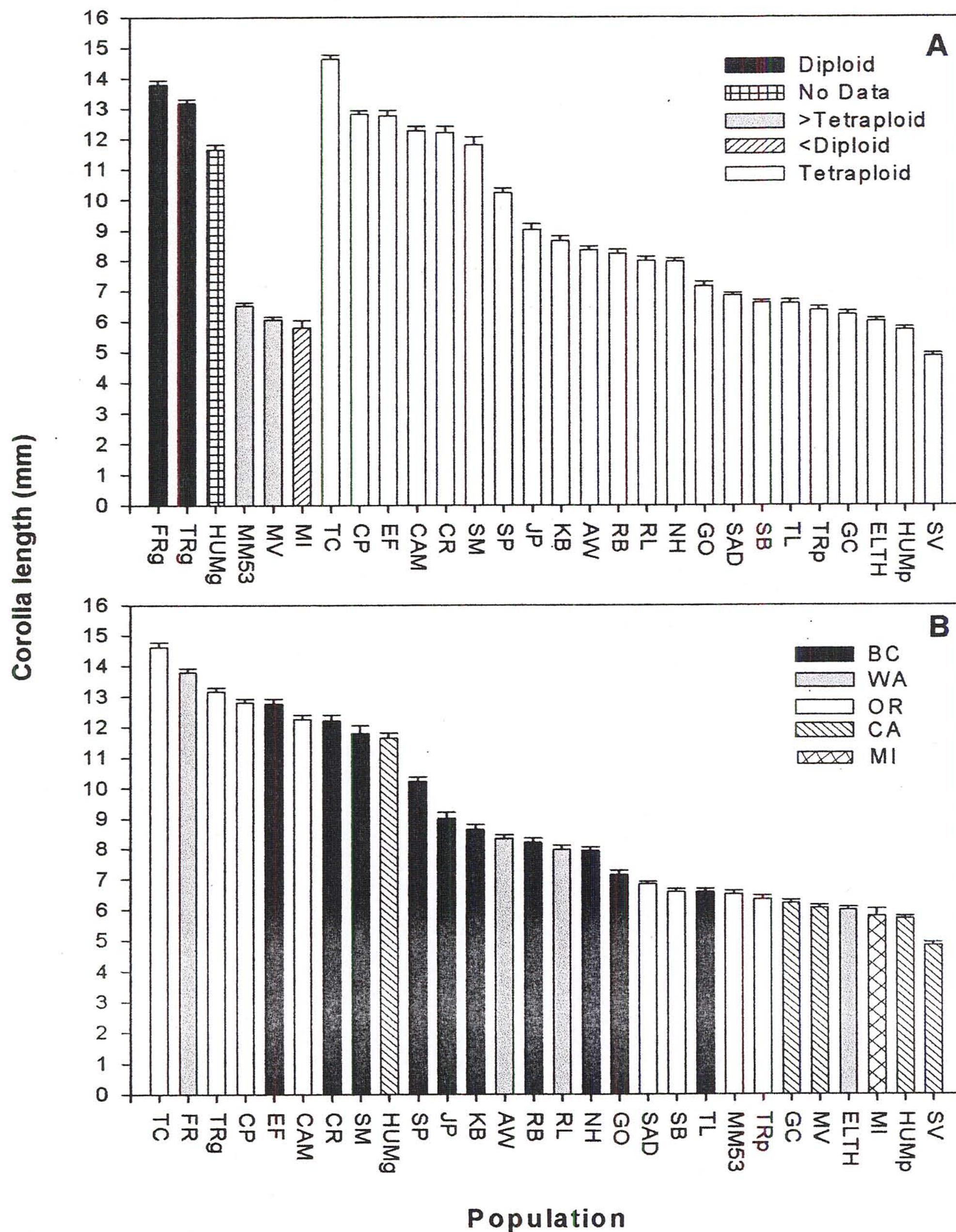

Figure 2-6 Mean corolla length + SE of *Collinsia* populations, arranged by decreasing corolla length within ploidy level (A); arranged by State/Province within decreasing corolla length (B). In A: >Tetraploid = 2C-DNA > 6.0pg; <Diploid = 2C-DNA <2.0pg. All data were collected from plants *in situ* as outlined in the methods section with the exception of MI. Corolla lengths for MI were measured on plants raised from wild collected seeds in an environmental chamber (see Chapter 3 for methods).

3: CHAPTER THREE: REPRODUCTIVE ISOLATION BETWEEN MORPHOLOGICALLY VARIABLE POPULATIONS OF *COLLINSIA PARVIFLORA* AND *C. GRANDIFLORA*.

3.1 Introduction

The study of reproductive isolation (RI) has long been recognized as a means for researchers to gain insight into the nature of species and the kinds of barriers important in their formation. In recent years, the focus of this research, particularly in botanical studies, has been to determine how barriers at multiple life stages contribute to overall isolation between closely related species (Coyne and Orr 1989, 1997, Ramsey et al. 2003, Kay 2006, Martin and Willis 2007, Scopece et al. 2007, Lowry et al. 2008, Widmer et al. 2009). Although many studies have found strong individual barriers to reproduction (Ramsey et al. 2003, Kay 2006, Martin and Willis 2007), it is rare for a single barrier to act in isolation (Lowry et al. 2008). Because reproductive barriers act sequentially over the lifespan, all things being equal, barriers expressed at earlier life stages will be more important to total RI than later acting barriers (Ramsey et al. 2003, Rieseberg and Willis 2007). Although most studies have found that both pre- and post-zygotic isolating mechanisms contribute to RI, one study (Costa et al. 2007) found that pre-zygotic barriers did not contribute to total RI between populations in the *Chamaecrista desvauxii* complex. In the majority of studies, even those that found strong and early acting individual barriers, multiple barriers tend to

make important relative contributions to total RI (Coyne and Orr 2004, Rieseberg and Willis 2007, Lowry et al 2008, Widmer et al. 2009). Thus, studies of RI must include potential barriers at multiple life stages to capture the true degree of isolation between species.

The strength of RI between two species can be indicative of their genetic relatedness. Coyne and Orr (1989, 1997) found a strong association between Nei's D, a genetic measure of time since divergence, and RI between 171 species pairs in the genus *Drosophila.* However, of three angiosperm genera examined in Moyle et al. (2004) only one showed a strong, significant relationship between RI and genetic distance. Scopece et al. (2007) examined RI and genetic distance among several species of specialist and generalist pollinated orchids and similarly found conflicting results. A significant association between RI and genetic distance was found for postzygotic barriers among orchid species with generalist pollinators (Scopece et al. 2007, 2008), but no such relationship was found for sexually deceptive orchids with specialist pollinators (2007). Another potential deviation from the relationship between RI and genetic distance is the expectation that RI will be high even between very recently diverged species when isolation is driven by a change in ploidy (Moyle et al. 2004, Ramsey and Schemske 1998). The production of polyploids is a special case that can result in virtually instantaneous speciation (Stebbins 1950). An estimate of the contribution of polyploidy to speciation in angiosperms by Otto and Whitton (2000) indicates that changes in ploidy are associated with a minimum of 2-4% of all speciation events in that group. Although some evidence has been found

supporting a relationship between RI and genetic distance in flowering plants, given that these results are not consistent across the genera studied, the interpretation of RI as an indicator of genetic relatedness between taxa should be approached with caution.

Collinsia parviflora and *C. grandiflora* are small and large-flowered sister species (Armbruster et al. 2002) that occur in Western North America from California to British Columbia and are identified primarily by their difference in flower size (Douglas et al. 2000). In the southern part of their range, Washington (WA), Oregon (OR) and California (CA) (hereafter referred to as US populations), large and small-flowered populations are found in both allopatry and sympatry. Although flower size variation among the US populations tends to be discontinuous, as would be expected based on the existing taxonomic classifications, ploidy differences between populations within a flower size class indicate that species division on this basis may be misleading. In the northern part of their range, on Vancouver Island and the Gulf Islands of British Columbia (hereafter referred to as BC populations), intermediate, large and small flower sizes occur such that the range of flower sizes varies continuously among populations (Ganders and Krause 1986, Elle and Carney 2003). Nonetheless, little intra-population variation in flower size is observed in any part of the range, and intermediate flower sizes are rare in the US (Chapter 2).

In addition to differences in the distributions of flower sizes between BC and US populations, there are also differences in ploidy. Ganders and Krause (1986) reported tetraploid status (2n=4x=28) for six BC populations including

those of small, intermediate and large flower sizes. These findings were in agreement with Taylor and Mulligan's (1968) report for a population in Northern BC, but contradicted earlier work by Garber (1956, 1958) who reported plants of both species to be diploid (2n=2x=14). Garber (1956, 1958) reported diploid status for the *C. parviflora* and *C. grandiflora* populations included in his study, however, the location of populations and the number of individual plants he sampled to arrive at this conclusion were not indicated. In the previous chapter I reported that four ploidy levels actually exist among populations sampled (Table 2-1). All BC populations had flow cytometric results consistent with tetraploidy, regardless of flower size, whereas the ploidy of US populations varied with location. Large-flowered populations growing in sympatry with small-flowered populations were all diploid, while allopatric populations were tetraploid. Small-flowered populations represented three levels of genomic content depending on location (Table 3-1).

The differences in floral and ploidy variation between the BC and US populations have made the taxonomic identity of the BC populations equivocal. Several possible factors may account for the unique flower size variation in BC. Although all the BC populations that have been examined are tetraploid, it is unknown whether they are allo- or autopolyploids. One possible explanation for the presence of intermediate flower sizes could be that the BC populations result from a history of hybridization between diploid *C. parviflora* and *C. grandiflora* followed by polyploidization and northward migration. Hybridization generally results in the production of offspring morphologically intermediate to their parent

species in the first generation followed by continuous range of phenotypes in subsequent generations (Grant 1981). Alternatively, BC populations may be autopolyploids. Given this scenario, it is possible that one or both species underwent polyploidization and northward migration. Because tetraploids have twice the genomic content of diploids, there are more alleles present for selection to act on and, therefore, opportunity for the production of more intra-specific phenotypic variation. In any case, the variation seen in BC appears to have arisen, at least in part, because of adaptation to local conditions. Previous research has shown that the shorter development times of small-flowered populations may be adaptive due to low water availability in these locations (Elle 2004). In addition, small-flowered plants gain a reproductive assurance benefit from selfing that is not realized by large-flowered plants (Elle and Carney 2003, Kennedy and Elle 2008).

Given the observed pattern of floral variation coupled with differences in ploidy and ecological conditions among flower sizes, this group of *Collinsia* is particularly interesting for studies of RI. Although several recent studies have examined total RI between plant taxa and the strength of the individual life stages contributing to it, only one such study (Husband and Sabara 2003) has included populations of different ploidy. The purpose of this research was to determine the degree of RI among populations of various flower size and ploidy from BC and the US. Although evidence from previous research has indicated that RI may not be directly associated with genetic divergence in all plant groups (Moyle et al. 2004, Scopece et al. 2007), the absence of RI between populations can

suggest a lack of divergence. Thus, determining patterns of RI can provide insight into species boundaries even in the absence of a direct relationship between RI and divergence.

My aim was to untangle the taxonomic relationships among morphologically variable populations of *Collinsia* from BC and the US. I performed reciprocal crosses among small and large-flowered populations from the US, among small, intermediate and large-flowered populations from BC, and between the populations of various flower sizes from BC and the US. I used methods proposed by Coyne and Orr (1989, 1997) and Ramsey et al. (2003) comparing fitness variables between intra- and inter-population crosses at several life stages, including both viability and fertility barriers, to calculate total intrinsic reproductive isolation between populations. Although many studies have shown that extrinsic prezygotic barriers make the strongest contribution to RI between closely related taxa (Ramsey et al. 2003, Kay 2006, Martin and Willis 2007), I focused on intrinsic barriers to reproduction because I was interested in determining the degree of genetic divergence among populations that, generally, are not likely to come into contact in nature. My specific goals were to determine: 1. If flower size variation is indicative of high levels of intrinsic RI among populations and 2. If BC populations show stronger RI with either of the two floral morphs that occur in the US.

3.2 Methods

3.2.1 Study System and Source Populations

I collected seeds from 40 plants per focal population (Table 3-1). Seeds were collected haphazardly from those plants retaining seeds at the time of collection (normally spaced >1m apart). Collections in the US were made in 2004 (western populations) and in Canada in 2005 (Table 3-1). Seeds from the MI population were provided from a bulk collection by S. Kalisz, University of Pittsburgh. Bulk seeds from the TG and TC populations (also provided by S. Kalisz) were planted due to the shortage of seeds available in our collections. Where possible I did not use the bulk-collected seeds as pollen recipients.

3.2.2 Initial Crossing Design

I planted seeds in 2006, staggering planting times to reflect the variation in time to sexual maturity between small and large-flowered plants (Elle 2004). Due to this variation in development time, populations were separated among different 48-cell flats. Where the identity of the maternal plant was known, seeds from different maternal families were planted in separate cells and marked accordingly to avoid making crosses among siblings. Growth conditions were initially 8 hr 20ºC light/16hr 10ºC dark, with daylight increased to 16hr eight weeks after the start of the experiment. To determine if differences in flower size observed among populations were due to genetic differences, I measured the width (across both banner petals) and length (from the tip of the keel to the base of the ovary) of the corolla of the first fully opened flower on each plant. These

data were compared to that collected from populations *in situ* (see Chapter 2, Methods).

For the crossing experiment, I selected individuals with large numbers of flower buds to be pollen recipients and moved them into a maternal flat eight weeks after germination. The remaining plants were designated as pollen donors and kept in a separate population-specific flat. In the crossing design below, I avoided using plants as pollen donors if they were related to pollen recipients (unknown for bulk samples from TG, TC, and MI)

I performed two experiments. Experiment 1 investigated the degree of RI between plants from large and small-flowered populations in the US. In this experiment, crosses were performed among plants from five populations comprising four different ploidy levels (TG = diploid, TC and TP = tetraploid, MI = 2C-DNA content < diploid, MV = 2C DNA content > tetraploid; see Table 2-1 in Chapter 2 for detailed information regarding ploidy). Experiment 2 was performed to determine the degree of reproductive isolation between populations of differing flower sizes within BC and between BC and US large and small-flowered populations. Among the five populations used in this experiment only two ploidy levels were included (EF, TC, JP and TL = tetraploid, MV = 2C DNA content > tetraploid). Each crossing group within an experiment included one plant from each of the populations to be inter-crossed. For the MV and TC populations, I selected pairs of siblings and assigned one sibling from each pair to each experiment. All crossing groups were rotated within the maternal flats and all flats were rotated around the environmental chamber daily to ensure that

no bias was introduced due to plant position within a flat or flat position within the chamber.

When maternal plants began to flower, I emasculated them in bud to prevent self-fertilization. I then waited 1-5 days for the stigma to become receptive (there is a visible change in stigma shape) before adding a mixture of pollen from 2-5 unrelated pollen donors with a clean toothpick. Pollen was added well in excess of the average ovule number (4.3-6.4 for the BC populations, Parachnowitsch and Elle 2004), to ensure that all ovules had an equal likelihood of being fertilized. This was repeated for each of the populations within the crossing group in haphazard order, depending on pollen availability. Where possible I did not use the same combination of pollen donors to pollinate more than one maternal plant per population. Maternal plants were checked daily for swelling of the ovary indicating seed capsule formation. Mature seed capsules were harvested and F1 seeds were counted and stored in paper envelopes for 2-6 months before planting.

3.2.3 Measurement of Fitness Components

Three seeds per family (where available) from all cross types were planted within one week in May 2007 in 48-cell flats (1037 seeds planted in total). Rearing conditions were identical to the parent generation. I used a stratified random planting design; each flat contained randomly arranged offspring of a single maternal population. Date of germination, survival to flowering and date of first flower were recorded for each F1 that germinated, and averaged among surviving family members to give one response value per family.

F1 Fertility was calculated as the average of % ovule viability and % pollen viability. To determine ovule viability I divided the number of seeds produced from the first flower after assisted self pollination (by wiggling a toothpick inside the corolla tube once the stigma was receptive and the pollen mature) by the number of ovules per bud, again averaging across members of a maternal family to produce a single response value per family. The second and third buds produced were collected and stored in 70% ethanol until counted, and a randomly selected bud was dissected and ovules counted. In cases where only one offspring within a family survived to flowering I counted both buds and took the average.

Pollen viability was measured as an indicator of fertility for each plant that survived to flower and averaged across survivors within a family. Methods for determining pollen viability were adapted from Kearns and Inouye (1993). I collected whichever two anthers were freshly dehisced from the four present in each flower, soaked them in 15μL 0.05% lactophenol-aniline solution for at least 3 hours and then vortexed samples for 3 minutes to suspend the pollen grains in the stain solution. I added 5μL of the cell suspension to a clean microscope slide and counted the number of stained (viable) and unstained (inviable) pollen grains along transects running the length of the cover slip. I counted a minimum of 200 pollen grains per sample unless the total number of grains was less than 200; if this occurred then I scanned the entire slide and counted as many pollen grains as possible. Pollen viability was calculated as the number of viable grains divided by the total number of pollen grains counted. In cases where pollen

viability was found to be below 0.20, I did not include ovule viability data in analyses. Because assisted self-pollination was utilized to form these crosses, in cases of low pollen viability, it was not possible to determine if reduced seed production was due to reduced pollen viability or reduced ovule viability.

3.2.4 Statistical Analysis

All statistical analyses were performed in SAS 9.1.3. To determine if the pattern of flower size differences among populations was similar between plants measured in the field and those grown in a common environment I conducted a Spearman rank correlation analysis. The MI population was not included in this analysis, as *in situ* measurements were not available.

To determine if variation existed among cross types for the four fitness components (life stages) used to calculate RI (seed production, germination, survival, and fertility) and how such variation was affected by parental populations, I performed a multivariate analysis of variance (MANOVA) with maternal population, paternal population and maternal x paternal population as main effects. I followed the multivariate analysis with univariate ANOVA to determine the effect of the specific combination of parental populations on the variation in the four individual fitness components measured and used Ryan-Einot-Gabriel-Welsch Multiple Range Tests to determine which combinations were statistically different from each other. To eliminate heteroscedasticity, seed production was square root transformed and germination and survival were arcsin square root transformed for both experiments (Sokal and Rolph 1995).

Calculation of total RI was based on methods from Coyne and Orr (1989, 1997) and Ramsey et al. (2003) but I compared mean fitness at four life stages to the mid-parent value to determine relative fitness rather than making comparisons to a single parental population. For this experiment, my interest was in determining the intrinsic barriers between populations that are separated by geographic distances that are likely beyond the limits of pollen and seed dispersal for these species. Given that I had no *a priori* hypothesis about the actual direction of gene flow between populations or the degree of maternal and paternal affect on fitness components, and that I expected among-population differences in the fitness components to bias results (Elle 2004), it was more appropriate to calculate the relative fitness of between-population crosses using mid-parent values rather than either the maternal or paternal population.

Life stages included in this experiment were seed production from the initial cross, germination of F1 seeds, survival to flowering of F1 seedlings and F1 fertility. The individual component of total RI was calculated at each life stage by subtracting the ratio of mean fitness of the between population cross to the mid-parent fitness value from one (Equation 1: n = life stage, W = mean fitness, W_{bar} = mean mid-parent fitness).

$$(1) \qquad RI_n = 1 - \left[\frac{W_{n\ between}}{\overline{W}_{n\ within\ population}} \right]$$

The absolute contribution of each life stage to total RI (Equation 2: AC = absolute contribution to total RI, n = life stage) and total RI (Equation 3: T = total RI, m =

total number of life stages included) were calculated according to equations outlined in Ramsey et al. (2003).

$$(2) \qquad AC_n = RI_n\left(1 - \sum_{i=1}^{n-1} AC_i\right)$$

$$(3) \qquad T = \sum_{i=1}^{m} AC_i$$

To calculate 95% confidence intervals around the total RI values, I re-sampled the data with replacement and recalculated RI 1000 times using a program written in R.

3.3 Results

3.3.1 Genetic Basis of Flower Size

Significant, positive rank correlations were found between plants grown in the field and those grown in the growth chamber for both corolla length (Spearman's rho=0.96, $P<0.001$, N=7) and width (rho=1.00, $P<0.0001$, N=7) indicating that the general pattern of flower size variation among populations did not differ between growth environments (Figure 3-1). This result indicates that among-population flower size differences have a strong genetic component, and although the environment may contribute to phenotype, it is not the root cause of inter-population variation.

3.3.2 Experiment #1

For crosses among small and large-flowered populations from the US, maternal ($F = 2.88$, $P < 0.001$) and paternal ($F = 1.75$, $P < 0.05$) population and

the interaction between the two ($F = 4.17$, $P < 0.0001$) had significant effects on the fitness components measured (seed production, F1 germination, survival and fertility) as evaluated with MANOVA. Univariate tests revealed that the flower size of the maternal population significantly affected seed production (Table 3-2) such that crosses made with small-flowered maternal plants tended to result in higher seed production than crosses using large-flowered maternal plants (Figure 3-2A). Mean seed production across all crossing combinations where the maternal plant was small-flowered was 1.19 ± 0.07(SE) while crosses using large-flowered maternal plants resulted in mean seed production of 0.41 ± 0.08(SE). No significant differences in the later F1 life stages of germination or survival to flowering were observed among crosses, but fertility was strongly affected by both parental populations individually and the interaction of the two (Table 3-2). Fertility tended to be higher in crosses between parental populations of similar ploidy compared with those between parents of disparate ploidy (Figure 3-2B). Mean fertility across all within a ploidy crossing combinations was 0.62 ± 0.04(SE), whereas, mean fertility for crosses between ploidy levels was only 0.28 ± 0.03(SE). There was also a trend, although less consistent, for crosses between populations of the same flower size to have higher fertility than crosses between populations of different flower size (Figure 3-2B). Mean fertility across all within flower size crossing combinations was 0.52 ± 0.04(SE), whereas, crosses between flower sizes resulted in a mean fertility of 0.34 ± 0.04(SE).

3.3.2.1 Reproductive Isolation

Total RI among populations was generally high (RI >0.50 in 90% of cross types), but tended to be more variable in crosses where the maternal population was small rather than large-flowered (Figure 3-3A; Pairs 2-7, Cross 2; Pairs 8-10, both crosses). There was also increased variation in the relative contribution of later life stages to total RI when small-flowered populations were maternal parents.

Crosses Between Ploidy Levels

Crosses were made between populations of different ploidy level both within and between flower sizes. Crosses between the two large-flowered populations resulted in total RI greater than 0.90 in both directions of cross (Figure 3-3A; Pair 1). This result was primarily driven by large reductions in seed production (>80%) compared to mid-parent values (Figure 3-3A; Pair 1). Of 21 total crosses between TC and TG, only two were successful, each producing a single seed that survived to reproductive maturity.

RI for crosses between both different ploidy level and flower size differed depending whether the maternal plant was small or large-flowered. When large-flowered maternal plants were crossed with small-flowered paternal plants RI was consistently high (>0.90 in all but one instance) regardless of the specific combination of ploidy levels (Figure 3-3A; Pairs 2, 3, 5-7, Cross 1). Reduced seed production was the primary barrier contributing to RI in all of these crosses. For crosses where small-flowered plants were maternal and large-flowered plants were paternal RI was still relatively high although the confidence intervals tended

to be much larger indicating greater variation among individual crosses within a cross type (Figure 3-3A; Pairs 2, 3, 5-7, Cross 2). Only the cross between MV and TC (the two higher ploidy levels) significantly differed in total RI between reciprocals (Figure 3-3A; Pair 3). Crosses between small-flowered maternal and large-flowered paternal populations generally differed from the reciprocal crosses in that later life stages, particularly fertility of the F1s, tended to be more important drivers of RI than seed production (Figure 3-3A; Pairs 2, 3, 5-7, Cross 2).

Crosses Within Ploidy Levels

Within this experiment there was only one combination of populations crossed that shared the same ploidy level. TC and TP are large and small-flowered populations, respectively, and crosses between them showed much stronger asymmetry than that seen for crosses between flower sizes that also differed in ploidy (described above). When the large-flowered population was maternal and the small paternal I observed complete RI, but in the reverse direction total RI was significantly lower (Figure 3-3A; Pair 4). Similar to the crosses between ploidy levels, total RI was due entirely to reduced seed production when large-flowered plants were maternal and F1 fertility was a much more important barrier when small-flowered plants were maternal (Figure 3-3A; Pair 4).

3.3.3 Experiment #2

Crosses among BC and US populations produced significant differences in fitness responses depending on maternal population ($F = 15.70$, $P < 0.0001$),

paternal population ($F = 6.52$, $P < 0.0001$) and the interaction of the two ($F = 7.35$, $P < 0.0001$). Univariate tests demonstrated a significant effect of maternal population on seed production (Table 3-3) such that crosses using maternal plants from populations with small or intermediate flower sizes tended to produce more seeds than those with large-flowered maternal plants (Figure 3-4A). Mean seed production was 1.95 ± 0.08(SE) across all combinations using maternal plants of intermediate flower size, 1.80 ± 0.06(SE) for crosses using small-flowered maternal plants and 1.21 ± 0.09(SE) for crosses using large-flowered maternal plants. There was also an interaction effect between parental populations such that large-flowered maternal plants crossed with small-flowered paternal plants resulted in significantly reduced seed set (Figure 3-4A, Last four columns). Again, no significant differences in F1 germination or survival to flowering were observed, but fertility was strongly affected by both parental populations and their interaction (Table 3-3). Fertility was generally high (>70%) and did not significantly differ among crosses between populations where both parents were of the same ploidy (Figure 3-4B). All crosses between the tetraploid populations and the diploid MV population, whether MV was maternal or paternal, resulted in significantly reduced fertility (Figure 3-4B). Mean fertility across all within ploidy crossing combinations was 0.84 ± 0.01(SE), while crosses between ploidy levels had a mean fertility of 0.31 ± 0.01(SE).

3.3.3.1 Reproductive Isolation

RI tended to vary much more among crosses between the BC and US populations than what was observed among the US populations in Experiment 1

(RI >0.50 in only 55% of cross types). Variation among cross types appeared to be dependant on a combination of influences including parental flower size, ploidy and the direction of cross.

Crosses Between Ploidy Levels

As in Experiment 1, crosses in this experiment were made both within and among flower sizes between populations of differing ploidy level. The only populations crossed that differed in ploidy but were of similar flower size were MV and TL, which are both small-flowered. Total RI was moderately high (0.68-0.79), but did not differ significantly between the reciprocal crosses in this case (Figure 3-3B; Pair 10). Fertility of the F1s was a strong contributing barrier to RI in both directions of cross.

For crosses between flower sizes, significant difference in total RI between reciprocals was found for only one crossing group (Figure 3-3B; Pairs 2, 3, 9). The most important life stage contributing to total RI differed among reciprocal crosses between large and small-flowered populations. For crosses where large-flowered populations were maternal, reduced seed production was the most important barrier while F1 fertility was generally more important when small-flowered populations were maternal (Figure 3-3B; Pairs 2, 3). However, for crosses between the populations with intermediate and small flower size (JP and MV), F1 fertility made a strong contribution to total RI in both directions of cross (Figure 3-3B; Pair 9).

Crosses Within Ploidy Level

In this experiment, crosses within ploidy level were made both within and between flower sizes, allowing for comparison. Only one combination of populations with both similar ploidy and flower size were crossed (EF and TC). The reciprocal crosses for this large-flowered pair did not differ from an RI of 0.0 in either direction of cross, indicating total crossability between these populations (Figure 3-3B; Pair 1).

Total RI was considerably more variable for crosses between flower sizes. Although significant differences between reciprocals for crosses between small and large flower sizes were found for only one crossing group (EF and TL), the large difference observed in total RI between the reciprocals of the other between-flower size cross (TC and TL), combined with wide confidence intervals, indicates that significant differences may exist, but due to the high variance between individual crosses the sample size would have to be increased to capture such a difference (Figure 3-3B; Pairs 4, 5). Unlike the between-ploidy crosses (described above), in both directions of cross between small- and large-flowered populations seed production was the most important life stage contributing to total RI (Figure 3-3B; Pairs 4, 5). F1 fertility did not make an important contribution to RI in either direction of cross. No significant difference in RI was found between any of the reciprocal crosses involving the population with intermediate-sized flowers (JP; Figure 3-3B; Pairs 6-8).

3.4 Discussion

At the outset of this experiment, I expected that if flower size was an effective indicator of species boundaries as described in Douglas et al. (2000) then RI among populations should reflect flower size variation. Based on their measures of RI between sympatric species, Coyne and Orr (1989, 1997) hypothesized that to prevent the collapse of species barriers upon secondary contact mean total RI must be greater than 0.903. I therefore expected that RI values between populations of different flower size should be high (>0.90) and those between populations of similar flower size should be lower (<0.90). My results did not correspond to this expectation. RI values varied markedly both within and between flower sizes indicating that some other trait may be important in predicting RI between populations. However, it should be noted that the threshold of 0.90 deduced by Coyne and Orr (1989, 1997) was meant to be applied to studies including both extrinsic and intrinsic pre- and post-zygotic barriers and may, therefore, be a conservative value for this study.

The production of polyploids is widely thought to be a significant factor in the creation of new species (Ramsey and Schemske 1998, Otto and Whitton 2000, Husband and Sabara 2003). The populations I chose from BC were among those already described as tetraploid by Ganders and Krause (1986), but the ploidy of the US populations was uncertain at the onset of the research. If variation in ploidy did exist between populations then I expected high RI values would result regardless of similarities in flower size (Levin 1978, Ramsey and Schemske 1998, Husband and Sabara 2003). For crosses both within and

between flower sizes, I found that differences in ploidy were more indicative of RI between populations than flower size.

3.4.1 Crosses Between Ploidy Levels

Crosses made between populations of different ploidy generally resulted in high RI values, which did not differ between reciprocals regardless of the flower sizes involved in the cross. For crosses both within and between flower sizes, I found a difference in the life stage that represented the strongest contribution toward total RI. Crosses between the two large-flowered populations of different ploidy and those between large-flowered maternal populations and small-flowered paternal populations resulted in a major decrease in seed production relative to mid-parent values. Crosses among small-flowered populations and between small-flowered maternal and large-flowered paternal populations were generally more likely to produce seeds that germinated and formed plants that survived to maturity. However, high levels of infertility in these offspring resulted in high overall RI values. Reduction in seed production due to abnormalities in endosperm development and infertility of the F1 offspring due to meiotic irregularities are both likely outcomes when crosses are made between plants of different ploidy (Ramsey and Schemske 1998). Reduction in fertility is common in the offspring of between ploidy matings due to the formation of multivalents that result in errors in chromosome pairing during meiosis (Ramsey and Schemske 1998).

3.4.2 Crosses Within Ploidy Level

Across both experiments, only the tetraploids were represented by more than a single population. The degree of RI between tetraploid populations varied considerably depending on the flower sizes involved in the cross. Unfortunately, only one combination of populations included in this experiment belonged to both the same ploidy and flower size class—the two large-flowered populations EF and TC. Crosses between these populations resulted in RI values that were not significantly different from zero indicating complete crossability. From this result, it appears clear that, at least, the large-flowered tetraploids are members of the same species.

Crosses between large and small-flowered tetraploid populations resulted in asymmetric RI. When large-flowered populations were maternal and small-flowered paternal, RI values were consistently high, but the reciprocal crosses tended to result in significantly lower levels of RI. Although significant differences between reciprocals was not observed for crosses between both ploidy and flower size as for crosses within ploidy and between flower size, there was a clear difference in the production of seeds depending on the direction of cross (described above). Possible explanations for the disparities in seed production between reciprocal crosses include genetic differences in fecundity, post-mating prezygotic differences in the ability of foreign pollen to germinate or grow pollen tubes to achieve ovule penetration and postzygotic differences in fruit abortion (Tiffin et al. 2001, Coyne and Orr 2004). Because I controlled for the fecundity differences between flower sizes by comparing inter-population crosses to the mid-parent values at all life stages this explanation is unsatisfactory.

Unfortunately, I have no data to determine whether the barrier to seed production was pre- or postzygotic.

It is possible that the asymmetries observed in RI are due to the inability of the pollen tubes formed by small-flowered pollen to grow long enough to reach the ovule of the large-flowered plants. There are dramatic differences in style length between plants from large and small-flowered populations. The large-flowered plants can have styles over 1cm long while the styles of small-flowered plants tend to be only 2-3mm long depending on the population (personal observation). Research in several genera of angiosperms has shown that seed production can be significantly reduced due to the failure of pollen from short-styled species to grow pollen tubes long enough to penetrate the ovule of long-styled species (Williams and Rouse 1988 and 1990, Tiffin et al. 2001, Coyne and Orr 2004, Lee et al. 2008). Further evidence supporting this mechanism is the fact that crosses between the intermediate sized flowers (JP), and the small and large-flowered populations of the same ploidy did not result in the asymmetry seen in the small/large-flowered combinations. Williams and Rouse (1990) found that when species of *Rhododendron* of intermediate style length were crossed with species of incompatible short and long style lengths seed production was not significantly different between inter- and intra-specific crosses. A comparison of pollen germination and tube growth from plants of different flower sizes on the pistils of plants from small and large-flowered populations should be performed to determine if one of these pre-zygotic barriers

is responsible for reduced seed production in crosses between small-flowered maternal and large-flowered maternal *Collinsia*.

3.4.3 Implications for Collinsia Taxonomy

My results suggest that taxonomic identification is significantly more complicated in this group than previously thought. Simple categorization of species based on flower size is not possible.

In the US, where I expected small and large-flowered populations to clearly fall into two separate diploid species, *C. parviflora* and *C. grandiflora*, I found variable ploidy and strong RI between populations within size classes. If species boundaries are indicated by a total RI value of 0.90 or greater, the two large-flowered US populations used in this experiment are separate species, as are some of the small-flowered populations (TP/MI and MI/TP had RI values not significantly different from 0.90 in both directions of cross while MV/TP had RI values lower than 0.80). Although crosses between flower sizes among the US populations resulted in strong isolation when large-flowered plants were maternal, crosses between populations with shared ploidy tended to have significantly weaker isolation in the reciprocal direction of cross. Because species which have asymmetric RI may be more likely to interbreed upon secondary contact compared to species with bilateral RI (Tiffin et al. 2001), even the high RI values observed in one direction of cross may not be indicative of actual species barriers that will be maintained after reintroduction.

In BC, crosses among populations generally resulted in low RI values, again with the exception of those between the large-flowered maternal and small-

flowered paternal populations. This low level of RI combined with the fact that all BC populations were found to be tetraploid indicates that populations in BC are likely all members of a single, morphologically variable species.

None of the crosses between BC and US populations resulted in RI values above 0.90, however relatively strong reciprocal barriers (RI>0.70) were found between all BC populations and the small-flowered US population (MV). Crosses between BC populations and the large-flowered US population (TC) generally resulted in relatively low isolation (RI<0.10) except in cases where large-flowered plants were maternal and small-flowered plants paternal. These results considered along with the high RI value between the two large-flowered populations in the US indicate that the TC population is likely closely related to the BC populations of all flower sizes, and that BC populations are likely reproductively isolated from TG as well.

In conclusion, I find that there are multiple biological species within this complex, but that they do not correspond to the previously described taxa. I have found compelling evidence to suggest that differences in ploidy, rather than flower sizes, are indicative of species boundaries. There appears to be a single tetraploid species with considerable variation in flower size that occurs from CA to BC for which I have proposed re-classification as *C. variabilis* (Chapter 2). The diploid and strictly large-flowered species found only in the US fits the existing taxonomic classification of *C. grandiflora*. Within the US, small-flowered tetraploids and large-flowered diploids co-occur but are strongly isolated from each other. Among the small-flowered populations in the US, there are multiple

ploidy levels, which show some degree of RI from other populations and may represent cryptic species.

It is important to bear in mind that this research is based on only four reproductive barriers, all of which may be post-zygotic. The importance of including many barriers in analysis of RI between populations has been stressed repeatedly in the literature (Coyne and Orr 1989, 1997, Husband and Sabara 2003, Ramsey et al. 2003, Kay 2006, Rieseberg and Willis 2007, Lowry et al. 2008, Widmer et al. 2009). However, the fact that this research was based on post-zygotic barriers and that prezygotic barriers have generally been found to make stronger contributions to total isolation, both within- and between-ploidy levels (Husband and Sabara 2003, Rieseberg and Willis 2007, Lowry et al. 2008, Widmer et al. 2009 but see Costa et al. 2007), suggests that my estimates of RI are conservative.

Given the existing ploidy variation among these populations and the inconsistency of the relationship between RI and genetic distance in flowering plants (Moyle et al. 2004, Scopece et al. 2007, 2008) it is important to consider that RI may not accurately reflect genetic divergence between members of this complex. Further research will be necessary to determine the genetic relationships among these morphologically variable populations.

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3.6 Tables

Table 3-1 Populations of *Collinsia* used in this study with their location, geographical grouping, flower size, and ploidy.

Population	Abbreviation	Location	Geographical Group	Flower Size	Ploidy
Elk Falls	EF	50º 02’34.9”N 125º 19’32.0”W	BC	Large	Tetraploid
Jack Point	JP	49° 09'29.9"N 123° 53'35.0"W	BC	Intermediate	Tetraploid
Thetis Lake	TL	48º 28’00.9”N 123° 27’58.6”W	BC	Small	Tetraploid
Michigan	MI	46º 52’24”N 87º 55’00”W	US	Small	2C-DNA <Diploid
Markleeville	MV	38° 41’36.8”N 119º 46’04.3”W	US	Small	2C-DNA >Tetraploid
Trout Creak	TC	44º24’02.2”N 122º 21’13.9”W	US	Large	Tetraploid
Table Rock grandiflora	TG	42º27’15.7”N 122º 57’01.0”W	US	Large	Diploid
Table Rock parviflora	TP	42º27’15.7”N 122º 57’01.0”W	US	Small	Tetraploid

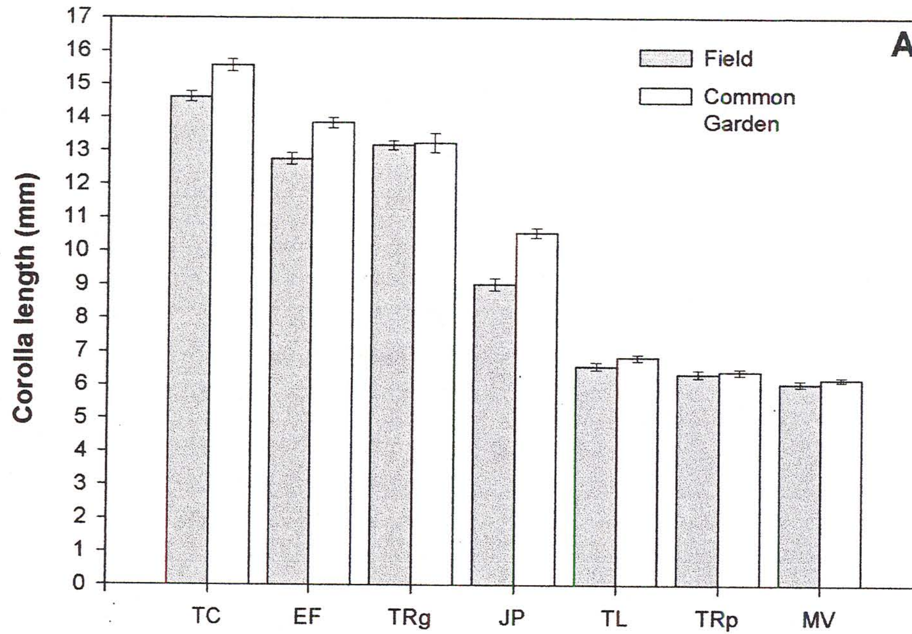
Table 3-2 Experiment 1. Analysis of variance testing the effects of maternal population, paternal population and the interaction of maternal and paternal population on seed production, F1 germination, F1 survival to flowering, and F1 fertility for 5 *Collinsia* populations of varying flower size from the US. Seed production was square root transformed and germination and survival were arcsine - square root transformed.

Source of Variation		Seed Production			Germination			Survival			Fertility		
	DF	MS	F	p	MS	F	p	MS	F	p	MS	F	p
Maternal Population	4	0.882	4.42	0.0026	0.030	0.50	0.7337	0.030	0.68	0.6100	0.123	6.25	0.0002
Paternal Population	4	0.192	0.96	0.4341	0.058	0.97	0.4304	0.022	0.48	0.7496	0.104	5.27	0.0007
Maternal x Paternal	12	0.278	1.39	0.1845	0.081	1.33	0.2133	0.021	0.47	0.9297	0.461	23.45	<0.0001
Error	92	0.200			0.060			0.045			0.020		

Table 3-3 Experiment 2. Analysis of variance testing the effects of maternal population, paternal population and the interaction of maternal and paternal population on seed production, F1 germination, F1 survival to flowering, and F1 fertility for 5 Collinsia populations of varying flower size from BC and the US. Seed production was square root transformed and germination and survival were arcsine - square root transformed.

Source of Variation		Seed Production			Germination			Survival			Fertility		
	DF	MS	F	p	MS	F	p	MS	F	p	MS	F	P
Maternal Population	4	1.942	12.64	<0.0001	0.012	1.33	0.2586	0.024	1.18	0.3189	0.831	65.84	<0.0001
Paternal Population	4	0.352	2.29	0.0617	0.002	0.27	0.8940	0.029	1.46	0.2152	0.324	25.67	<0.0001
Maternal x Paternal	16	0.588	3.82	<0.0001	0.007	0.83	0.6470	0.021	1.05	0.4071	0.528	41.84	<0.0001
Error	193	0.154			0.009			0.200			0.013		

3.7 Figures



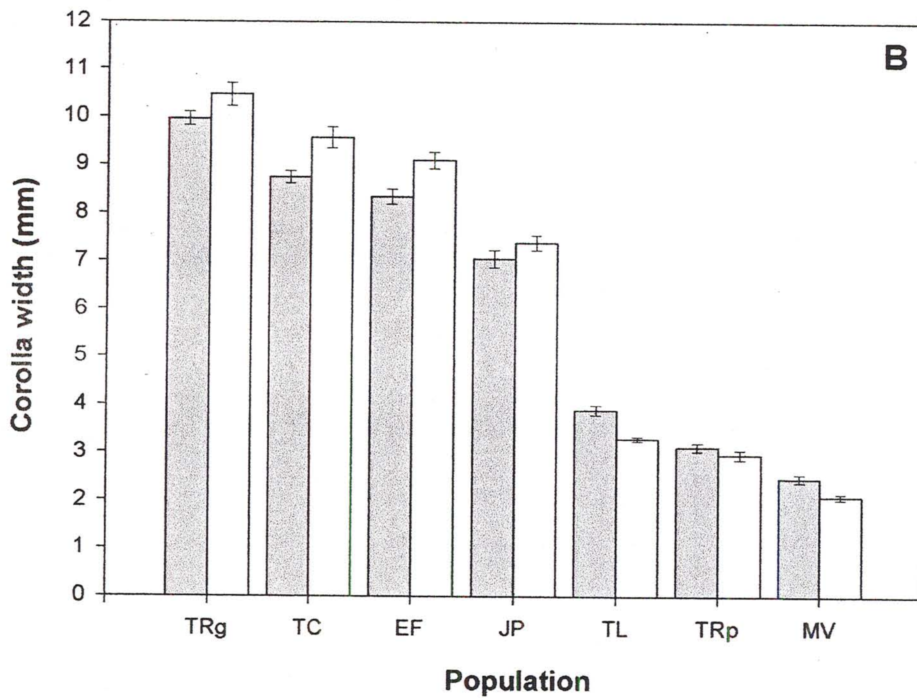


Figure 3-1 Variation in mean corolla length (A) and width (B) ± SE among plants measured from 7 *Collinsia* populations in situ and grown in a common environment. Data were square root transformed for analysis to correct for heteroscedasticity; untransformed data are shown here.

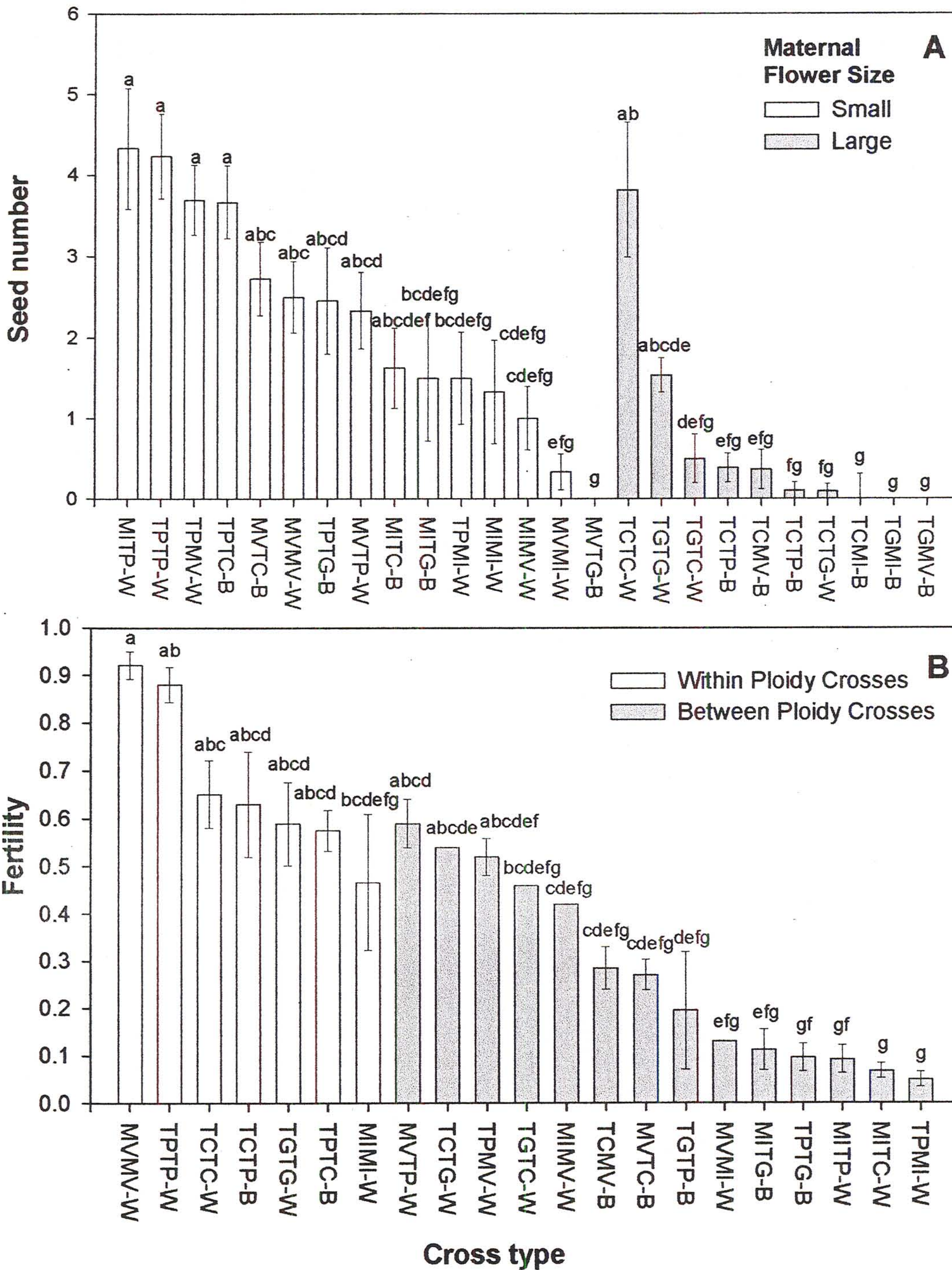
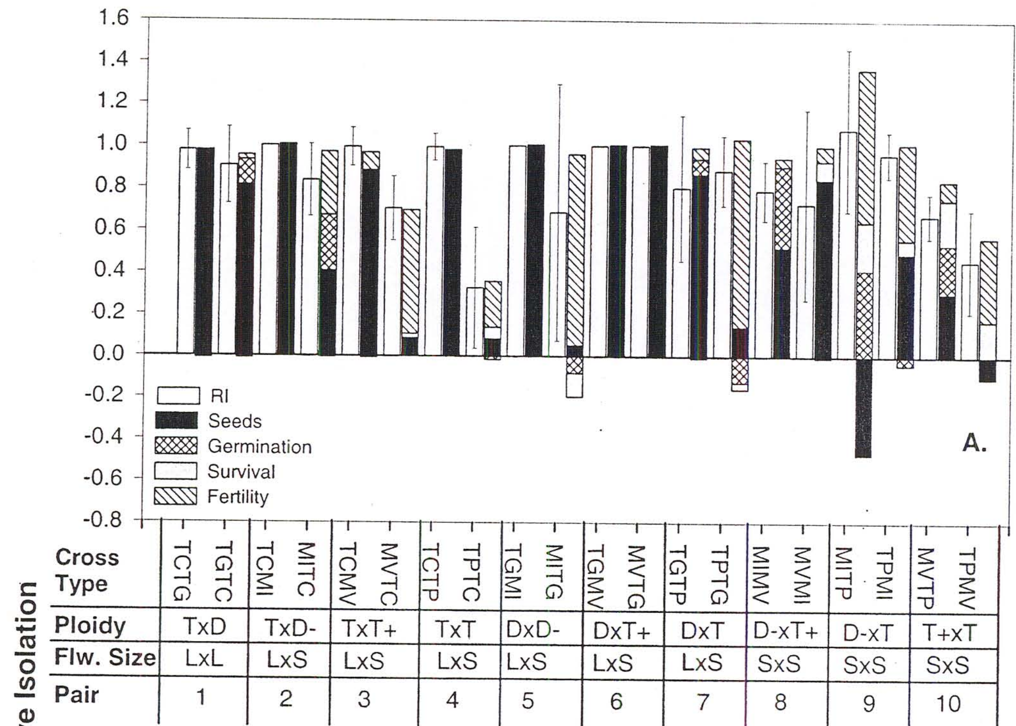
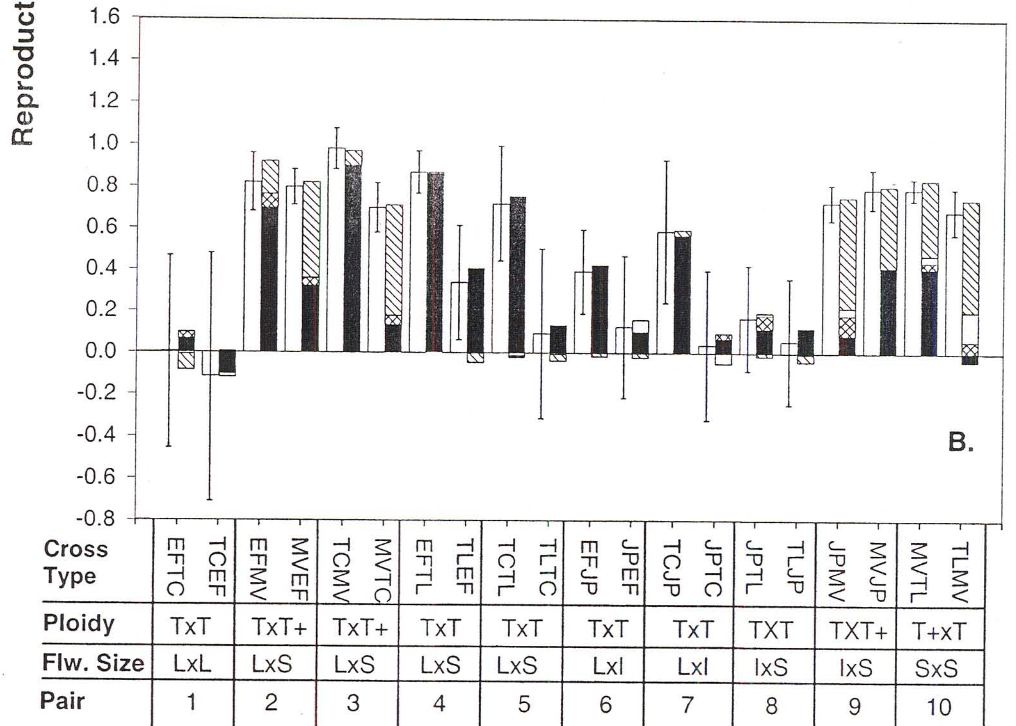


Figure 3-2 Experiment 1: Mean seed production (A) and mean fertility (B) ± SE for 21 populations of *Collinsia*. Maternal parent is listed first. In column labels, W = cross within flower size; B = cross between flower sizes. Columns with the same letter are not significantly different from each other. Seed production data were square root transformed to correct for heteroscedasticity; untransformed data are shown here.

Figure 3-3 Reproductive isolation and relative contributions of individual reproductive barriers to total isolation among populations of *Collinsia* of varying flower size and ploidy from the US, Experiment 1 (A) and from BC and the US Experiment 2 (B). Error bars represent 95% confidence intervals. The stacked columns represent the relative contribution of each life stage to total RI based on calculations made from the collected data. Because the white columns represent bootstrap estimates of total RI, the value of total RI may differ between the white columns and the stacked columns. Cross types are listed maternal plant first. Ploidy and flower size are listed as maternal x paternal plant for the first cross listed; reciprocal crosses are the reverse order. Ploidy codes: D=diploid, D- =2C-DNA < diploid, T=tetraploid, T+ =2C-DNA > tetraploid. Flower size codes: S = small, L = large, I = intermediate. Pairs of reciprocal crosses are grouped by pair number. The first cross in each pair will be referred to as Cross 1 and the second, Cross 2.


Reproductive Isolation
RI
Seeds
Germination
Survival
Fertility
A.
Cross Type
Ploidy
Flw. Size
Pair
TCTG TGTC TCMI MITC TCMV MVTC TCTP TPTC TGMI MITG TGMV MVTG TGTP TPTG MIMV MVMI MITP TPMI MVTP TPMV
TxD TxD- TxT+ TxT DxD- DxT+ DxT D-xT+ D-xT T+xT
LxL LxS LxS LxS LxS LxS LxS SxS SxS SxS
1 2 3 4 5 6 7 8 9 10


B.
Cross Type
Ploidy
Flw. Size
Pair
EFTC TCEF EFMV MVEF TCMV MVTC EFTL TLEF TCTL TLTC EFJP JPEF TCJP JPTC JPTL TLJP JPMV MVJP MVTL TLMV
TxT TxT+ TxT+ TxT TxT TxT TxT TXT TXT+ T+xT
LxL LxS LxS LxS LxS Lxl Lxl IxS IxS SxS
1 2 3 4 5 6 7 8 9 10

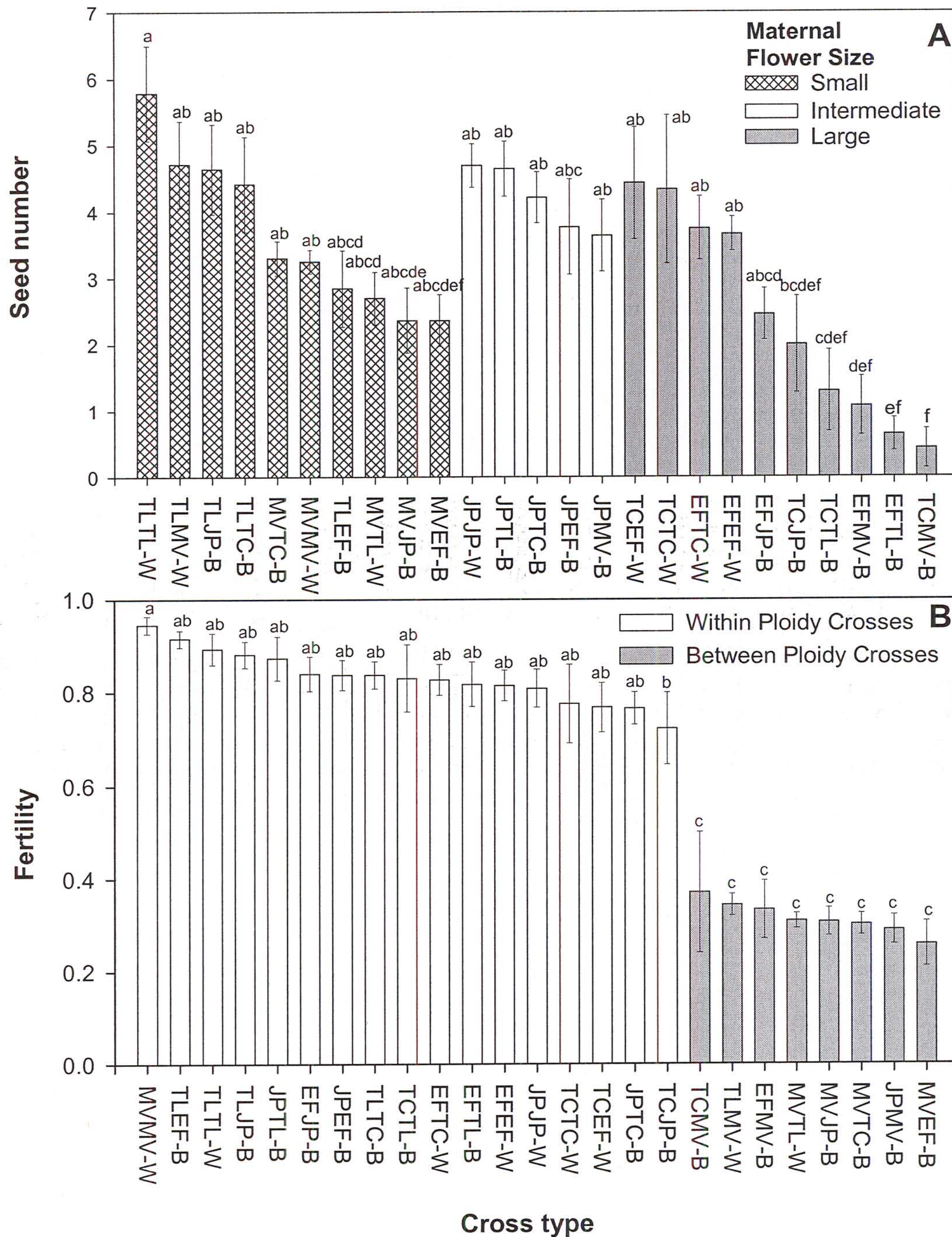


Figure 3-4 Experiment 2: Mean seed production (A) and mean fertility (B) ± SE for crosses among 25 populations of *Collinsia*. Maternal parent is listed first. In column labels, W = cross within flower size; B = cross between flower sizes. Columns with the same letter are not significantly different from each other. Seed production data were square root transformed to correct for heteroscedasticity; untransformed data are shown here.

4: CHAPTER FOUR: GENERAL DISCUSSION

Speciation occurs when morphological, physiological, genetic, or genomic differences between groups cause barriers to the production of viable, fertile offspring. Variation in floral morphology is a particularly important factor in the evolution of reproductive isolation in plants because it is directly linked to reproductive success. Polyploidization also plays an important role in plant evolution because changes in ploidy result in the immediate production of strong reproductive barriers and can result in nearly instantaneous speciation (Stebbins 1950). The purpose of this research was to quantify flower size and ploidy variation among populations of small and large-flowered *Collinsia* occurring in the western region of North America from BC to CA and to determine how this variation affects inter-population reproduction and, thus, species barriers.

In Chapter Two, I showed that the populations of *Collinsia* included in this study are comprised of multiple ploidy levels and a continuous array of flower sizes that do not strictly conform to the two previously described small and large-flowered species, *C. parviflora* and *C. grandiflora*. I found a large group of tetraploid populations that occur throughout the range and exhibit continuous, genetically based variation in flower size, one group of large-flowered diploid populations that co-occur with small-flowered tetraploid populations, one small-flowered population with a 2C-DNA content lower than that of the diploids and two other small-flowered populations with 2C-DNA content higher than that of the

tetraploids. Based on these differences in genomic content and the known difficulties in reproduction between plants of different ploidy (Stebbins 1950) I concluded that the taxonomy of this section of the genus *Collinsia* should be re-evaluated to classify species based on differences in genomic content rather than flower size. I recommended that a new species, *C. variabilis,* be added to the section to recognize the morphologically variable tetraploid group.

In Chapter Three I described the results of two crossing experiments conducted using plants grown from seed collected from populations throughout the study area and representing three flower size classes (small, intermediate and large) and all four levels of genomic content. The purpose of these experiments was to determine if populations of varying flower size and ploidy are separate biological species. Based on the results I concluded that populations with different ploidy or 2C-DNA content are strongly reproductively isolated from each other. A combination of hybrid inviability and infertility contributed to this isolation suggesting that when ploidy differs among populations, they are distinct biological species, as predicted in Chapter Two.

All of the BC populations included in this study were found to be tetraploid and, those that were included in the reciprocal crossing experiment, were not significantly isolated from tetraploid populations in the US—within a common flower size class. However, RI was found to be asymmetric when large-flowered tetraploids were crossed with small-flowered tetraploids. When large-flowered plants were maternal, RI was high due to reduced seed production, but the in the

reciprocal crosses RI was much lower due to relatively higher levels of seed production.

When asymmetric RI is found between populations, theory predicts that it is more likely that RI will break down than prezygotic isolating factors will arise upon secondary contact (Tiffin et al. 2001). In this situation genes are predicted to introgress directionally from the population which is more resistant to accepting foreign gametes (in this case, large-flowered plants) into the less resistant population (small-flowered plants) (Tiffin et al. 2001). It is possible that, given the asymmetry in RI between small- and large-flowered tetraploids, we could see a collapse of the reproductive barriers upon secondary contact. However, the strength of prezygotic isolation due to mating system differences between flower sizes may slow this process. Because large-flowered plants are thought to be primarily outcrossing (Kennedy and Elle 2008a), it might be expected that their genes would introgress into the small-flowered group, however, a problem exists with this hypothesis. Due to the demonstrated preference of pollinators for large-flowered plants and the fact that small-flowered plants have been shown to be strongly dependent on the reproductive assurance value of selfing (Elle and Carney 2003, Kennedy and Elle 2008b), it is not clear that cross pollination would occur at a frequency high enough to promote the collapse of reproductive barriers. Given the challenges of overcoming this mating system barrier between flower sizes, I predict that some degree of asymmetric isolation would probably persist between small- and large-flowered plants even upon secondary contact.

The large-flowered, US, diploid population, TRg, was found to be diploid and to fit the morphological characters describing *C. grandiflora*. The upper confidence limits of the RI values for all crosses involving these diploids surpassed the 0.90 threshold proposed by Coyne and Orr (1989, 1997), indicating total isolation and likely distinct species status. I also observed that all diploid plants occurred in sympatry with small-flowered tetraploid populations. In spite of this close proximity, no intermediate floral forms were found, indicating that if hybridization does occur, the hybrids do not survive to maturity.

Assessing the species status for the small-flowered populations with genomic content falling outside the range of either diploid or tetraploid values is somewhat more problematic. Total RI surpassed the 0.90 threshold for all crosses involving the MI population indicating that it may be a separate species from both the diploid and tetraploid populations with which it was crossed. However, as only a single population was surveyed from outside the Pacific Coast region it is not possible to determine if this population represents a larger interbreeding group or just a few aberrant individuals.

Crosses between tetraploids and the higher ploidy population, MV, resulted in somewhat more relaxed RI. Although RI was over 0.90 when crosses were made between MV pollen donors and large-flowered tetraploid maternal plants, the reciprocal crosses (as well as crosses between MV and small and intermediate flower sizes) resulted in moderate RI (generally about 0.70 with upper confidence limits not crossing the 0.90 threshold). This increase in RI for crosses where large-flowered plants are maternal and small-flowered paternal

was also seen in crosses among tetraploids and may be due to the mechanical incompatibilities between short pollen tubes and long styles described in Chapter 3. The degree of isolation between MV and populations of different ploidy level is certainly enough to cause a reproductive disadvantage for mixed compared to within cytotype matings, however, a sizable proportion of offspring with at least partial fertility would be produced. Given that the reproductive barriers are considerably "leaky" for crosses between MV and other cytotypes it is difficult to predict whether they would remain isolated upon secondary contact.

Although the results of this research do not answer the question of how ploidy and flower size variation evolved in this group of Collinsia, but they do help to generate some interesting hypotheses. At the end of Chapter 3, I suggested a potential pathway by which the diploids and tetraploids may have arisen. I hypothesized that tetraploids arose in sympatry with large-flowered diploids and were selected to be small and selfing to avoid inter-cytotype mating. I then suggested that the tetraploid populations expanded and colonized new environments where they evolved variation in flower size in response to selection imposed by local ecological conditions including pollinator preference and moisture availability. Although this hypothesis is plausible, other pathways may also have led to the variation in ploidy and flower size we see today. For example, it is possible that ploidy variation arose in allopatry and that the disparity in flower sizes observed at sympatric sites is due to reproductive character displacement upon secondary contact between diploids and tetraploids.

The hypothesis that flower size variation arose as a result of hybridization, as proposed in Chapter 2, is unlikely because the one diploid large-flowered population used in this experiment was totally isolated from all three small-flowered populations (RI not significantly different from 1.0 for all cross combinations). It is unlikely that hybridization could have occurred frequently enough to establish the continuously variable tetraploid group (including the BC populations). The alternative explanation, that the BC (and US tetraploid) populations arose as a result of polyploidization in a single species followed by migration and local adaptation is much more likely, given these results. It should be noted, however, that in spite of extensive sampling throughout the study area, I failed to find any small-flowered diploid populations. It is possible that if diploid *C. parviflora* historically occurred in sympatry with the large-flowered diploid populations, then allotetraploids could have been produced. The results of the crosses between EF/TL, TC/TL, and TC/TRP indicate that RI is only strong in one crossing direction when crosses are made between populations of different flower size within a ploidy level. If small-flowered tetraploids were able to out-compete the small-flowered diploids at the sympatric sites, then that may explain why we only see small-flowered tetraploids in sympatry with the large-flowered diploids.

In order to determine how the among population variation in this group actually evolved, molecular analysis would be required to determine the genetic relationships among populations. Armbruster et al. (2002) produced a phylogeny of the genus *Collinsia* indicating that the small- and large-flowered *C. grandiflora*

and *C. parviflora* were sister species, however, all sample plants used in the Armbruster study were collected from populations in CA. Understanding the relationships between the various ploidy and flower sizes in this group of *Collinsia* would require a significant expansion of the phylogeny to include populations from throughout the geographic range.

In conclusion, I have found that this section of the genus Collinsia represents a broadly variable group of populations that do not conform to the previously proposed taxonomy. It is clear from this work that a revision of the taxonomy accounting for the continuous nature of floral variation as well as the disparities in genomic content and reproductive compatibilities is required.

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