ECOLOGICAL AND EVOLUTIONARY FACTORS THAT INFLUENCE SPECIES BOUNDARIES IN *COLLINSIA*

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Understanding the factors that contribute to the origin and maintenance of species, and elucidating the mechanisms that influence species' distribution across the landscape are two goals that are fundamental to evolutionary biology and ecology. I combined field and laboratory experiments, a robust phylogeny, and species distribution data from herbaria to test a series of hypotheses that address variation in the distribution of species, and, the evolution and maintenance of reproductive isolating barriers, key components for understanding speciation. I used species in the genus *Collinisa* to test the following main hypotheses: 1) An extension of Baker's Law: Among similar aged sister-taxa pairs, species more proficient at autonomous self-fertilization should be better colonizers and thus should have larger range sizes than their sister-taxa that are less proficient at autonomous selfing, 2) Bateson-Dobzhansky-Muller (BDM) model of reproductive isolation: Intrinsic postzygotic isolation barriers increase as divergence time increases among species, and 3) Reinforcement of reproductive isolation: early selfing can evolve in response to heterospecific pollen receipt, and may thus act as a prezygotic reproductive isolating barrier that is reinforced in sympatry. I found that species most proficient at selfing had significantly larger range sizes than their sister-taxa that were less proficient at selfing. Thus, mating system did explain differences in the ranges sizes of similar aged sister-taxa. To address the second hypothesis, I first tested for allopatric speciation in this genus, and found strong support for allopatric speciation in the California clade of *Collinsia*. But I did not find strong support overall, likely because of large range-shifts in the northeastern clade, which obscured the

expected pattern of increasing range overlap with increasing divergence time. In support of the BDM model, I found that post-mating pre- and postzygotic isolation increased with increasing divergence time. And finally, in support of the final prediction, I found that when *C. rattanii* was sympatric with *C. linearis* it self-pollinated at a significantly earlier stage, suggesting that earlier selfing may be acting to reinforce of reproductive isolation in sympatry.

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PREFACE

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1.0 INTRODUCTION

Elucidating evolutionary processes that lead to the origin and maintenance of new species is of paramount importance for understanding the diversity of life on earth. Speciation, the process by which distinct lineages arise, is fundamentally related to the formation of isolating barriers (Mayr 1942). How isolating barriers between species arise and are maintained is a subject that has been of considerable interest to evolutionary biologists ever since Darwin (1859) established the framework for understanding the origin of biological diversity. Despite the importance of understanding the mechanisms that lead to reproductive isolation and eventually to speciation, the study of speciation lagged for many years, partially due to a lack of specific hypotheses and mechanisms for how patterns observed in the nature could be achieved via the genetic mechanisms discovered throughout the twentieth century (Coyne and Orr 2004). Seminal work by Dobzhansky (1937) and Muller (1940), illuminated our understanding of intrinsic postzygotic reproductive isolating barrires among species. The concept of how incompatibilities may accumulate between allopatric lineages through time, was first proposed by Bateson in (1909), but was forgotten until it was re-discovered by Orr in 1996 (Coyne and Orr, 2004). Recently, this area was further explored in a series of papers by Orr (1995) and Coyne and Orr (1989, 1997), who tested several of the predictions of Dobzhansky (1937) and Muller (1940) and examined how pre- and post-mating isolating barriers evolve through time across an entire group of related species. Their work had implications for determining which barriers (pre- or post-zygotic) were important in speciation, and which were the result of selection against the production of unfit hybrid offspring (reinforcement). Their book, entitled

Speciation (2004), was the first synthesis on speciation since Grant wrote *Plant Speciation* (1981) over twenty years prior. The work by Coyne and Orr strongly motivated my dissertation.

My dissertation work tackles a series of questions regarding how species' barriers arise among diverging plant taxa, how barriers to hybridization are maintained when species live in sympatry, and how mating system characteristics can act as reproductive isolating barriers and may help explain variation in the geographic range sizes of species. I integrate field, laboratory, and experimental greenhouse studies to examine a series of questions related to the evolution of reproductive isolation, the geographic distribution of species, and the geographic mode of speciation in a monophyletic group of annual plants species in the genus *Collinsia*. For my dissertation, I was fortunate to be able to make use of the phylogeny developed by Dr. Bruce Baldwin (University of California – Berkeley) in collaboration with Dr. Susan Kalisz and Dr. Scott Armbruster, and other resources related to their collaboration.

In Chapter 2, I address the role of mating system in explaining the variation in geographic range sizes of *Collinsia* species. Baker's Law (Baker 1955) states that species that are self compatible should be better colonizers than species are self-incompatible, because self-compatible species, particularly those that are proficient at autonomous self-pollination, are more likely to establish breeding populations after a founding event, even if that founding event only involves one individual. I test an extension of this idea, predicting that *Collinsia* species proficient at self-pollination will have larger ranges sizes than species that are not proficient at self-pollination. Although many studies on the ecological impacts of selfing simply use flower size as a proxy for selfing ability (assuming smaller flowered species are more likely to be selfers than larger flowered species), this assumption is often untested. Using six large-flowered, small-flowered sister-taxa pairs in *Collinsia,* I tested the idea that smaller flowered species were more

proficient selfers than large-flowered species and that smaller flowered species have larger geographic and elevational range sizes relative to their large-flowered sister taxa. I grew the twelve taxa in the greenhouse using field-collected seeds. I quantified a variety of metrics to document that the sister taxa truly differed in their flower sizes. I measured a series of floral developmental variables likely to be important to autonomous selfing ability including stigma-anther contact, stigma receptivity, floral time available for selfing, and the ability of unmanipulated flowers to make fruit. Using these data, I then related each species' overall autonomous selfing ability to its geographic range size, as measured from collection records compiled from a number of herbaria. I found the species most proficient at selfing had the largest geographic ranges (but not larger elevational ranges). These results argue for an extension of Baker's Law, suggesting that selfing species are better colonizers, and as a result, on average have large geographic ranges. This chapter has been submitted to the journal *New Phytologist* for a special issue on plant adaptations and is co-authored with one of my undergraduate assistants who contributed significantly to the estimates of geographic range sizes, Jake Slyder, and my dissertation advisor, Susan Kalisz.

In Chapter 3, I focus on testing two prominent hypotheses regarding the formation and maintenance of reproductive isolation, the Bateson-Dobzhansky-Müller (BDM) model of speciation (Bateson 1909, Dobzhansky 1937, Müller 1940, Orr 1995, Coyne and Orr 2004) and the Reinforcement Model (Wallace 1889, Dobzhansky 1941, Fisher 1930, Grant 1963, Grant 1966, Coyne and Orr 1989, Noor 1999). This chapter has three central elements. First, using the geographic range sizes I estimated for *Collinsia* species in the previous chapter, as well as estimates I made for the remaining *Collinsia* species, I test whether there is evidence that *Collinsia* species diverged in allopatry. Allopatric divergence is a fundamental assumption of

both the BDM and Reinforcements models, yet this assumption is rarely tested in empirical studies. I find evidence that many *Collinsia* species have diverged in allopatry, particularly in the diverse clade of species that are primarily found in California. This result provides strong evidence that *Collinsia* is a suitable system to tests these prominent models of reproductive isolation. Second, using extensive experimental greenhouse crosses involving almost all species in the *Collinsia* phylogeny, I look for evidence of BDM incompatibilities between species, and relate the degree of reproductive isolation to diverge time. My divergence time estimates come from a robust, four-locus, time-calibrated molecular phylogeny constructed by Dr. Bruce Baldwin and colleagues. Using measures of prezygotic, postzygotic, and total isolation, I find strong evidence in support of the BDM model of speciation. This work is one of only a handful of empirical studies of plants that have found evidence for a pattern consistent with an accumulation of BDM incompatibilities with increasing divergence time, mirroring the results from a variety of animal systems. In addition, I grew and crossed F1 individuals to flowering, then let these plants autonomously self fertilize to produce F2 progeny to help distinguish between reproductive isolation driven by BDM incompatibilities versus chromosomal rearrangements, as these two processes have different predicted effects on the viability of F1 and F2 hybrids. I also measured the degree of asymmetry in reproductive isolation between species pairs, as strong asymmetry has been widely documented in hybrid crosses. If BDM incompatibilities are primarily driven by interactions between nuclear loci, then there is no *a priori* expectation for reproductive isolation to be particularly asymmetric. A few species pairs showed asymmetry in reproductive isolation, but on the whole across *Collinsia*, isolation was largely symmetric. Finally, to address the Reinforcement model, I assessed the degree of reproductive isolation between pairs of species that are found in allopatry or in sympatry, testing

the prediction that species that have come into secondary contact after divergence in allopatry should show stronger prezygotic reproductive isolation than species that have remained allopatric. I do not find strong evidence of reinforcement in *Collinsia*, and I discuss the possible causes and implications of this result. This chapter will be submitted to the journal *Evolution*, and will be co-authored with my dissertation advisor Susan Kalisz.

In Chapter 4, I examine the strength of prezygotic reproductive barriers between two sympatric *Collinsia* species: *C. linearis* and *C. rattanii*. Specifically, I examine the degree to which these species overlap in geographic and elevational range. Where *C. linearis* and *C. rattanii* are sympatric, I quantified the overlap in flowering time between species and the degree to which pollinators move within and between species. Finally, in a greenhouse experiment, I examined the timing of self-pollination in sympatric and allopatric populations of both species. This was done to determine if selfing acts as an isolating barrier where these species co-occur. To test this, I looked for evidence of divergence in the mating system when *C. linearis* and *C. rattanii* occur in sympatry relative to where they are allopatric. Specifically, I expected that heterospecific pollen flow from *C. linearis* to *C. rattanii* would select for earlier selfing in *C. rattanii* when it is sympatric with *C. linearis*. My field results revealed that sympatric populations of these two species exhibit substantial overlap in flowering phenology and share pollinators, indicating non-trivial opportunities for heterospecific pollen flow. As predicted, I found that the timing of selfing was significantly earlier for *C. rattanii* when in sympatry with *C. linearis*, relative to populations in allopatry. These results suggest that mating system may be an important component of prezygotic isolation for these species.

Lastly, in Chapter 5 I briefly synthesize my results and discuss the how my dissertation work adds to the body of knowledge on reproductive isolation, speciation, and how mating

system influences variation in the geographic distribution of plant species and acts as a reproductive isolating barrier.

2.0 AUTONOMOUS SELFING ABILITY EXPLAINS DIFFERENCES IN RANGE SIZE AMONG SISTER-TAXA PAIRS OF *COLLINSIA* (PLANTAGINACEAE): AN EXTENSION OF BAKER'S LAW

2.1 ABSTRACT

Species with greater selfing ability are predicted to be better adapted for colonizing new habitat (Baker's Law). We tested an expansion of this hypothesis: that species proficient at autonomous selfing have larger range sizes than their less proficient sister taxa. We also tested competing hypotheses regarding propagule pressure (seed number) and niche breadth on range size. We measured floral traits affecting the proficiency of autonomous selfing and calculated propagule pressure for six sister-taxa pairs in the clade *Collinsia*. We tested for the hypothesized effects of these variables on elevational distribution and range size. We found that species most proficient at selfing had significantly larger range sizes than their sister-taxa that were less proficient at selfing. Species proficient at autonomous selfing occupied a higher mean elevation than their sister taxa, but they did not differ in their total elevational range. Propagule pressure did not affect range size. Our results have general implications and extend Baker's Law, suggesting that species proficient at autonomous selfing are better adapted to establish new populations, and thus can more readily expand their range. Autonomous selfing ability may play a vital role in explaining variance in range size among other species.

2.2 INTRODUCTION

Why some plant species are narrowly distributed and others are widespread remains a fundamental question in the fields of ecology, evolution, biogeography, and conservation biology (Brown et al. 1996, Gaston 1998, Holt and Keitt 2005). Differences in the distribution of species have been attributed to a variety of factors including local and regional habitat conditions (e.g., geographic barriers, habitat availability, and species interactions; reviewed in Brown et al. 1996, Gaston 1996); historical factors such as species age (Willis 1922, Paul et al. 2009); species-level traits including dispersal and establishment (Böhning-Gaese et al. 2006), fecundity (Lockwood 2005), niche breadth (McNaughton and Wolf 1970, Brändle et al. 2003), local abundance (Brown 1984, Lawton 1993), environmental or physiological tolerance (Brown et al. 1996, Pither 2002), and mating system (Henslow 1879, Lowry and Lester 2006). Of these possible influences on plant species distributions, mating system is of special interest because it has long been held as a primary determinant of a species' success in establishing a breeding population in a novel location (e.g. Baker 1955, Baker 1967, Stebbins 1957, Lloyd 1980). Specifically, the ability to autonomously self-fertilize when mate availability is low has repeatedly been suggested as a key adaptive trait that increases the likelihood of successful colonization, establishment, and population spread (e.g., Baker 1955, Baker 1967, Stebbins 1957, Lloyd 1980, Pannell and Barrett 1998, Flinn 2006). Since species that can autonomously self-fertilize are more likely to establish new breeding colonies, we hypothesize that they may also be expected to have larger range sizes.

The relationship between range size and mating system was proposed over a century ago by Henslow (1879), who noted that most weedy plant species are self-fertilizing and that the most widely distributed plants in Great Britain were also self-fertilizing. Thus, his general observations directly linked mating system with high colonization rates and large range size.

This relationship remained mostly unaddressed until 1955, when Herbert Baker published a paper in *Evolution* entitled "Self Compatibility and Establishment after Long-Distance Dispersal." Baker proposed that a single propagule from a self-compatible (hermaphrodite) species was more likely to establish a viable population after long-distance dispersal than a self-incompatible (or dioecious) species, which would require at least two propagules to arrive at the same time and place. Baker noted that a high proportion of self-compatible species (or populations) were found in isolated locations (e.g., oceanic islands, isolated ponds; Baker 1955, Longhurst 1955). This pattern was strongly supported by Stebbins (1957 and references therein) and thought to be so general that Stebbins (1957) elevated it to the status of law (Baker's Law). Subsequent comparative studies show a greater proportion of selfing species and greater propensity for selfing on islands compared to the mainland sites and confirm these observations: Galapagos (McMullen 1987), Hawaii (References in Baker 1967 and Carr et al. 1986), New Zealand (Webb and Kelly 1993), Juan Fernandez Islands (Anderson et al. 2001, Bernardello et al. 2001), Channel Islands (Schueller 2004). In addition, shifts from dioecy to cosexuality (Cox 1989, Sytsma and Smith 1991, Pannell 1997), and heterostyly to homostyly (Barrett and Shore 1987, Barrett et al. 1989) were also noted after long-distance dispersal, which further suggests that traits that facilitate autonomous selfing increase the probability of establishment after dispersal.

More recently, the generality of the link between colonization and mating system has been expanded. While Baker (1957, 1967) focuses on the benefits of selfing after long-distance dispersal, Pannell and Barrett (1998) argue that the premise of Baker's Law should apply to any dispersal distance, stating, "… solitary selfers will always be more successful than obligate outcrossers in founding new colonies" (Pannell and Barrett 1998, p. 657). In fact, increased

selfing ability in colonizing species has been noted in several studies (Henslow 1879, Baker 1974, Price and Jain 1981, Lloyd 1980, Kelly 1996, Schueller 2004, Flinn 2006). Species that can self-pollinate are predicted to be better at colonizing habitat with few pollinators or mates (Stebbins 1950, Stebbins 1957). Further, species that differ in mating system often differ in seed production (Primack 1987). Differences in seed production, termed propagule pressure, have been shown to be an important trait affecting range expansion (reviewed in Lockwood et al. 2005, Colautti et al. 2006). Thus both increased seed production and increased selfing may both influence range expansion. The logical extension from the empirical and theoretical investigations described above is that species that are proficient at autonomous selfing and that produce numerous seeds, should be better colonizers in general, and thus should have larger range sizes than species that are self-incompatible or otherwise poor autonomous selfers, or produce few seeds.

In contrast to the above prediction, highly selfing species could have relatively smaller range sizes than outcrossing species due to low genetic diversity (Lowry and Lester 2006). Because selfing species typically have lower levels of genetic diversity than outcrossing species (e.g., Hamrick and Godt 1996), populations founded by selfed individuals will have lower genetic diversity than those founded by outcrossed individuals (e.g. Carlquist 1966, Crawford et al. 2008). Lack of genetic diversity may limit the ability of a selfing species to adapt to novel environments (Stebbins 1957, Levin 1968, McNaughton and Wolf 1970, Hedrick et al. 1976, Pound et al. 2004), limiting colonization of new habitats, and thus constrain range size (McNaughton and Wolf 1970, Moldenke 1975, Brändle et al. 2003). Species with narrow niche-widths are predicted to have small range sizes (McNaughton and Wolf 1970) but this has been rarely test in plants. In comparisons of 31 species of weedy plant species, those with narrow

germination niche-requirements were shown to have smaller range sizes than those with broader germination requirements (Brändle et al. 2003). However, a similar study examining germination niche-width found equivocal results (Thomson and Ceriani 2003). Lowry and Lester (2006) found that polyploid species have larger range sizes than diploid species, which is consistent with the prediction that species with greater genetic diversity (e.g., outcrossed or polyploid species) will have larger range sizes. If we assume that typical selfing species have lower genetic diversity than outcrossing species, then we might predict that selfing species will have smaller range sizes.

Clearly autonomous selfing ability has the potential to strongly influence range size, however, the direction of influence is difficult to predict given the contrasting hypotheses above. In addition, because multiple factors are expected to influence the range size over the evolutionary history of a species, phylogenetically controlled comparisons are required to correctly assess the effect of mating system on range size. If species' niches are relatively constrained over evolutionary time (i.e. phylogenetic niche conservatism; Wiens and Graham, 2005), closely related species are more likely to occupy similar habitats and overlap in traits than distantly related species (Harvey and Pagel 1991). Thus, if sister species differ in range size, we can test for key traits that have lead to those differences.

Flower size is often assumed to be diagnostic of a species' or population's mating system (Takabayashi and Morrell 2001), but this assumption is not often verified. Here we quantify mating-system traits that are widely expected to influence autonomous selfing ability (flower size, herkogamy, dichogamy, stigmatic receptivity and autonomous seed set) of sister-taxa pairs of *Collinsia* (Plantaginaceae) to determine the effects of mating system on range size. If increased ability for autonomous selfing results in greater colonization and establishment of

populations, then we expect selfing species to have larger ranges sizes than outcrossing species. In contrast, if low genetic diversity limits population expansion, then we expect that selfing species will have smaller ranges sizes than outcrossing species. In this study, we quantify three dimensions of species' ranges, their total geographic extent of occurrence (Gaston 2003, maximum spread across the landscape as measured by minimum convex polygons), their elevational range size (total elevational spread) and elevational mean. We contrasted these range size metrics between sister-taxa pairs that differed significantly in their autonomous selfing ability. Finally, we compared propagule pressure between sister-taxa pairs.

2.3 METHODS

2.3.1 Study System

Collinsia is an excellent model system for this study because all members of the genus share similar life history and pollination traits (annual, bee-pollinated, native herbs) and occupy similar habitats (Neese 1993, Armbruster et al. 2002). Importantly, all species are self-compatible, but because of differences in floral size and development, are expected to differ widely in their ability to autonomously self-pollinate (Armbruster et al. 2002). The genus *Collinsia* is a monophyletic group of plants comprised of ~22 species (Baldwin et al., unpub. data) that are exclusively short-lived winter or spring annuals native to North America. A new robust molecular phylogeny for *Collinsia* (Baldwin et al., in prep.) allows us to identify sister-taxa pairs for comparison. The center of diversity for *Collinsia* is central California, but many of the western species extend into Oregon, Washington, and British Columbia. In addition, three

Collinsia species occur in eastern North America: *Collinsia parviflora* extends from Californai north to British Columbia and Alaska and east to Michigan and Ontario while *C. verna* and *C. violaceae* are found exclusively in the eastern and central portions of the United States, respectively.

All species of *Collinsia* have zygomorphic flowers with a 5-lobed calyx and a 2-lipped corolla with a folded keel petal containing four stamens that develop sequentially. As in Kalisz et al. (1999), we define the developmental stage of a flower relative to the number of mature stamens exhibiting anther dehiscence (e.g. Stage 1=1 anther dehisced) in this study. Ovule number varies widely among species (ranging from 2 to 26 ovules/ovary, Armbruster et al. 2002; Kalisz and Randle, unpub. data) and can vary among populations within species (Kalisz and Randle, unpub. data). Flowers are borne in whorls with display sizes ranging from ~2 to 22 simultaneously open flowers in species across the genus. Bees are the primary visitors of all *Collinsia* species (Rust and Clement 1977, Kalisz and Vogler 2003, A. Randle, pers. obs.) even those considered highly selfing (A. Randle, unpub. data). Although all *Collinsia* species are self-compatible, proficiency at autonomous self-pollination varies among the taxa. *Collinsia* species have passively dispersed seeds although some species are described as having winged seeds (Neese 1993), including *C. sparsiflora* (all varieties), *C. tinctoria* and *C. rattanii*.

2.3.2 Selection of Species Pairs

We aimed to include sister-taxa pairs that were likely to differ in selfing ability. Because flower size is often used as a proxy for mating system (e.g., Grant 1958, Jain 1976, Takabayashi and Morrell 2001), we selected sister taxa pairs within the genus *Collinsia* that differed significantly in flower size. To do this, we measured a suite of floral size and shape traits (3

plants/population; 1-3 populations/species) across all *Collinsia* species. Flowers were collected from plants raised from field-collected seed under optimal conditions in the greenhouse facilities at the University of Pittsburgh. One mature flower from each plant was collected and preserved in 70% ethanol for up to 24 hrs before photographing. Lateral view images of all flowers were made using a Hitachi KP-D50 digital camera affixed to a Nikon SMZ800 dissecting microscope. Images were captured using Optimus 6.5 image analysis software and stored for subsequent analysis. To describe the shape and size of flowers, we generated a shape-model template map in MATLAB, using the 'Point Model Editor' in the AAM Toolbox (Hanna 2006, Whibley 2006). For *Collinsia*, our template consists of 76 points (8 primary and 68 secondary points). These points were placed onto the scaled digital image of each flower at predefined intervals and locations to capture the shape and size information.

To analyze the floral variation among species, we conducted a principal components analysis (PCA) using the AAMToolbox (Hanna 2006). Because we are interested in species level comparisons, we used data from all available populations (1-3 populations/species) in our analysis. The first three principal components (PC) described ~90% of the variation in floral shape and size across all taxa. PC1 corresponded to overall floral size, and accounted for 75% of the total variation among species. We used the PC1 scores of individual taxa to test for significant differences in floral size between sister taxa. In this study, sister taxa refers to both sister species or sister varieties, thus we first tested for significant differences in floral size between the most closely related sister-taxa level in our phylogeny (e.g., variety). If flower sizes were not significantly different between varieties, we pooled the varieties' data and compared flower size between sister species. Because sample sizes were generally small (n=3-9 flowers/species), we used both an independent t-test and Mann-Whitney U-test to compare

flower size between sister taxa. Results did not differ between the two analyses. We found 6 pairs of sister taxa that differed significantly in flower size ($p<0.05$): 1) C. *parryi and C. concolor*, 2) *C. sparsiflora v. sparsiflora* and *C. sparsiflora v. arvensis*, 3) *C. bartsiifolia* [*including v. bartsiifolia & v. davidsonii*] and *C. corymbosa*, 4) *C. rattanii* and *C. linearis*, 5) *C. parviflora* and *C. grandiflora*, and 6) *C. torreyi v. wrightii* and *C. torreyi v. torreyi* (Fig. 2.1).

We compared floral traits among sister-taxa pairs that are known to correlate with species mating system, specifically the ability to autonomously self-pollinate. These traits include: stage of stigma-anther contact, stage of stigma receptivity, autonomous fruit set, and proportion of floral life where the receptive stigma was in contact with the anthers. Species that are self-compatible can avoid self-pollination by spatial or temporal separation of male and female reproductive parts (herkogamy and dichogamy, respectively) or both. Highly selfing species, proficient at autonomous autogamy, reduce herkogamy and dichogamy early in floral development whereas outcrossing species reduce herkogamy and dichogamy late in floral development or not at all (e.g., Lloyd 1980, Bertin and Newman 1993, Schoen et al. 1996, Kalisz et al. 1999, Armbruster et al. 2002, Lloyd 1992, Takebayashi and Morell 2001, Kalisz and Vogler 2003). In addition to mating system traits, we also measured propagule pressure as average seed production, mean elevation of each species, and elevational range size (methods below).

2.3.3 Stigma-Anther Contact

To determine the timing of stigma-anther contact (S-AC), we scored the developmental stage of S-AC using one of two methods. First, *Collinsia* species of the focal sister-taxa were grown from field-collected seeds under optimal conditions in greenhouse facilities at the

University of Pittsburgh. S-AC was determined by depressing the lower petals of each sampled flower and noting the location of the stigma in relation to the position of the dehisced anthers using either fresh or preserved flowers (2 flowers in each stage on 5 plants/populations from 1-3 populations/species (N=40-120 flowers/species fresh sample; 20-40 flowers/species preserved samples). A subset of flowers never made S-AC during stages 1-4 and these flowers were given a score of 5. Second, in a subset of species (*C. concolor, C, grandiflora, C. linearis, C. rattanii, C. torreyi v. torreyi*) an additional measure of S-AC was made by scoring presence/absence pollen on stigmas at each developmental stage. One flower in each stage was collected from 6 individuals of each species listed above. To avoid accidental pollination, the two lateral petals were fastened to the sticky side of a Post-It Note™ (Kalisz et al. 1999). This allows the corolla to be depressed, the keel petal to be opened and the floral stage to be determined. Flowers still attached to the Post-It Note were placed under a dissecting microscope and the style was removed using a pair of fine dissecting scissors. The styles were mounted in a 1:1 solution of glycerol and 1% acetocarmine stain and examined for the presence of pollen with a compound microscope (100X). We calculated the mean stage of S-AC for each individual plant (S-AC contact method) or population (pollen on stigma method). For the five species where both measures of S-AC were available, we pooled the data and compared the mean stage of S-AC between sister-taxa pairs.

2.3.4 Stigmatic Receptivity

The stage of stigmatic receptivity is positively correlated with the stage when pollen tubes are first detected growing through the styles of *Collinsia* species (Armbruster et al. 2002). The timing of stigmatic receptivity was determined by testing for stigmatic peroxidase activity

(Kearns and Inouye 1993, Kalisz et al. 1999) across the four stages of flower development. Styles were excised from 2 fresh flowers in each stage from 5 plants per species (2 flowers x 4 stages x 5 plants=40 flowers/species). Stigmas were examined for the presence of pollen, and were only chosen if no pollen was present, as pollen on the stigma can result in false positives (Kearns and Inouye 1993). Styles were placed on glass slides with 3% hydrogen peroxide and examined under a light microscope. If bubbling occurred within 2-3 minutes, the stigma was scored as receptive. Data was collected from plants grown in the greenhouse facilities at the University of Pittsburgh under optimal conditions or from a natural population in the field (*C. concolor* only). The mean stage of stigmatic receptivity was calculated for each individual plant and a grand mean was calculated for the species.

2.3.5 Autonomous selfing ability

The relative % ovules fertilized provides the best measure of autonomous autogamy ability because it scales seed production to the total number of seeds possible. However, we found that for several species in our study, ovule number is highly variable among individuals within a population. Because we did not measure ovule number for each plant used in our study and the mean value was not accurate, we used the ability of an unmanipulated flower to make a fruit as our estimate of autonomous autogamy proficiency. We marked the calyx or subtending leaf of three flowers on each plant with non-toxic fabric paint (n=3 flowers/plant x (6 to 12) plants/species =18-36 flowers/species). Marked flowers were monitored after corolla abscission to determine if a fruit containing at least one seed was produced. We had sufficient data to estimate the autonomous autogamy for 10 of our 12 species.

2.3.6 Proportion of Total Floral Life When Autonomous Selfing Can Occur

To determine the proportion of total floral life when autonomous selfing can occur, we first determined floral longevity for each species. Six plants from each of 1-3 populations per species were grown together in a Conviron PGW36 growth chamber under optimal conditions. On each plant, 6 flower buds were marked with a unique color of non-toxic fabric paint on the calyx or subtending leaf (n=6 flowers x 6 plants x (1 to 3 populations/species) = 36-108 flowers/species). Flowers were checked daily at ~0900h and ~1500h, and the stage of development (as described above) was noted for each flower at each time period until the corolla abscised. Because some stage transitions occurred rapidly, we did not always capture each stage for each individual flower with our sampling scheme. Thus to calculate total floral longevity for each individual, we calculated the average duration of each stage across all flowers/individual. We took the grand mean of the duration of each stage across individuals for each species, and summed these values across each stage to obtain the mean total floral longevity per species. To determine the proportion of a flowers life that autonomous selfing can occur, we determined the mean stage at which the stigma was receptive *and* the stigma and anthers were in contact (receptive stigma-anther contact) for each species. We then used the data on the proportion of time each species spent in each developmental stage to calculate the total proportion of floral life that a flower was able to autonomously self pollinate.

2.3.7 Propagule pressure

We estimated propagule pressure for each species by the multiplying the average seed number per fruit by the median daily floral display size of each species. Average optimal seed

production per fruit was quantified using 3 flowers per plant from 6-12 plants/species. Each flower was uniquely marked with non-toxic fabric paint on the calyx or subtending leaf. Anthers were removed from the immature marked flowers, and hand pollinations were conducted with outcrossed pollen three times during the time period when the stigmas were receptive. Fruits were collected, and the number of seeds per fruit was counted. The mean number of seeds per fruit was determined for each individual. Flowers that failed to make fruit (0 seeds) were excluded from the analysis, as they were likely due lack of successful pollinations. Because of low germination of *C. torreyi* v. *wrightii* and *C. torreyi* v. *torreyi*, we used the published maximum seed number/fruit for these species (2 seeds/fruit for both). The median daily floral display size was determined from photographs of plants in the field found at the Calflora (www.calflora.org) and USDAPlants (plants.usda.gov) websites. Propagule pressure for each species was calculated as the maximum outcrossed seed number multiplied by median daily floral display size.

2.3.8 Species collections and range-size projections

To determine the range size for each species, we searched and collated latitude and longitude data for each *Collinsia* species from 25 herbaria collections using the Jepson's Consortium of California Herbaria (ucjeps.berkeley.edu/consortium/), Global Biodiversity Information Facility (www.gbif.net), Oregon Flora Project (http://www.oregonflora.org/atlas.php), and BC E-Flora (Klinkenberg 2008, http://www.eflora.bc.ca/). We converted all location data from these collections to the NAD 27 coordinate system and used ArcGIS (9.2) to create point shapefiles for each species. The shapefiles were then projected in North America Albers Equal Area Conical Projection in order to preserve accuracy for area. The number of points contained in each species'

shapefile varied considerably (9-954, median 38). The points included were collected over a 126-year period (1881-2007). Hawth's Tools extensions for ArcGIS 9 were used to create minimum convex polygons (MCP) about the points. Polygons for each species were clipped where they extended over water. Bruce Baldwin, curator of the UC Berkeley Jepson Herbarium validated all point location data used in our maps. For species with multiple varieties, we only used collections where the variety was identified. To calculate range size for each species, we used X Tools Pro (5.2) to calculate the square kilometers of each polygon.

2.3.9 Elevational Range Sizes

Elevation data for each species was extracted from the Worldclim altitude layer (www.worldclim.org; ~1 km^2 resolution) with Diva-GIS (Hijmans et al. 2001) using our geo-referenced herbarium collection records (above). We measured niche width as the elevational range (i.e., max-min elevation) occupied by small- and large-flowered sister taxa.

2.3.10 Statistical Tests

Differences in means for sister taxa in the traits flower size, stigma-anther contact, stage of stigmatic receptivity, autonomous selfing ability, and mean elevation were each compared with an independent t-test and if samples sizes were small, with a Mann-Whitney U test. In all cases, results of these two tests did not differ and only results from t-test were used. To test for the general pattern of differences across all species pairs, we conducted a combined probability test using a Z-transform (Whitlock 2005) of the p values from the above t-tests. Differences between sister pairs in the "proportion of total floral life when autonomous selfing can occur"

was compared across sister-taxa pairs with a paired t-test. Elevational range and propagule pressure were compared using Wilcoxon's sign-rank tests.

2.4 RESULTS

2.4.1 Flower size and mating system

Flower size differed significantly for all six sister-taxa pairs [C. *sparsiflora* v. *sparsiflora* and *C. sparsiflora* v. *arvensis* (t=4.02, df=7, p=0.0051), *C. parryi* and *C. concolor* (t=17.1, df=4, p=0.001), *C. bartsiifolia* and *C. corymbosa* (t=3.88, df=13, p=0.0019), *C. rattanii* and *C. linearis* (t=18.4, df=14, p=0.0001), *C. parviflora* and *C. grandiflora* (t=21.4, df=9, p=0.0001), and *C. torreyi* v. *wrightii* and *C. torreyi* v. *torreyi* (t=13.7, df=7, p=0.001), Fig. 2.1]. To determine if flowers size is a good proxy for mating system for these species, we compared a suite of traits known to correlate with autonomous autogamy ability. Overall, we found that relative to the large-flowered species of our study, the small flowered sister species had stigmatic receptive at a significantly earlier stage (Z=2.96, n=6, p=0.002; Fig. 2.2a); had significantly earlier stigma-anther contact (Z=4.67, n=6, p=0.0001; Fig. 2.2b). In 4 of 6 sister taxa pairs, small flowered species spent a greater proportion of their floral lifespan with the receptive stigma in contact with anthers that were shedding pollen (Fig. 2.2c). However, in a paired t-test across all six pairs of sister-taxa, this difference was only marginally significant (t=2.23, df=5, p=0.08). Our autonomous fruit-set data complement these floral developmental results. We found that, overall, small flowered species were significantly better at autonomously selfing and producing fruit in the absence of pollinators than their large flowered sister-taxa (Z=3.12, n=5, p<0.001; Fig. 2.2d).

Therefore, relative flower size of sister taxa is a good estimator of their relative autonomous selfing ability in *Collinsia*.

2.4.2 Flower Size (Mating System), Propagule Pressure, Occupation of Marginal Habitat (High Elevation), Elevational and Geographic Range Size

Because propagule pressure has been linked to increased colonization rates, we examined differences in propagule pressure between sister-taxa pairs. In 5 of 6 sister-taxa pair comparisons, large flowered species produced more seeds per capita than small flowered species, however across all pairs, this difference was not significant (Wilcoxon's sign rank: small mean =14.12, large mean= 35.56; W=13, n=6, $p>0.05$; Fig. 2.3). This result suggests that in most, but not all *Collinsia*, large flowered taxa exert greater propagule pressure than their small flowered sister taxa, but across all species, there is no significant difference.

The mean elevation of small flowered species was significantly greater than their large flowered sister taxa (Z=6.26, n=6, p=0.0001; Fig. 2.4). However, small and large flowered sister taxa did not differ significantly in the mean elevation range size (e.g. elevational niche-width) (Wilcoxon's sign rank; W=7, n=6, p=0.2813). Finally, in all cases, small flowered species, which have significantly greater autonomous selfing ability, have significantly larger range sizes than their large flowered sister-taxa (Wilcoxon's sign rank; W=21, n=6, $p<0.030$; Fig. 2.5).

2.5 DISCUSSION

Our analysis of range size for *Collinsia* sister taxa supports the hypothesis that species that are more proficient at autonomous self-pollination have larger range sizes than their less proficient sister taxa. In all six sister-pair comparisons, the better autonomous selfing species had larger range sizes. To our knowledge, our analysis is only the second phylogenetically controlled test of the effects of mating system on range size. Surprisingly, our results contrast with those of Lowry and Lester (2006), who in a pair-wise comparison of sister taxa of *Clarkia* concluded that outcrossing species have larger range sizes than selfing species. It is unclear why *Collinsia* and *Clarkia* would differ in this regard. Both genera are annual plants that occur in similar regions of North America (Neese 1993, Lewis 1993). *Clarkia* differs from *Collinsia* in the number of polyploid species in the genus, but these species were removed from their sister-pair analysis. Also, the *Clarkia* phylogeny was not as well resolved as the *Collinsia* phylogeny, containing many polytomies, which may have influenced their results. Finally, many factors influence range size (Brown et al. 1996, Gaston 1996), and the benefits of selfing to colonization may play a relatively smaller role in *Clarkia*. As Pannell and Barrett's (1989) model suggests, if *Clarkia* species are common across the landscape, or if they have a seed bank, or if they experience relatively high inbreeding depression (Holtsford 1996), the benefits of selfing (e.g., reproduction assurance) may not be realized. Our results for *Collinsia* extend Baker's Law (Baker 1955, Stebbins 1957) beyond the comparison of self-compatible and self-incompatible species to include a wider spectrum of the mating system.

Despite the fact that many studies find seed number or propagule pressure to be important in colonizing species (e.g., Lockwood et al. 2005, Colautti et al. 2006) we did not find that species with greater propagule pressure achieved larger range sizes. In our analysis, all five of

the six sister taxa with low autonomous selfing ability had higher propagule pressure than their sister-taxa, yet all had smaller range-sizes.

Our results also did not support what Lowry and Lester (2006) termed the Niche-breadth hypothesis. In contrast to their predictions, highly-selfing species (with purportedly low genetic diversity) had larger range sizes than their outcrossing sister taxa. In addition, we found no difference between sister taxa in elevational range size (i.e., magnitude of elevational range occupied), which we used as a proxy for one dimension of niche breadth. Our data suggest that species with higher autonomous selfing ability did not suffer narrower niche-breadth or reduced range size as a result of their mating system. The autonomous selfing species may lack genetic variation, but may maintain beneficial gene complexes that confer an advantage for colonizing new habitat, which are not broken up by sexual reproduction (Stebbins 1957). Alternatively, although the proficient autonomous selfers have a higher propensity for selfing in the absence of mates or pollinators, they are not strictly selfing in the long run, so they may not differ substantially in genetic diversity from their sister taxa. Supporting this idea, Kalisz (unpub. data) found that in some populations of the smallest-flowered *Collinsia* species, outcrossing rates calculated for wild-produced progeny arrays estimated with microsatellite markers can reach as high as 40-82%. These surprisingly high outcrossing rates suggest that the ability to autonomously self may buy time for the establishment of dense populations that are attractive to pollinators despite their small flower size. This idea is supported by observations of pollinators regularly visiting the small-flowered species *C. parviflora* and *C. rattanii* in the field (A. Randle, unpub. data).

We compared the mean elevation range between sister-taxa pairs to determine if small-flowered, highly selfing-species were more likely to inhabit high elevation sites than their large

flowered sister-taxa. Because all *Collinsia* species are annuals, with short life-cycles and some level of self compatibility, all *Collinsia* species should be relatively good at colonizing open, unsaturated, or temporary habitat with few mates or pollinators (Lloyd, 1980). However, those that are most proficient at selfing should be best at moving into these sites. High elevation sites have been shown to have lower abundance of pollinators (Kalin-Arroyo et al. 1985, Malo and Baonza 2002), and may represent harsher environments than lower elevations sites. Stressful environments, especially those with water stress, are known to favor reduced allocation to flower size in *Polemonium viscosum* (Galen 1999, 2000), *Epilobium angustifolia* (Carroll et al. 2001) and *Rosmarinus officinalis* (Herrera 2005). The small flowered *Collinsia* species occupied higher mean elevations than their large flowered sister taxa. One interpretation is that the more highly selfing-species are able to move into more marginal-high elevation sites in part because they are also better at coping with a stressful environment. Conversely, autonomous selfing may be a byproduct of correlated selection on flower size (Takabayashi and Morrell 2002).

One assumption that we make is that sister species arise with similar range sizes. There is little evidence that range size is heritable (Webb and Gaston 2003, but see Waldron 2007) and thus the range sizes of sister taxa are not necessarily expected to be similar. Differences in range size among sister taxa could be affected by the mode of speciation (Paul and Tonsor, 2008). In the case of peripatric speciation the two sister species would begin with very different range sizes. Here, the small flowered selfing species are most likely to evolve at the periphery of the large-flowered outcrossing species' range (Lloyd 1965, Herlihy and Eckert 2005, Moeller and Geber 2007). If this were true, then we would expect small-flowered species to have on average smaller range sizes than their large-flowered sister taxa. This is clearly not the case for *Collinsia*. Alternatively, many peripheral populations of the 'selfing species' may evolve at the

range margin of the large-flowered outcrossing species, resulting in a multiple 'selfing species' that are more closely related to their outcrossing sister-taxa progenitor than they are to each other. This would result in a ring of small flowered (selfing) daughter species surrounding the range of the outcrossing species. This could give the appearance of a larger range size for the small-flowered sister taxa using our methods of delineating range size. However, this does not appear to be the case with *Collinsia,* as geographic distributions of the sister-taxa pairs do not fit this pattern and the small flowered taxa are found throughout their range, not just at the margins of their large-flowered sister-taxa (Fig. 2.3). With a vicariance event, it is unlikely that sister taxa would arise with similar range sizes (Glazier 1987, Price et al. 1997, Gaston and Chown 1999; Barraclough and Vogler 2000, Webb et al. 2001). Thus, as in peripheral isolates, sister-taxa may begin with very different range sizes, but which taxa has the small vs. the large range size should be random with respect to traits of the species. Regardless of starting conditions for range size when the *Collinsia* sister taxa split, our data show that over evolutionary time, the small-flowered selfing species obtain a larger range size than the large-flowered outcrossing species for all 6 sister-taxa pairs.

Although our data across all 6 species-pairs support the general pattern of larger range sizes for small-flowered selfing species, the difference in range size between *C. bartsiifolia* and *C. corymbosa* is likely driven by additional ecological factors. While *C. bartsiifolia* and *C. corymbosa* differ significantly in their flower size, and thus meet the criteria for inclusion in our study, *C. bartsiifolia* (the smaller flower species) has relatively large flowers within the genus (Fig. 2.1) and lacks many of the selfing traits of other small flowered species (Fig. 2.2 a,b,c). Interestingly, *C. bartsiifolia* did have significantly greater autonomous fruit set than *C. corymbosa*. More importantly, unlike our other sister-taxa comparisons, *C. bartsiifolia* and *C.*

corymbosa inhabit strikingly different habitats. *C. corymbosa* is restricted to Pacific Ocean coastal dunes while *C. bartsiifolia* inhabits a broader range of habitat, including open sandy places (Neese 1993). The limited distribution of *C. corymbosa* could be attributable to many factors, but little is known about the ecology of this endangered species. Thus, the large range size of *C. bartsiifolia* may be in part due to its relative proficiency at self-pollination, but we do not think that this is the only factor driving the huge difference in range size between these two sister-taxa (Fig. 2.5). Rather, *C. corymbosa* is likely restricted in its range size by unmeasured ecological or physiological factors.

2.5.1 Conclusions

In summary, we attribute the majority of the differences in range size between sister taxa to differences in mating system and conclude that those species most proficient at autonomous selfing are best at establishing populations, and thus can more readily expand their range. Understanding the factors that contribute to range size differences among species is important to conservation. Factors that lead to range expansion are intimately related to processes that facilitate the establishment and spread of invasive and weedy species (Baker 1974, Mack et al. 2000). Likewise, factors that result in range contraction or limit expansion are critically important, as range size is inversely related to the probability of extinction (McKinney 1997, Purvis et al. 2000, Jones et al. 2003, Gaston and Fuller 2008). Our data clearly support the importance of mating system, particularly autonomous selfing ability, as an adaptive trait that influences establishment success in a novel location. Species that can autonomously self-fertilize and establish new breeding colonies, but switch to outcrossing as their numbers increase locally, may be an additional adaptive explanation for the prevalence of mixed mating. Hence, mating

system traits related to autonomous selfing may play a vital role in explaining variance in range size amongst species. Future studies that explore the relationship between mating system and range size in a variety of other taxa will help shed light on the generality of this pattern.

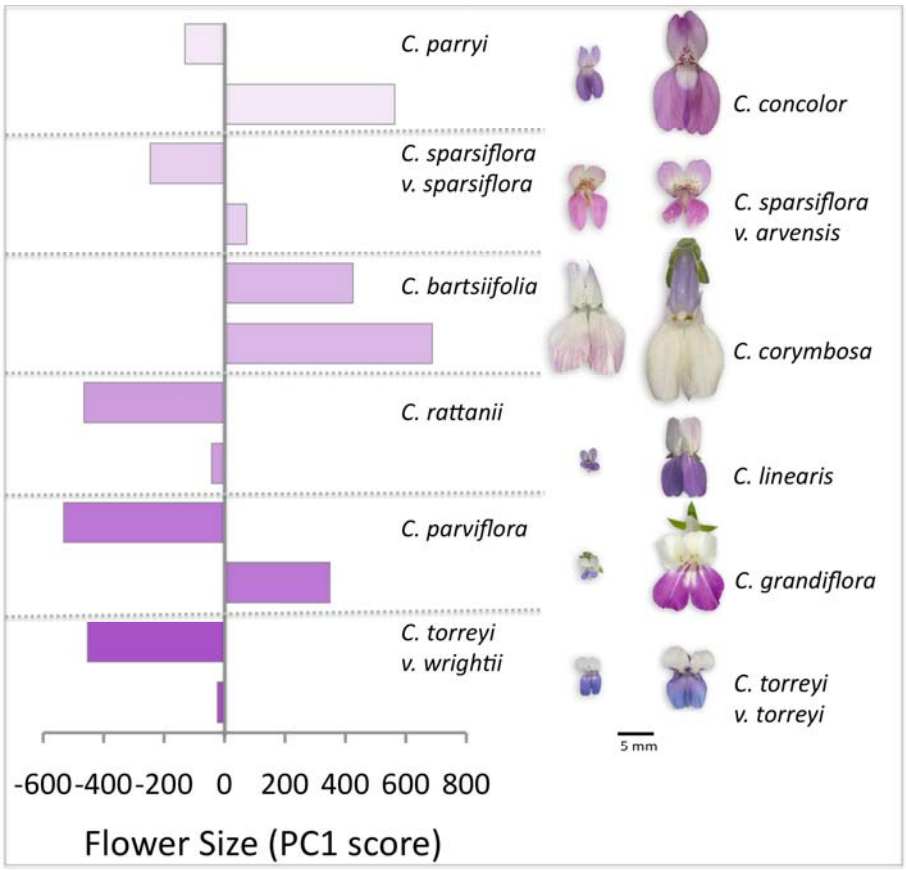

Figure 2.1. Six sister-taxa pairs in the genus *Collinsia* that differ significantly in flower size identified by principle component analysis. PC1 explains ~78% of the variation in flower size among all *Collinsia* species. Negative vs. positive PC1 scores indicate species with smaller vs. larger flowers than the genus average PC1=0, respectively. Note: species pairs arranged by relative divergence times, with the *C. torreyi* pair being the oldest taxon pair.

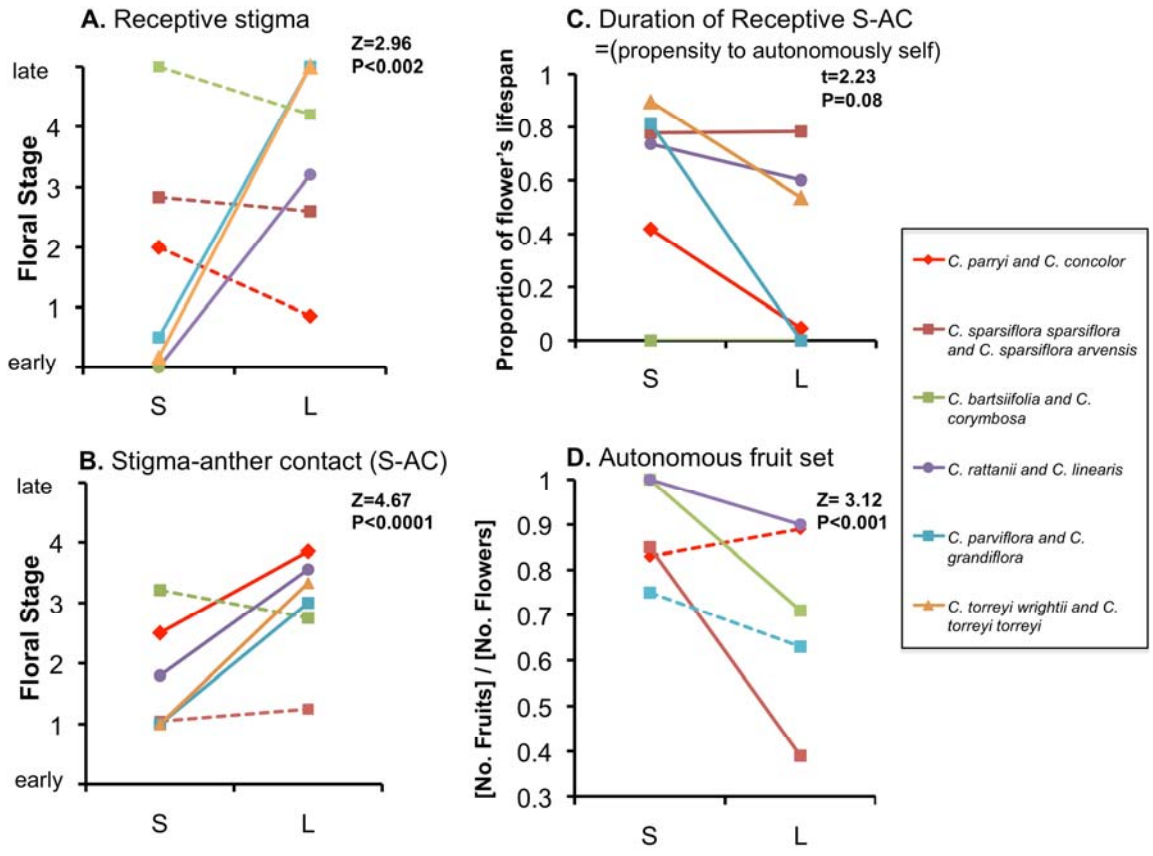

Figure 2.2. Overall, small-flowered sister species have traits associated with higher autonomous selfing ability and have higher rates of autonomous fruit production relative to their sister. Panels A-D present mean species value for each trait. Sister pairs connected by solid lines indicate that the taxa differed significantly in mean values; dotted lines indicate no significant difference between sister taxa for trait mean. In Panels A-B, floral stage=number of dehisced anthers within a flower (see text for details). Within individual flowers and relative to their large flowered sister, small flowered sister taxa exhibit: A. stigmatic receptivity at a significantly earlier stage, B. stigma-anther contact (S-AC) at a significantly earlier stage, C. in 4 of 6 species pairs, small flowered taxa spend a greater proportion of floral lifespan with the receptive stigma in contact with dehisced anthers (RS-AC) and D. significantly higher rates of autonomous fruit

set. A Z-test (Z) or paired t-test (t) was performed across species pairs to test for overall relationship of flower size and each autonomous selfing trait.

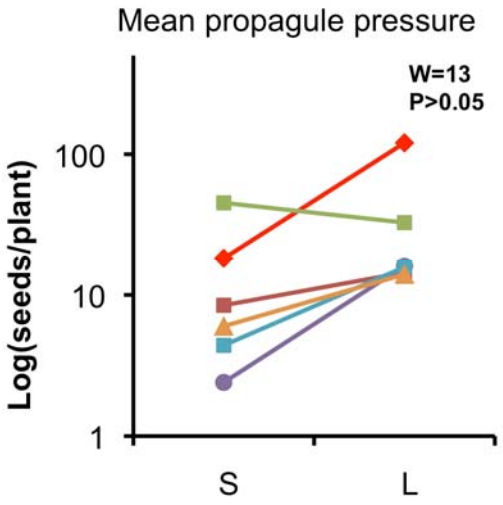

Figure 2.3. On average, large-flowered species less proficient at autonomous selfing produce more seeds relative to their sister taxa, suggesting higher propagule pressure in the less selfing species. However when tested across all pairs, this difference was not significant. Wilcoxon sign-rank test (W) performed across species pairs to test for overall relationship of flower size and propagule pressure. Species pair legend as in Figure 2.2.

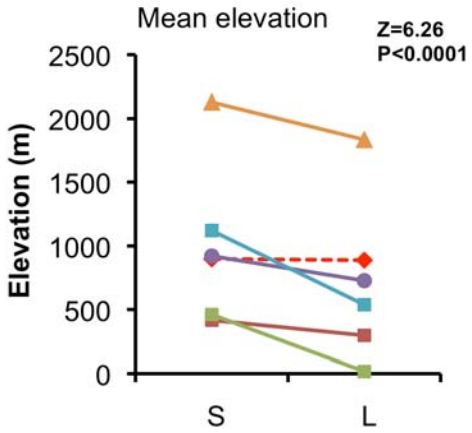

Figure 2.4. Small-flowered species proficient at autonomous selfing are found at significantly higher elevations relative to their sister. Sister pairs connected by solid lines indicate that the taxa differed significantly in mean values; dotted lines indicate no significant difference between sister taxa for trait mean. Z-test (Z) performed across species pairs to test for overall relationship of flower size and mean elevation. Species pair legend as in Figure 2.2.

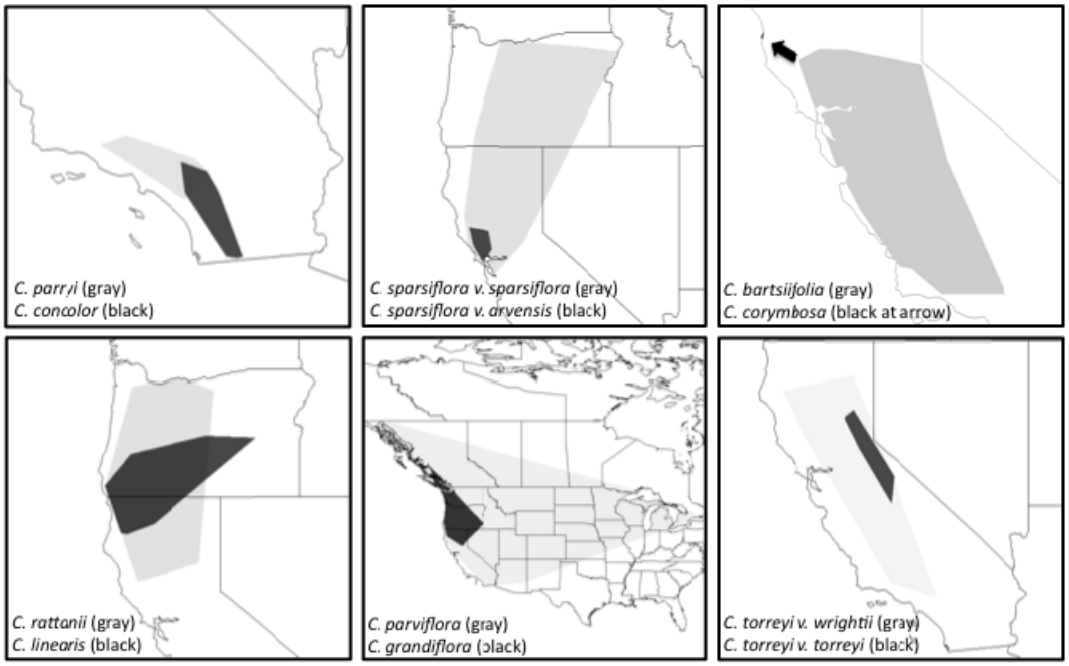

Figure 2.5. Range maps for the six sister-taxa pairs in the genus *Collinsia* that differ significantly in flower size and autonomous selfing ability. Wilcoxon sign rank test (W) performed across species pairs to test for overall relationship of autonomous selfing ability and range size.

3.0 PATTERNS OF REPRODUCTIVE ISOLATION AMONG SPECIES IN THE GENUS *COLLINSIA*

3.1 ABSTRACT

Central to the study of evolution is an understanding of the process of speciation. A variety of factors, both intrinsic and extrinsic, may be responsible for reproductive isolation between species. A prominent model to explain why allopatrically diverging species become reproductively isolated is the Batson-Dobzhansky-Müller (BDM) model of reproductive isolation. This model posits that, as divergence time between species increases, so should the degree of reproductive isolation, due to an accumulation of genetic incompatibilities between species. If species that diverged allopatrically come into secondary contact, natural selection against maladaptive hybrids may enhance prezygotic reproductive barriers between species, a process known as Reinforcement. There is ample evidence in support of both the BDM and Reinforcement models of reproductive isolation in animal systems, but results from the few studies in plants, particularly of BDM, have been largely equivocal. The assumption of allopatric divergence, key to both models, is likely a reasonable assumption for many taxa, yet has rarely been explicitly tested in previous studies of these models. In this study, I investigated the geographic mode of speciation and the pattern of intrinsic post-mating reproductive isolation among species in the genus *Collinsia*. I combined data from a dated molecular phylogeny,

species' geographic ranges, and pairwise greenhouse crosses of the majority of *Collinsia* species to investigate if the BDM and Reinforcement models provide viable explanations for reproductive isolation in *Collinsia*. I found evidence for allopatric speciation in this genus, driven primarily by a strong positive correlation between range overlap and divergence time in the California clade. I found that post-mating pre- and postzygotic isolation increased with increasing divergence time, as predicted by the BDM model of reproductive isolation, and that pre- and postzygotic isolation evolved at similar rates. In contrast, I found no evidence of reinforcement of post-mating prezygotic isolating barriers among sympatric species. My crossing studies revealed that asymmetry in reproductive isolation occurred in 30% of reciprocal hybrid crosses. Overall, this study provides strong evidence for patterns of reproductive isolation over time that are consistent with the BDM model, suggesting that a buildup of genetic incompatibilities in diverging species may be important in driving reproductive isolation in this group. However, I did not find evidence that reinforcement is important to reproductive isolation in *Collinsia*. I discuss my results in light of previous studies on both plants and animals, and suggest future directions for the study of reproductive isolation.

3.2 INTRODUCTION

Ever since Darwin's Origin of Species (1859), biologists have endeavored to understand the relative importance of the ecological and evolutionary factors that contribute to the formation of new species. However, the very definition of a species and which factors contribute most to the creation and maintenance of species remains highly debated (e.g., Abbott et al. 2008 and references therein). Ernst Mayr's (1942) Biological Species Concept (BSC), which defines a

species as "groups of interbreeding natural populations that are reproductively isolated from other such groups", remains the most widely accepted species concept to date (Schemske 2000, Coyne and Orr 2004). The BSC essentially equates the origin of new species with barriers to hybridization because it requires that species be reproductively isolated from one another (Orr 1995, Coyne and Orr 2004). Therefore, identifying the intrinsic and extrinsic barriers that prevent taxa from hybridizing are critical to our understanding of how new species form and how species are maintained in the presence of conspecific gene flow (Orr 1995; but see Nosil 2008 and Mallet 2008).

Reproductive isolation can be driven by a wide variety of factors (Coyne and Orr 2004), both intrinsic and extrinsic, but common to all isolating factors is that they result in barriers to continued hybridization between species. Barriers to hybridization are partitioned into pre and post-mating isolating barriers, with pre-mating barriers (geography, behavior, phenology, etc.) often argued to be more important in the initial divergence of species' lineages (e.g., Schemske and Bradshaw 1999, Schemske 2000, Ramsey et al. 2003, Antonovics 2006, Martin and Willis 2007). Post-mating barriers are further partitioned into pre-and postzygotic isolation (e.g., Moyle et al. 2004), and are predicted to increase with increasing divergence time among lineages (Bateson 1909, Dobzhansky 1937; Müller 1940; Coyne and Orr 1989, 1997). Intrinsic post-mating barriers may also be important in the origin of new species, and particularly in the maintenance of species in cases where pre-mating isolation is weak (Scopece et al. 2007, but see Sobel and Randle in press, Cozzolino and Scopece 2008, Scopece et al. 2008). Here I focus on the evolution of intrinsic post-mating (prezygotic and postzygotic) isolating barriers among species in the genus *Collinsia*, a group of annual plant species known to occur frequently in sympatry with congeners (Randle, Ch. 1), and when found in sympatry, to overlap in flower

phenology and pollinator visitors (Randle, Ch 3; Randle unpubl. data). Thus, for some *Collinsia* species, intrinsic post-mating reproductive isolating barriers may be important in species formation, and are certainly important in the maintenance of species' boundaries when they are sympatric and if pre-mating isolation barriers are weak.

Postzygotic isolation via hybrid sterility or inviability is predicted to increase as a result of the accumulation of between-locus incompatibilities among hybridizing diploid taxa, which increase as divergence time increases (reviewed in Orr 2005). This mechanism to explain hybrid sterility and inviability was first proposed by Bateson (1909), but was verbally formalized by Dobzhansky (1937) and Müller (1940), and mathematically formalized by Orr (1995). It is currently known in the literature as the Bateson-Dobzhansky-Müller (BDM) model of speciation (Coyne and Orr 2004), and it explains the presence of hybrid inviability or sterility (postzygotic isolation) among divergent taxa without either species having to pass through an intermediate maladaptive hybrid state (Dobzhansky 1937, Müller 1940, Orr 1995). In the BDM model, incompatibilities among taxa arise because of allopatric divergence (via drift or natural selection) among lineages, where a substitution arises in one lineage that is neutral or advantageous in its 'native' genetic background, but is deleterious in the 'foreign' genetic background of its sister taxa (Dobzhansky 1937, Müller 1940, Orr and Turrelli 2001, Orr 1995). One prediction of BDM model is that the number of incompatibilities will increase as the square of the divergence time between species; $E[I]=(kt)^2/2$, where I is the total number of incompatibilities, k is the rate of substitutions and t is the time since divergence (Orr 1995, Orr and Turrelli 2001). Thus, a doubling of genetic divergence time causes a fourfold increase in the number of incompatibilities. In animals, many studies have found patterns of increasing reproductive isolation with increasing genetic divergence (a proxy for divergence time), consistent with the

predictions of the BDM model of incompatibility: *Drosophila* (Orr 1987, Orr and Coyne 1989, Pantazidis et al. 1993, Presgraves 2003), fish (Mendelson 2003, Bolnick and Near 2005), frogs (Sasa et al 1998), sea urchins (Zigler et al. 2005), birds (Price and Bouvier 2002, Lijtmaer et al. 2003). In plants, patterns of post-mating isolation may differ from that of animals due to differences between plants and animals in a number of traits (Grant 1976) including mating systems, for example, increased frequency of selfing, apomixes, and vegetative reproduction (Grant 1976, Levin 2000), increased frequency of polyploidy (Orr 1990, Moyle et al. 2004, Tate et al. 2005), or increased isolation in plants by chromosomal rearrangement (Reiseberg 2001, but see Lowry et al. 2008). Examples in plants of between-locus incompatibilities in hybrid crosses include crop species like rice (Matsubara et al. 2003) and cotton (Gerstel 1954), and non-crop species or groups such as *Crepis* (Hollingshead 1930), *Mimulus* (Christie and Macnair 1984, 1987, Fishman and Willis 2001, 2006), the Caryophyllaceae (Weller and Sakai 2001), and *Ceratopteris* (Nakazato et al. 2007). However, there are only two published studies that I am aware of that examine the relationship between reproductive isolation and divergence time across plant genera (Moyle et al. 2004, Scopece et al. 2008), and data from these studies are equivocal with regard to the pattern of reproductive isolation. Moyle et al. (2004) found that in *Silene*, all measures of post-mating reproductive isolation increased with increasing divergence time among species. However similar patterns of isolation were not found for the two other taxa examined, *Glycine* and *Streptanthus* (Moyle et al. 2004). Scopece et al. (2008) found a strong positive relationship between postzygotic reproductive isolation and divergence time in orchids, thus more data are needed to understand these processes in plants. Our system provides a particularly powerful test because of our use of a strongly supported, time-calibrated molecular phylogeny

(Baldwin et al. in prep), dense sampling of sister taxa pairs, and explicit tests of a key assumption of BDM model (allopatric divergence, see below).

Previous work on the evolution of reproductive isolating barriers have found that in many plant and animal systems, prezygotic isolating barriers evolve faster than postzygotic isolating barriers (Gleason and Ritchie 1998, Coyne and Orr 1989, 1997; Mendelson 2003, Wolf et al. 2001, Ramsey et al. 2003). However, these studies typically focus on pre-mating prezygotic isolation, rather than post-mating prezygotic isolation. Distinguishing between these two phases of prezygotic isolation is important, as differences in the rates of evolution in pre-and post-zygotic isolation are often attributed to reinforcement of reproductive barriers among sympatric species. The frequency of reinforcement may differ between pre-mating and post-mating prezygotic isolation. The BDM model does not specifically address intrinsic post-mating prezygotic incompatibilities among divergent lineages. However, it may be reasonable to suspect that intrinsic prezygotic incompatibilities arise in a similar manner as those described by the BDM model for intrinsic post-zygotic isolation, and that incompatibilities between pollen and ovule or pollen and stigma accumulate at a similar rate as other BDM incompatibilities. If this is the case, I would expect a comparable pattern of increasing incompatibility among sister taxa pairs with increasing divergence time for intrinsic post-mating prezygotic and postzygotic isolation. However, there is evidence that proteins on the egg and sperm (or pollen and ovule), involved in species recognition, can evolve rapidly (Swanson and Vacquire 1995, Swanson et al. 2001, Swanson and Vacquire 2002, Swanson and Vacquire 2005), which may result in more rapid evolution of post-mating prezygotic isolation compared to postzygotic isolation.

Reinforcement of reproductive barriers can also result in prezygotic isolation evolving faster that postzygotic isolation. Reinforcement is defined as the evolution of enhanced

reproductive isolation via natural selection to avoid the production of costly maladaptive hybrids, and refers specifically to species that have come into secondary contact after allopatric speciation (Wallace 1889, Dobzhansky 1941, Fisher 1930, Grant 1963, Grant 1966, Coyne and Orr 1989, Noor 1999). Reinforcement of reproductive barriers is predicted to occur more frequently in short-lived organisms for which any loss of reproductive output is particularly disadvantageous (Dobzhansky 1958, Stebbins 1958, Grant 1966). Evidence of reinforcement has been demonstrated by patterns of stronger prezygotic barriers to reproduction among sympatric species than allopatric species with similar divergence times (Coyne and Orr 1989, Howard 1993, reviewed in Noor 1999) and in experiments where selection against hybrids has resulted in ethological barriers to reproduction (Koopman 1950, Wallace 1954, Knight et al. 1956).

Patterns of reinforcement have primarily been found in animal species (reviewed in Hostert 1997). However, evidence of divergent selection on floral phenotype among sympatric species is not uncommon, and includes divergence in floral morphology (Levin and Kerster 1966, Levin 1985, Armbruster et al. 1994, Caruso 2000, Kephart and Theiss 2003, Miyake and Inoue 2003) and floral phenology (Petit et al. 1997, Soliva and Widmer 1999, Antonovics 2006). Divergence in floral phenotypes can arise via direct selection to reduce competitive interactions for pollinator services (Fishman and Wyatt 1999, Levin and Anderson 1970) that indirectly reduces the transfer of pollen between species, or via direct selection for reduced heterospecific pollen flow (i.e., reinforcement). Here I look for evidence of post-mating prezygotic isolation by comparing the strength of prezygotic isolation between sympatric and allopatric species of *Collinsia.* It is possible that because effective ethological barriers are not available to plants, they may evolve strong post-mating prezygotic barriers (i.e., pollen-stigma and pollen-style incompatibility) (but see Servedio 2001). Thus, selection for post-mating, prezygoic isolation

could result from direct selection against the formation of unfit hybrids (i.e., reinforcement), and may actually be a better test of reinforcement because it would not be confounded with divergence in floral characters due to competition for pollinators. Thus, if reinforcement of reproductive barriers occurs in *Collinsia*, I expect post-mating, prezygotic barriers to be stronger among sympatric species than allopatric species.

The BDM model of speciation predicts that crosses between species pairs should be largely symmetrical (Tiffin et al. 2001), as there is no *a priori* expectation that nuclear incompatibilities should arise at a faster rate in one sister lineage than another. Thus, if intrinsic reproductive isolation in *Collinsia* were due largely to BDM nuclear incompatibilities I would expect species pairs not to differ in the strength of reproductive isolation. However, in plants, asymmetry in reproductive isolation among species pairs is common (Grant 1954, Lewis and Crowe 1958, Rick 1963, Kiang and Hamrick 1978, Tiffin et al. 2001, Turelli and Moyle 2007), and may be due to interactions between maternally inherited and nuclear genomes (Turelli and Moyle 2007) or to differences in flower size or pollen tube length (Emms et al. 1996), or mating system (Brandvain and Haig 2005, Martin and Willis 2007).

A fundamental assumption of both the BDM model and the Reinforcement model is that species initially diverge in allopatry, however, this key assumption is almost never specifically addressed in empirical studies. To explicitly test the mode of speciation in *Collinsia,* I employed the method of Barraclough and Vogler (2000), which uses the geographic range of each species, their phylogenetic relatedness, and the estimated age of each species and clade to assess the mode of speciation. Although allopatric speciation is generally thought to be the most likely mode of speciation (Mayr 1942), the high degree of sympatry in *Collinsia* and the importance of

this underlying assumption to both the BDM and reinforcement models prompted us to rigorously test the assumption of allopatric speciation.

Here I use species in the genus *Collinsia* as a model system to test a suite of predictions regarding the relative importance of various factors that may be responsible for reproductive isolation in this group. I examined whether intrinsic pre- and postzygotic isolation increase with increasing divergence time among species, as predicted by the BDM model of speciation. I investigated whether post-mating prezygotic isolation evolves at a similar or faster rate than postzygotic isolation, and whether there is evidence of reinforcement of post-mating prezygotic isolation among sympatric taxa compared to allopatric taxa. Finally, I quantify reproductive isolation at multiple developmental stages in 16 reciprocally crossed species pairs to determine the degree of asymmetry in these sympatric and allopatric taxa.

3.3 METHODS

3.3.1 Study System

The genus *Collinsia* is a monophyletic group comprised of ~22 species of self-compatible winter or spring annual herbaceous species. The center of diversity for *Collinsia* is California and western North America, however, three species are found in eastern North America: *Collinsia parviflora*, which ranges north to British Columbia and Alaska and eastward into Michigan and Ontario, and *C. verna* and *C. violaceae*, which are found exclusively in the eastern half to the mid-western portions of the US, respectively. More than 50% of *Collinsia* have broadly overlapping ranges and many species co-occur in nature, overlap in flowering time, and

share pollinators (Randle, pers. obs., Chapter 4). All *Collinsia* species are pollinated by bees (Rust and Clement 1977, Neese 1993, Kalisz and Vogler 2003) and essentially all species are diploid (n=7; Neese 1993) with rare polyploid populations identified in *C. grandiflora* and *C. heterophylla* (E. Elle pers. com and B. Baldwin pers. com). Early work with this genus by Garber (1975 and references therein) revealed that many species of *Collinsia* are interfertile when hand-pollinated in the greenhouse (Garber 1975). Recent work by Baldwin et al. (in prep.) has resulted in a well-resolved molecular phylogeny. Thus, this genus is ideal for examining how the relative strength of intrinsic post-mating reproductive isolating barriers is expressed between species pairs that differ in their relative divergence times. In our study, post-mating reproductive isolation includes both prezygotic (pollen-pistal and pollen-style interactions) and postzygotic isolation.

3.3.2 Phylogeny and Divergence Time

In our analyses, I use the phylogenetic relationships among species of *Collinsia* and their relative divergence times that was determined by Baldwin et al. (in prep). The tree was inferred using Bayesian inference (MrBayes, Ronquist and Huelsenbeck 2003) of a four-locus dataset including both nuclear and chloroplast loci (rDNA, ITS and ETS +*trn*K intron + CYC1) rooted within the tribe Chelonieae (*Chelone*, *Keckiella*, *Penstemon*). Divergence times were estimated using penalized likelihood (in r8s, Sanderson 2003) with a 15 Ma basal calibration (onset of major summer drying trend in western North America, Axelrod 1986). Divergence times ranged from > 0.1 to 15 Ma (Figure 3.1). Therefore, I was able to choose species with a wide range of divergence times across the entire phylogeny. In total, my hybrid crosses represent 15 nodes in the phylogeny, which encompass 72% of all possible nodes.

3.3.3 Mode of Speciation

One key assumption of the BDM model is that speciation occurred in allopatry, and current sympatric distributions are the result of range expansion and secondary contact. While it has been argued that the majority of speciation is likely to be allopatric (Mayr 1963), I know of no BDM studies that have tested for mode of speciation. Here, I estimate the geographic mode of speciation for *Collinsia* using the geographic range size and range overlap of each clade and the age of each node in the phylogeny using the methods of Barraclough and Vogler (2000). The age of each *Collinsia* clade, at each node, was assigned using the dates from the *Collinsia* phylogeny (Fig. 3.1; Baldwin et al. in prep). The range size of each species and each clade was determined from on-line herbaria collections including Jepson's Consortium of California Herbaria (ucjeps.berkeley.edu/consortium), Global Biodiversity Information Facility (www.gbif.net), Oregon Flora Project, (http://www.oregonflora.org/atlas.php), and BC E-Flora (http://www.eflora.bc.ca; Klinkenberg 2008). I converted all location data from these collections to the NAD 27 coordinate system and used ArcGIS (9.2) to project these data into a North America Albers Equal Area Conical map projection. I then used Hawth's Tools extensions for ArcGIS 9 to create minimum convex polygons (MCP) for each species and clade in the *Collinsia* phylogeny. Polygons were clipped where they extended over water. Finally, I used X Tools Pro (5.2) to calculate the range size and range overlap of each clade at each node. Because the *Collinsia* phylogeny is made up of two major clades that are separated by a deep and well-supported node (10.2 Ma) that differ substantially in their geographic distribution (one primarily California species and the other primarily Northern and Eastern species), I analyzed the relationship between range overlap (sympatry) and divergence time in three ways: for each major clade separately (CA; NE), and using all *Collinsia* species. Sympatry was calculated as the [area

of overlap of two clades (for each node in the phylogeny)]/[range size of the smaller of the two clades] (Chesser and Zink 1994; Barraclough and Vogler, 2000). Thus sympatry values range from 0 (no range overlap) to 1 (one clade's range completely within the range of the other). If the general pattern of speciation in this group is allopatric, then using these methods I expect to find a pattern of increasing sympatry with increasing divergence time because, over time, species expand their range and come into contact secondarily. In contrast, if sympatric speciation were the likely mode, then I would expect decreasing sympatry with increasing divergence time because young species completely overlap in range size, but over time, range expansion results in less sympatry (Barraclough and Vogler, 2000). I analyzed the relationship between sympatry and clade age with a two-tailed Spearman's rank test.

3.3.4 Hybrid Crosses

I collected naturally pollinated seeds of 22 *Collinsia* taxa in Springs of 2004 and 2005 and grew them to flowering in the greenhouse facilities at the U. Pittsburgh in 2005 and 2006. In the Winters of 2005 and 2006, I conducted controlled heterospecific crosses between 35 *Collinsia* taxa pairs, representing the majority of nodes across the entire *Collinsia* phylogeny and ranging in divergence time from <0.1 Ma to 11 Ma (Table 3.1, Fig. 3.1). Crosses were selected based both on the availability of co-flowering taxa growing in the greenhouse and on our desire to maximize crosses between clades at each node of the *Collinsia* phylogeny. Thirty-four of the 35 taxa pairs crossed included reciprocal crosses (i.e., each species served as both dam and sire) totaling 17 full reciprocal crosses (Note: reciprocal crosses were not conducted between *C. sparsiflora v. sparsiflora* X *C. sparsiflora v. collina*; additionally, *C. torreyi v. wrightii* and *C. torreyi v. torreyi* were only examined for prezygotic isolation). For each cross, I used an average

of 9 dams (range 2-28) and conducted an average of 20 pollinations per treatment (range 6-58; Table 3.1). In total, I performed 1377 pollinations among parental taxa, including both conspecific and heterospecific crosses to produce F1s.

3.3.4.1 F1 Crosses

I emasculated pairs of flower buds prior to anther dehiscence on parental plants and randomly assigned buds to receive either conspecific or heterospecific pollen. Flowers were marked with fabric paint on the sepals and subtending leaves with a unique color to denote the pollen treatment. Pollen (from a mix of 2-3 donor plants) was applied to receptive stigmas 2-4 days after emasculation. Only flowers occurring on whorls three or higher were used in our pollinations to standardized the developmental state of the flowers. For each parental cross I scored stage-specific fitness at seven life-stages (Fruit production, F1 seed number, F1 germination rate, F1 pollen viability, F2 seed number, F2 germination rate, and F2 pollen viability; details of data collection are provided below, *Stage-specific fitness measurements*). Because I pollinated multiple flowers on each dam with either conspecific or heterospecific pollen, I pooled the data at each stage for each dam by cross type to calculate mean by dam for each cross type.

3.3.4.2 F2 Crosses

F1 progeny from conspecific and heterospecific parental crosses that survived until flowering were allowed to autonomously produce selfed seed (F2). If an F1 conspecific or heterospecific individual failed to autonomously produce fruit, I hand pollinated all open flowers on that plant with self-pollen collected from its open flowers.

3.3.5 Stage-Specific Fitness Measurements

3.3.5.1 Fruit Production

Subsequent to the hand pollinations of parental plants, I checked each marked and pollinated flower every 2-3 days and scored them for fruit formation (0,1). For each dam and cross type (heterospecific or conspecific) involved in the production of F1 seeds, fruit production was calculated as the proportion of pollinations that resulted in fruit production.

3.3.5.2 Seeds Per Fruit

All marked flowers on parental plants that formed F1 fruits were allowed to mature, each fruit was collected individually, and the number of seeds per fruit was counted. For F1 selfed plants (F2 fruits), mature fruits were collected at random, and the number of seeds per fruit was counted.

3.3.5.3 Germination

The 4665 F1 seeds produced by our conspecific and heterospecific crosses in 2004 and 2005 were planted in 2006 and grown to flowering in the greenhouse facilities at the U. of Pittsburgh. I monitored seedling trays daily to determine the proportion of seeds that germinated. A subset of seeds from each cross per dam were transplanted and grown to flowering. In 2007 I planted 3111 F2 selfed-seeds produced by selfing the F1 progeny (hybrids and conspecifics). I monitored seedling trays daily to determine the proportion of planted F2 seeds that germinated. I transplanted a subset of the F2 seedlings for each cross per dam, and grew them to flowering.

3.3.5.4 F1 and F2 Pollen Viability

I collected four undehisced anthers from each F1 (2007) and F2 (2008) progeny, placed them in a 0.5 ml microcentrifuge tube and placed the tubes inside a desiccation chamber. Tubes were left open for 1-2 days to allow anthers to dehisce. I then added 0.5 ml of aniline blue-lactophenol (Kearns and Inouye 1993) to each tube and sonicated each for 2 minutes to release the pollen from the anthers and preserve the sample. To determine pollen viability, I re-suspended pollen within the microcentrofuge tubes by both carefully crushing preserved anthers with a pestle (to release all pollen) and then vortexing each tube for ~30 seconds. Two samples from each tube were taken with a disposable micropipette, and one drop from each sample was placed on a haemocytometer and covered with a cover slide. For each sample, I counted the total number of pollen grains, and the number of viable pollen grains within a 2.6 mm^2 area under a compound light microscope (magnification =10X). Pollen grains were scored as viable if they were turgid and darkly stained (Willis 1999, Fishman and Willis 2001). Non-viable pollen stained weakly or not at all and appeared concave. I calculated the percentage of pollen grains that were viable for each conspecific and heterospecific cross.

For all 7 stages above for each of the 35 crosses, I expressed the fitness of the heterospecific cross relative to that of the conspecific cross by dividing the mean fitness measure for the heterospecific cross by the mean fitness measure for the conspecific cross at each stage. In each comparison, the species used in the conspecific cross was the same species as the dam in the heterospecific cross. If this value was greater than 1 (i.e., the hybrids did better than the conspecific progeny) I truncated the value to 1. Reproductive isolation at each stage was defined as (1-relative fitness of heterospecific progeny) (Coyne and Orr 1989, 1997, Ramsey et al. 2003, Moyle et al. 2004, Scopece et al. 2008).

3.3.6 Reproductive Isolation and Divergence Time

I tested the hypothesis that reproductive isolation increases as divergence time increases, and determined if the relative rates of reproductive isolation differed between pre- and post-zygotic barriers. I quantified the relationship between reproductive isolation in four ways: using all crosses in our experiment (phylogenetically uncorrected), using the mean values of the reciprocal crosses (phylogenetically uncorrected), using the phylogenetically corrected mean values of the reciprocal crosses (Coyne and Orr 1989, 1997; Moyle et al. 2004), and using the mean values of reciprocal crosses (strictly independent; Felsenstein 1985). For each of these four ways, I regressed the data for three different types of isolation: prezygotic, postzygotic, and total on divergence time.

3.3.6.1 Prezygotic Isolation

Post-mating prezygotic isolation was scored across dams as the proportion of fruit formed by heterospecific crosses relative to conspecific crosses for each species pair = [1-[(% of heterospecific pollinations that formed fruit)/(% of conspecific pollinations that formed fruit)]]. I assume that failure to induce fruit formation is due to either to interactions between the pollen and the stigma, pistil, or gynoecium (Moyle et al. 2004).

3.3.6.2 Postzygotic Isolation

Post-mating, postzygotic isolation was calculated as the sum of the absolute contribution (AC) of reproductive isolation at each stage subsequent to fruit formation. Because early stages reduce the amount of isolation available to later stages, I accounted for the reduction in total isolation at each stage (Ramsey et al. 2003). To do this, I calculated postzygotic reproductive

isolation as follows: $AC_1 = RI_1$; $AC_2 = RI_2$ (1- AC_1); $AC_3 = RI_3$ [1-(RC_1 + RC_2)], … such that total postzygotic isolation across all stages = RI_n (1-$\sum RI_i$).

3.3.6.3 Total Reproductive Isolation

Total reproductive isolation was calculated the same as postzygotic isolation, with the addition of our measure of pre-zygotic isolation as the first of the seven stages. I used Kendall's rank correlation to examine the relationship between divergence time and prezygotic, postzygotic, and total isolation for all four datasets (individual crosses and mean of reciprocal crosses- phylogenetically uncorrected; mean of reciprocal crosses-phylogenetically uncorrected; mean of reciprocal crosses-phylogenetically corrected, and mean of reciprocal crosses-strictly independent).

3.3.7 Relative Rates of Prezygotic verses Postzygotic Isolation

To compare the relative rates of reproductive isolation for prezygotic and postzygotic barriers, I compared the regression coefficients (+/- 95% CI) of pre- and post-zygotic isolation on divergence time. For these analyses I used only the strictly independent data, which is the most conservative test, and as in Moyle et al. (2004) I assumed that diverging species started as a single interbreeding population, thus I constrained the intercept to zero.

3.3.8 Reinforcement of Reproductive Barriers

Examination of the reinforcement of reproductive barriers was done in two ways. First, I scored species pairs as either 0 (no overlap in range = allopatric) or 1 (any overlap range =

sympatric) and compared the regression coefficient of prezygotic isolation on divergence time for allopatric and sympatric species. I predict that reinforcement of reproductive isolation will result in stronger prezygotic isolation in sympatric verses allopatric populations. In addition, I tested for a correlation between the strength of prezygotic isolation and percent range overlap with a partial correlation analysis, controlling for species age. I expect that reinforcement will be stronger among species that overlap more in their range, thus if reinforcement of prezygotic isolation occurs in this group, it should increase with increasing range overlap.

3.3.9 Asymmetry in Reproductive Isolation

To examine the degree of asymmetry between each of the reciprocal crosses across the 16 species pairs in our study, I plotted the absolute contribution (AC) of each stage of isolation I measured for each species pair. For each cross at each stage, I normalized the contribution to reproductive isolation by the conspecific cross of the seed parent.

3.3.10 Statistical Analysis

Because of the non-normal distribution of the data, and in order to make data comparable to early work on the mode of speciation, I used a Spearman's Rank Correlation to examine the relationship between range overlap and divergence time (SPSS 16.0, SPSS Inc. 2007). Likewise, to make this work comparable to earlier work (Coyne and Orr 1989, 1997, Moyle et al. 2004) I used Kendall tau rank correlation to examine the relationship between reproductive isolation and divergence time (SPSS Inc. 2007). To compare the relative rates of prezygotic reproductive

isolation in sympatry vs. allopatry, and between prezygotic and postzygotic isolating barriers I compared the slopes of linear regressions (SAS 9.2, SAS 2007).

3.4 RESULTS

3.4.1 Geographic Mode of Speciation

The BDM model of speciation assumes that species have undergone divergence via drift or selection while in allopatry. As expected under allopatric speciation, I found that across all nodes in the *Collinsia* phylogeny, the area of overlap (sympatry) increases with divergence time (Fig. 3.2A) and the correlation between sympatry and divergence time was marginally significant (two-tailed test Spearman rank; Rho=0.42, df=18, p=0.065). However, in the separate analyses of the Northeastern (NE) clade and the California (CA) clade (Fig. 3.2B and 3.2C) the NE clade shows no relationship between range overlap and divergence time whereas the CA clade shows a strong positive correlation with sympatry and divergence time (two-tailed tests, Spearman rank; NE: Rho=0.198, df=5, p=0.67 vs. CA: Rho=0.777, df=11, p=0.002). Thus, across the entire phylogeny, I find a weakly positive correlation between sympatry and divergence time because of the strong relationship in the CA clade. The CA clade pattern is consistent with our expectations for allopatric speciation, while I cannot make any conclusions about the geographic mode of speciation for the NE clade.

3.4.2 Reproductive Isolation and Divergence Time

Intrinsic post-mating reproductive isolation was strongly positively correlated with divergence time for prezygotic, postzygotic, and total isolation for all data sets examined (Table 3.2; Fig. 3.3). This is consistent with the expectation that BDM incompatibilities increase with increasing divergence time among lineages.

3.4.3 Relative Rates of Reproductive Isolation

Consistent with expectations of the BDM model, both prezygotic and postzygotic isolation increase with increasing divergence at similar rates (Fig. 3.4; prezygotic isolation R^2=0.754; t=4.63, df=1, p=0.0024; postzygotic isolation R^2=0.786; t=4.69, df=1 p=0.0033).

3.4.4 Reinforcement of Reproductive Barriers

There was little evidence of reinforcement of reproductive barriers among sympatric species (scored as any degree of range overlap) compared to allopatric species (scored as no range overlap) (Fig. 3.5; allopatric R^2=0.931, t=9.74(1) p=0.0001; sympatric isolation R^2=0.875,t=7.49(1) p=0.0001). The slope of the regression of prezygotic isolation on divergence time was consistently higher among sympatric species than among allopatric species (Fig. 3.5), but the 95% CI of the regression lines overlap. Finally, I found no correlation between the strength of prezygotic isolation and area of range overlap (i.e. degree of sympatry) controlling for species age, (partial correlation analysis; r=0.079, df=15, p=0.381). This lack of correlation

suggests that selection for reinforcement of intrinsic prezygotic isolating barriers is not occurring.

3.4.5 Asymmetry Among Species Pairs in Reproductive Isolation

The majority of species pairs showed similar levels of isolation (69%). Across all 34 reciprocal crosses, only 12 hybrid crosses survived until the F2 generation, and most of these were from species that diverged less than 4 Ma (Fig. 3.1). In only two of the 16 hybrid crosses (12.5%) did I find asymmetry in reproductive isolation in the later stages (F1 germ-F2 pollen viability; Fig. 3.6), and both crosses involved taxa that were very recently diverged (<0.1 Ma: *C. bartsiifolia v. bartsiifolia* X *C. bartsiifolia v. davidsonii* and *C. parryi* X *C. concolor*). In three of 16 hybrid crosses (19%), prezygotic isolation differed substantially among species pairs, with one species almost completely isolated at the fruit formation stage, while the other species made fruits, but either had low germination success (*C. childii* X *C. sparsiflora v. sparsiflora* and *C. grandiflora* X *C. multicolor*), or low F1 pollen viability (*C. multicolor* X *C. heterophylla*; Fig. 3.6).

3.5 DISCUSSION

Work on *Collinsia* in the 1960's and 1970's by Garber (summarized in Garber 1975) revealed that many *Collinisa* species could hybridize and make viable F1 progeny. In a series of cytological studies over many years, Garber conducted ~ 90 hybrid crosses among 17 *Collinsia* taxa, and found that half resulted in hybrid offspring, and of those approximately 20 were fertile

(Garber 1975). His approach of 'experimental taxonomy', using hybrid crosses to determine the relatedness of species is essentially the reciprocal experiment to the one I conducted here. For Garber's assumption was that species that can hybridize are likely to be close relatives, whereas our assumption is that because species are close relatives, they should have fewer barriers to isolation, and thus should hybridize well. For the crosses that I have in common with Garber, our data are qualitatively similar with one exception. He found that crosses between *C. linearis* and *C. rattanii* were unsuccessful, whereas I find these two species to be highly interfertile.

Using species in the genus *Collinsia*, I tested two prominent hypotheses related to the evolution of post-mating reproductive isolation in plants, the BDM model of reproductive isolation and the reinforcement model. Our data are consistent with the BDM model of isolation for both post-mating prezygotic and postzygotic isolation, which did not differ in their evolutionary rates. However, I found no evidence for stronger reinforcement of post-mating prezygotic isolating barriers in sympatric compared to allopatric species pairs of similar age, as would be expected if reinforcement was important in maintaining species' boundaries.

3.5.1 Mode of Speciation

The BDM and Reinforcement models both assume that species diverged in allopatry, thus I tested for the geographic mode of speciation in the genus *Collinsia.* The method I used to test for the geographic mode of speciation employs a species-level phylogeny, estimates of divergence time, and the estimates of the geographic range of species and clades to compare the pattern of range overlap (sympatry) through time (Barraclough and Vogler 2000). I found a weak, positive correlation between the degree of sympatry and divergence time across all nodes of the *Collinsia* species phylogeny. However, when I looked at the two most prominent clades

of *Collinsia* separately (NE and CA clade), I found a strong positive correlation between sympatry and divergence time in the California clade, suggesting allopatric speciation, but no relationship between sympatry and divergence time in the Northeastern clade. Thus our data show strong evidence of allopatric speciation in the CA clade, but no conclusions about mode of speciation can be drawn from the NE clade. Barraclough and Vogler (2000) suggest that large shifts in range size will obscure any pattern of mode of speciation. In the NE clade, this indeed appears to be the case (Fig. 3.2). *Collinsia parviflora*, in the NE clade, is the most widespread *Collinsia* species, with a range that includes western states, Canada, and several eastern states. Thus clearly this species, which is relatively young (Fig. 3.1), has undergone substantial range expansion in a very short amount of time, relative to other *Collinsia* species. Overall our data is consistent with allopatric speciation for the entire *Collinsia* clade and for the CA clade, but across all species this relationship is not strong. This method of determining the mode of speciation has been strongly criticized due the difficulty of inferring ancestral range sizes from extant species, and because ranges size are dynamic and can change quickly (expand or contract) over time (Losos and Glor 2003 and references therein). However, recent studies have shown a general pattern of increasing range size with increasing divergence time (Paul and Tonsor 2009, and Paul et al. 2009), which is an assumption of this approach. In addition, it is generally assumed that allopatric speciation is the norm, and that sympatric and peripatric speciation are rare (Mayr 1963, Wiley and Mayden 1985), thus our attempt here was to support that assumption with complementary evidence. So while this analysis does not conclusively indicate that *Collinsia* species diverged in allopatry, it adds further support to that general assumption.

3.5.2 Reproductive Isolation and Divergence Time

I found that both post-mating prezygotic and postzygotic isolation increased with increasing divergence time among species. This is consistent with what was found in *Silene* by Moyle et al. (2004) and in orchids by Scopece et al. (2008). Thus, *Collinsia* species appear to accumulate genetic incompatibilities over time, in a manner consistent with the predictions of BDM model of reproductive isolation. For post-zygotic isolation and total isolation, regardless of which data set was used (e.g., individual crosses uncorrected, mean of reciprocal crosses uncorrected, mean of reciprocal crosses corrected, or mean of crosses strictly independent) I found that reproductive isolation increased rapidly, and that total isolation was nearly complete by four million years. Results from hybrid crosses by Garber (1975) also support this result. The steepness at the beginning of the curve is consistent with a "snowball effect" (Orr 1995), which states that isolation should increase rapidly, at approximately the square of the divergence time until it reaches an asymptote. Our measure of post-mating prezygotic isolation also increased with increasing divergence time, but the steepness of the slope was not as great, which is the opposite of what is predicted for prezygotic isolation. However, when I compared the relative rates of post-mating prezygotic and postzygotic isolation for the strictly independent data set, I found that the slopes of the lines did not differ significantly. Thus I found no difference in the rate of prezygotic verses postzygotic isolation. Studies showing that prezygotic isolation evolves faster than postzygotic isolation are typically done in animals (e.g., Blair 1964, Gleason and Ritchie 1998, Coyne and Orr 1989, 1997; Mendelson 2003), or if in plants (e.g., Ramsey et al. 2003) include earlier pre-mating isolating barriers than the one I tested here. Perhaps our results would be different if I included earlier stages of prezygotic isolation such as pollinator

isolation or flower phenology. Our results were similar to what Moyle et al. (2004) found *Silene* or *Glycine*.

Overall our data is consistent with the accumulation of BDM incompatibilities between divergent lineages over time. However, it is possible that chromosomal rearrangements (Rieseberg 2001) and/or changes in chromosome number (Rieseberg 1997, Mallet 2007) could also cause reproductive isolation among *Collinsia* species. Large chromosomal rearrangements are likely to result in abrupt changes in incompatibility among species (Fishman and Willis 2001), as opposed to incremental increases in isolation with increasing divergence time. However, if chromosomal rearrangements are small or few in number among closely related species, and increase in magnitude and number between more distantly related species, then the pattern of increased reproductive isolation with time could be similar to that predicted by the BDM model. In the crosses conducted by Garber (1975) he found from 0-3 heterozygous paracentric inversions in 18 of the interspecific hybrid crosses. Thus, it appears that small chromosomal rearrangements do occur when *Collinsia* hybridize, but they act in a similar manner as BDM incompatibilities. Experimental crosses in conjunction with some form of gene mapping could help reveal the underlying genetic architecture of these species and help elucidate what mechanisms are responsible for the patterns I observed between divergence time and isolation. Although I did not have the opportunity to do this, it could be a powerful approach in the future (see Fishman and Willis 2001). One way to test for chromosomal rearrangement vs. BDM incompatibilities is to compare the relative fitness of the F1 and F2 hybrids (Fishman and Willis 2001). In chromosomal rearrangement, the F2 hybrids are expected to regain some fitness and are predicted to have greater viability than the F1 hybrids. In contrast, BDM incompatibilities predict that F2 hybrids should have equal or lower fitness than F1 hybrids

(Fishman and Willis 2001, Fishman and Stratton 2004). Unfortunately, I could not do this test, because our F2 hybrids were self-pollinated, thus a comparison between the fitness of F1 and F2 hybrids would be potentially confounded. However, the F2 parental controls were also self-pollinated in the F2 generation, so I can consider the relative fitness of each hybrid relative to the parental. Of the few species that made F2 hybrids, isolation increased between the F1 and F2 stages (i.e., F2 had lower relative fitness; Fig. 3.6). Although this is not a definitive test of BDM vs. chromosomal rearrangement, it does show that our data is consistent with the predictions of BDM model.

In experimental hybrid crosses, Garber (1975) was able to produce polyploids from both distantly and closely related species, thus it is possible that new species of *Collinsia* could arise via polyploid speciation. However, there is little evidence of polyploids in natural populations, and polyploids are only known from some populations of *C. heterophylla* and *C. grandiflora* (pers. con. B. Baldwin and E. Ellie). Hence, it seems unlikely that polyploidy is a major contributor to patterns of speciation in *Collinsia*.

3.5.3 Reinforcement of Reproductive Barriers

Reinforcement of reproductive barriers occurs when species come in to contact that are not completely reproductively isolated, and selection acts against the production of unfit hybrid offspring (Reviewed in Noor 1999). Reinforcement results in stronger prezygotic isolation among sympatric species relative to allopatric species of the same age. I looked for evidence of reinforcement of reproductive barriers in two ways. The first measure of reinforcement was similar to the methods of Coyne and Orr (1989, 1997) and Moyle et al. (2004) where I categorized species that overlap in any part of their range as sympatric, and those that did not

overlap as allopatric. I looked for evidence of increased post-mating prezygotic isolation in sympatric species compared to allopatric species over time. Although the slope of the regression for the sympatric species pairs was steeper, suggesting greater prezygotic isolation, the difference between sympatric and allopatric species pairs was not significant. I also examined that relationship between the area of range overlap among species pairs and the degree of reproductive isolation, controlling for species age. Our expectation was that reinforcement of reproductive barriers would be greater the more species overlap in range. However, I found no significant relationship between range overlap and post-mating prezygotic isolation. Evidence for increased post-mating, prezygotic barriers to reproduction in sympatry relative to allopatry in plants is not common, but was found in two separate groups of *Gilia* taxa (Grant 1966). Our data again were similar to that of Moyle et al. (2004) who found no evidence of reinforcement in *Glycine* and *Silene.* However, one potential problem with these and earlier analyses using these same methods, is that reinforcement of reproductive barriers may only occur in populations where gene flow between species was historically high. Thus, looking for evidence of reinforcement at the species level rather than at the population level may be problematic. In addition, without knowing what the potential for gene flow was in the populations where these species were collected, it may not be surprising that I did not detect reinforcement post-mating prezygotic isolation. Thus it remains equivocal whether or not reinforcement of reproductive barriers occurs and is important at the post-mating prezygotic stage in *Collinsia*.

3.5.4 Asymmetry in Reproductive Isolation

Asymmetry in post-mating reproductive isolation among reciprocally crossed taxa is common [Tiffin et al. 2001, Turelli and Moyle 2007 (and references therein), Takami et al. 2007,

Lowry et al. 2008]. In hybrid crosses between species with heterogametic sex determination, the heterogametic sex is more likely to be sterile or inviable, as predicted by Haldane's Rule (reviewed in Orr 1997). However, asymmetry is not predicted to occur between species crosses if only BDM nuclear incompatibilities (or chromosomal rearrangements) are responsible for post-mating reproductive isolation (Tiffin et al. 2001). In species that do not have heterogametic sex determination, other factors, such as differences in flower size or pollen tube length (Emms et al. 1996) and mating system (Brandvain and Haig 2005, Martin and Willis 2007) can influence hybridization success in species pairs differentially. For example, pollen from small flowered species may have shorter or slower pollen tubes, and may not be able to successfully reach the ovules of a larger flowered species (Emms et al. 1996). Also, highly selfing species may have pollen that experience little pollen competition, and thus are less vigorous than pollen from a highly outcrossing species (Brandvain and Haig 2005). Finally, asymmetric reproductive isolation can occur when incompatibilities occur between maternally inherited and nuclear genetic factors, (Darwins Corollary, Turelli and Moyle 2007, Bolnick et al. 2008). In *Collinsia,* I found that the majority of reciprocal hybrid crosses were not strongly asymmetric in reproductive isolation. However, three species pairs differed greatly in the early stages of post-mating isolation suggesting factors other than nuclear BDM incompatibilities are contributing to species isolation for these pairs. For these three species pairs, post-mating prezygotic isolation strongly differed, suggesting that in one direction, pollen-stigma or pollen-ovule interactions were important (Fig. 3.6). These species pairs did not differ in flower size or mating system (Randle Ch. 1, Kalisz unpubl. data). In the two youngest species pairs, reproductive isolation differed in later stages, which may be the result of differences in maternally inherited and nuclear genetic factors (Turelli and Moyle 2007, Bolnick et al. 2008).

3.5.5 Conclusions

Collinsia species appear to have diverged in allopatry, which makes them an excellent group to test both the evolution of BDM post-mating incompatibilities and reinforcement of reproductive barriers. Post-mating reproductive isolating barriers increased with increasing divergence time at similar rates for both pre and postzygotic isolating barriers. I found no evidence of reinforcement of post-mating prezygotic reproductive barriers in sympatric populations, and I found few species pairs that showed strong asymmetry in reproductive isolation. Where these differences were strong; they involved very early stages of reproductive isolation, suggesting negative interactions between pollen and stigma or ovule in one direction.

This work adds significantly to the growing body of literature elucidating patterns of reproductive isolation in plants. It is one of only three studies that examines the pattern of post-mating reproductive isolation across an entire group of related plant species, and demonstrates that plants likely exhibit similar patterns to animals with regard to increasing reproductive isolation with increasing divergence time. However the relative rates of pre-and post-zygotic isolation and reinforcement of reproductive barriers do not appear to be similar to what has been found in animals systems, but clearly more work needs to be done here. Finally, detailed genetic work that can distinguish BDM incompatibilities from small chromosomal rearrangements, would greatly contribute to our understanding of postzytogic reproductive isolation in this group.

Table 3.1. Correlations between reproductive isolation and divergence time for three levels of reproductive isolation (RI; prezygotic, postzygotic, and total). Data was analyzed in 4 ways: 1) each hybrid cross considered separately (Full data set; not corrected), 2) mean RI of reciprocal pairs (Full data set, not corrected), 3) mean RI of reciprocal pairs (phylogenetically corrected), and 4) mean RI of reciprocal pairs (strictly independent). All analyses showed that RI was positively correlated with divergence time at all stages. **C. sparsiflora* v. *sparsiflora* was combined with *C. sparsiflora* v. *arvensis* for the cross with *C. sparsiflora* v. *colina*- no reciprocal cross was done for this species pair, thus it's not included in the "mean of reciprocal cross" analyses. ***C. torrey v. torreyi* and *C. torreyi v. wrightii* were only examined at the prezygotic stage because of poor germination for both conspecific and heterospecific crosses.

Reproductive Isolation	N	Kendall's τ	P-value
Total Isolation*			
Full data set (All single crosses)	33	0.721	**0.005**
Full dataset mean of all reciprocal crosses	16	0.587	**0.025**
Mean of reciprocal crosses (Phylogenetically corrected)	14	0.695	**0.01**
Mean of reciprocal crosses (Strictly independent)	9	0.833	**0.01**
Prezygotic Isolation**			
Full dataset (All single crosses)	35	0.671	**0.05**
Full dataset mean of all reciprocal crosses	17	0.776	**0.01**
Mean of reciprocal crosses (Phylogenetically corrected)	15	0.765	**0.01**
Mean of reciprocal crosses (Strictly independent)	8	0.63	**0.05**
Postzygotic Isolation			
Full dataset (All single crosses)	33	0.686	**0.01**
Full dataset mean of all reciprocal crosses	16	0.701	**0.01**

Reproductive Isolation	N	Kendall's τ	P-value
Postzygotic Isolation (cont.)			
Mean of reciprocal crosses (Phylogenetically corrected)	14	0.75	**0.05**
Mean of reciprocal crosses (Strictly independent)	7	0.671	**0.01**

Table 3.2. Divergence time, population, and sample size for the initial conspecific and heterospecific crosses of *Collinsia*.

D-time (Ma)	Species (dam)	Species (sire)	Pop (dam)	Pop (sire)	# of Faml.	# of Crosses
0	*C. bartsiifolia v. bartsiifolia*	*C. bartsiifolia v. bartsiifolia*	OWSLV & RH	OWSLV & RH	3	11
0	*C. bartsiifolia v. bartsiifolia*	*C. bartsiifolia v. davidsonii*	OWSLV & RH	FHL	3	12
0	*C. bartsiifolia v. davidsonii*	*C. bartsiifolia v. bartsiifolia*	FHL	OWSLV & RH	3	19
0	*C. bartsiifolia v. davidsonii*	*C. bartsiifolia v. davidsonii*	FHL	FHL	3	12
0	*C. concolor*	*C. concolor*	Kristen's	Kristens	6	12
0	*C. concolor*	*C. parryi*	Kristens	UNK (Kristens)	6	12
0	*C. parryi*	*C. concolor*	UNK (Kristens)	kristens	9	9
0	*C. parryi*	*C. parryi*	UNK (Kristens)	UNK (Kristens)	11	11
0.1	*C. sparsiflora v. sparsiflora*	*C. sparsiflora v. sparsiflora*	MRWV	MRWV	8	16
0.1	*C. sparsiflora v. sparsiflora*	*C. sparsiflora v. colina*	MRWV	855	4	8
0.6	*C. bartsiifolia v. bartsiifolia*	*C. bartsiifolia v. bartsiifolia*	OWSLV & QHR	OWSLV & QHR	3	6
0.6	*C. bartsiifolia v. bartsiifolia*	*C. corymbosa*	OWSLV & QHR	MD	5	10
0.6	*C. corymbosa*	*C. bartsiifolia v. bartsiifolia*	MD	OWSLV & QHR	4	8

D-time (Ma)	Species (dam)	Species (sire)	Pop (dam)	Pop (sire)	# of Faml.	# of Crosses
0.6	*C. corymbosa*	*C. corymbosa*	MD	MD	4	8
0.9	*C. rattanii*	*C. rattanii*	AG500	AG500	14	30
0.9	*C. rattanii*	*C. linearis*	AG500	AG500	28	58
0.9	*C. linearis*	*C. rattanii*	AG500	AG500	13	20
0.9	*C. linearis*	*C. linearis*	AG500	AG500	13	17
1.7	*C. antonina*	*C. antonina*	ILR	ILR	13	29
1.7	*C. antonina*	*C. concolor*	ILR	R3R	13	26
1.7	*C. concolor*	*C. antonina*	R3R	ILR	13	29
1.7	*C. concolor*	*C. concolor*	R3R	R3R	13	32
2.5	*C. multicolor*	*C. multicolor*	CSR	CSR	5	10
2.5	*C. multicolor*	*C. heterophylla*	CSR	SC-D & HBR	5	10
2.5	*C. heterophylla*	*C. multicolor*	SC-D & HBR	CSR	5	9
2.5	*C. heterophylla*	*C. heterophylla*	SC-D & HBR	SC-D & HBR	5	9
2.8	*C. verna*	*C. verna*	Western PA	Western PA	10	32

D-time (Ma)	Species (dam)	Species (sire)	Pop (dam)	Pop (sire)	# of Faml.	# of Crosses
2.8	*C. verna*	*C. violacea*	Western PA	Western PA	10	31
2.8	*C. violacea*	*C. verna*	Western PA	Western PA	14	14
2.8	*C. violacea*	*C. violacea*	Western PA	Western PA	17	17
2.8	*C. heterophylla*	*C. heterophylla*	ARSF	ARSF	10	29
2.8	*C. heterophylla*	*C. corymbosa*	ARSF	MD	10	30
2.8	*C. corymbosa*	*C. heterophylla*	MD	ARSF	10	20
2.8	*C. corymbosa*	*C. corymbosa*	MD	MD	10	20
2.8	*C. multicolor*	*C. multicolor*	CSR	CSR	10	26
2.8	*C. multicolor*	*C. bartsiifolia v. davidsonii*	CSR	FHL	10	28
2.8	*C. bartsiifolia v. davidsonii*	*C. multicolor*	FHL	CSR	10	35
2.8	*C. bartsiifolia v. davidsonii*	*C. bartsiifolia v. davidsonii*	FHL	FHL	10	39
3.2	*C. corymbosa*	*C. corymbosa*	MD	MD	5	10
3.2	*C. corymbosa*	*C. tinctoria*	MD	MDCCA	5	10
3.2	*C. tinctoria*	*C. corymbosa*	MDCCA	MD	9	17

D-time (Ma)	Species (dam)	Species (sire)	Pop (dam)	Pop (sire)	# of Faml.	# of Crosses
3.2	*C. tinctoria*	*C. tinctoria*	MDCCA	MDCCA	11	21
3.8	*C. greenei*	*C. greenei*	HSRA	HSRA	2	4
3.8	*C. greenei*	*C. heterophylla*	HSRA	HBR & SC-D	2	4
3.8	*C. heterophylla*	*C. greenei*	HBR & SC-D	HSRA	3	5
3.8	*C. heterophylla*	*C. heterophylla*	HBR & SC-D	HBR & SC-D	3	5
5.7	*C. childii*	*C. childii*	BCSB	BCSB	16	24
5.7	*C. childii*	*C. sparsiflora v. sparsiflora*	BCSB	LRBV	14	24
5.7	*C. sparsiflora v. sparsiflora*	*C. childii*	LRBV	BCSB	14	16
5.7	*C. sparsiflora v. sparsiflora*	*C. sparsiflora*	LRBV	LRBV	15	19

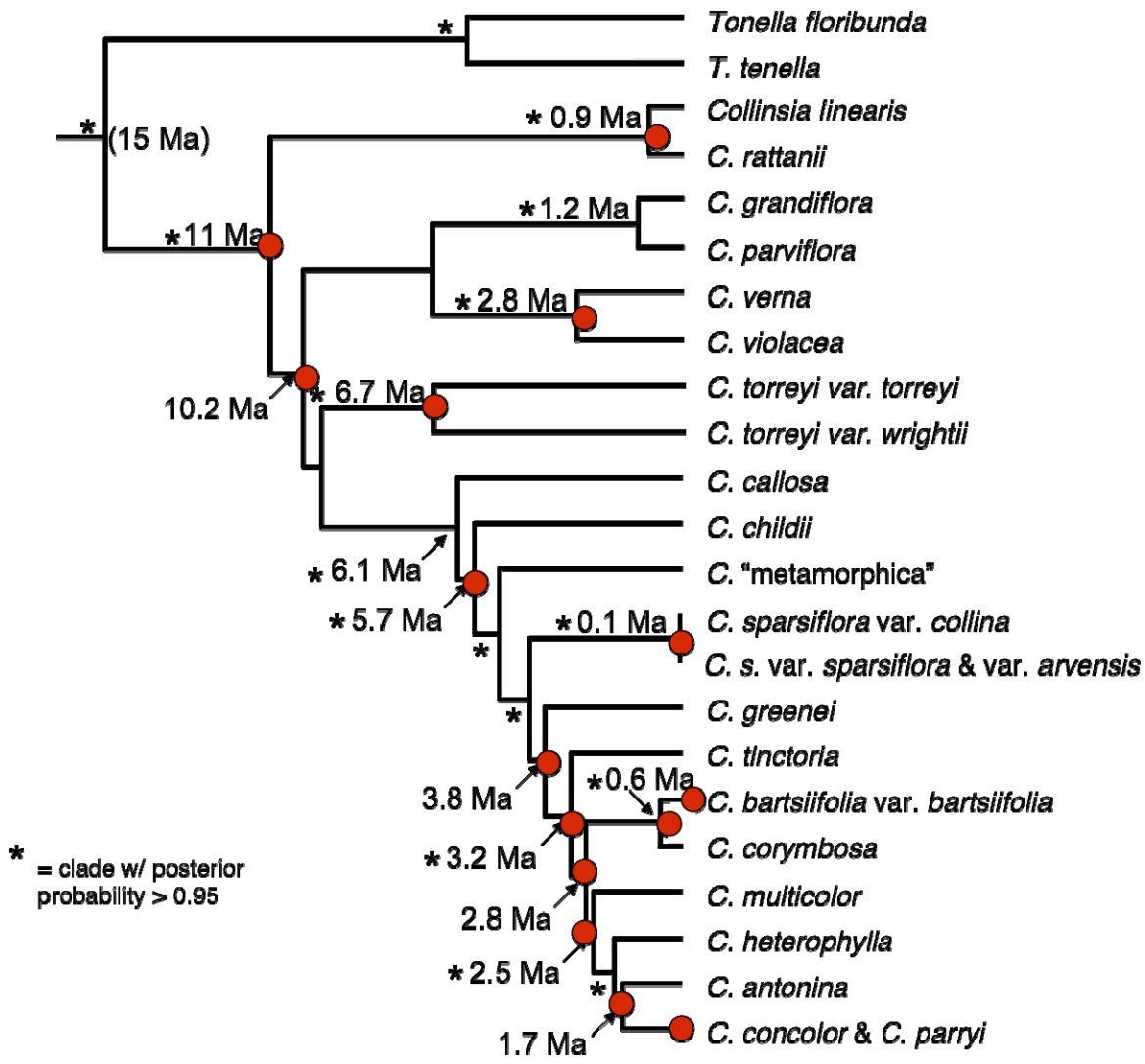

Figure 3.1. Chronogram of tribe Collinsieae (Bayesian tree of rDNA ITS and ETS +*trn*K intron + CYC1). The Collinsieae clade is rooted with tribe Chelonieae (*Chelone*, *Keckiella*, *Penstemon*). Divergence times were estimated using penalized likelihood (in r8s) and a basal calibration of 15 Ma, which is earliest onset of the drying trend in western North America (Axelrod 1986); (Baldwin et al., in prep). Dots represent nodes in the phylogeny that were used in hybrid crosses conducted for these analyses and represent 72% of all possible nodes across the phylogeny.

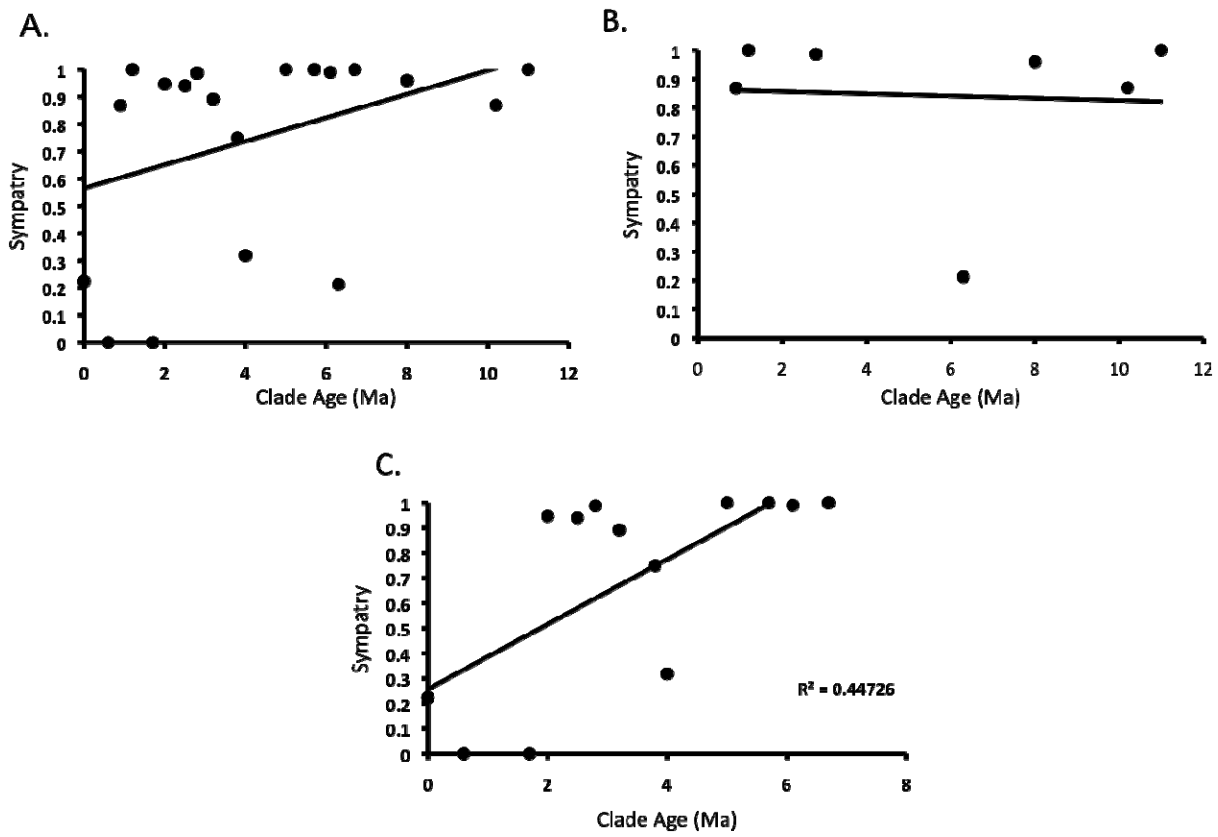

Figure 3.2. Estimates of the geographic mode of speciation for *Collinsia* using the relationship between the degree of sympatry and clade age across all nodes of the *Collinsia* phylogeny. A positive relationship is consistent with the expectation for allopatric speciation, whereas a negative relationship is consistent with the expectation for sympatric speciation. For this analyses, the three varieties of *C. sparsiflora* (var. *sparsiflora,* var. *arvensis*, and var. *colina),* and the two varieties of *C. bartsiifolia* (var. *bartsiifolia* and var. *davidsonii*) were combined into a single taxa because of their recent divergence times. In contrast, varieties of *C. torreyi* (var. wrightii and var. latifolia) were considered separately as they diverged 6.7 Ma (see Figure 1). Graphs depict the relationship between the area of sympatry and clade age in (A) all nodes of the *Collinisa* phylogeny, (B) all nodes in the monophyletic "Northern and Eastern" (NE) Clade, and (C) all nodes in the monophyletic "California" (CA) Clade.

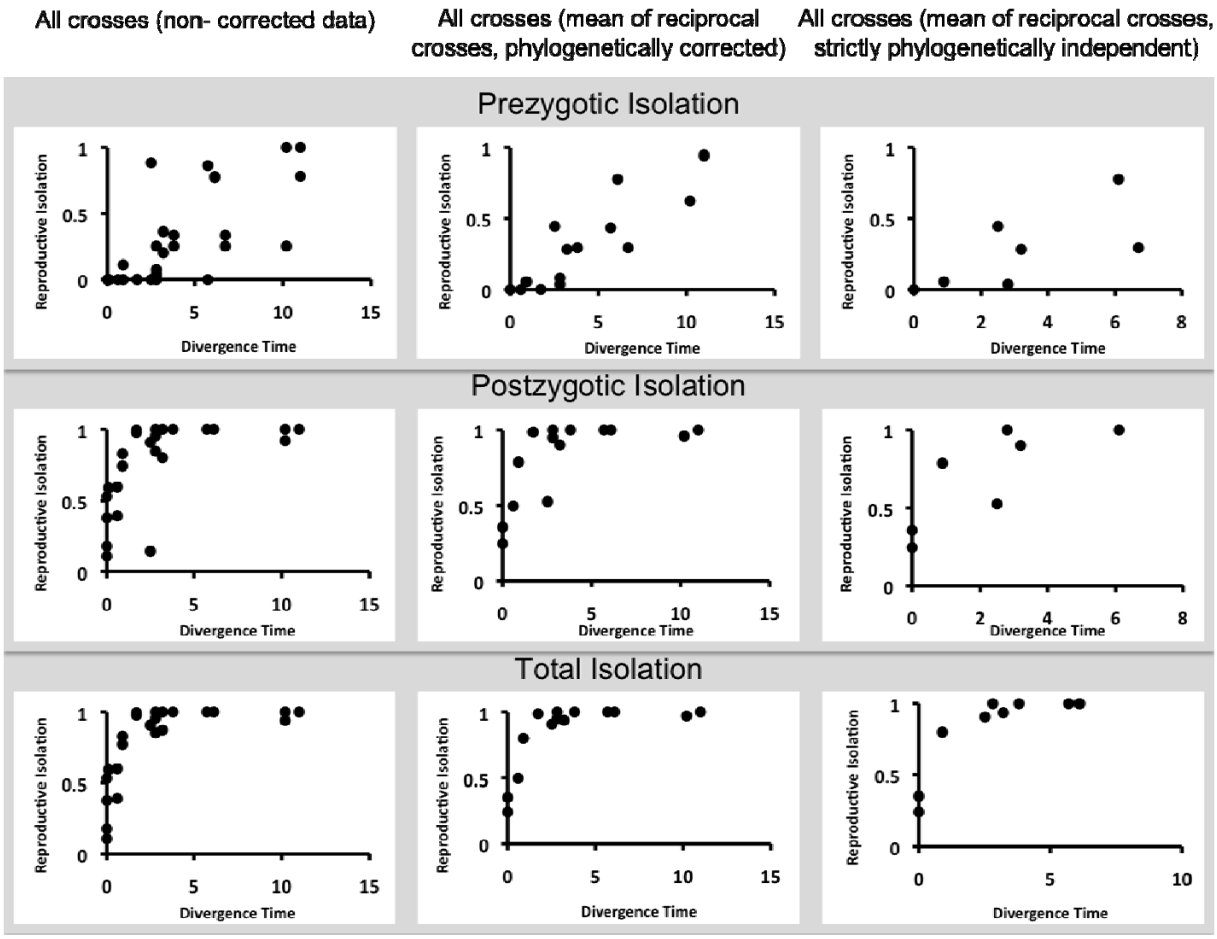

Figure 3.3. The relationship between reproductive isolation and divergence time for 17 species pairs of *Collinsia* for in pre-, post-, and total isolation (for the full dataset, the phylogenetically corrected data set (means of reciprocal crosses), and the strictly independent (mean of reciprocal crosses).

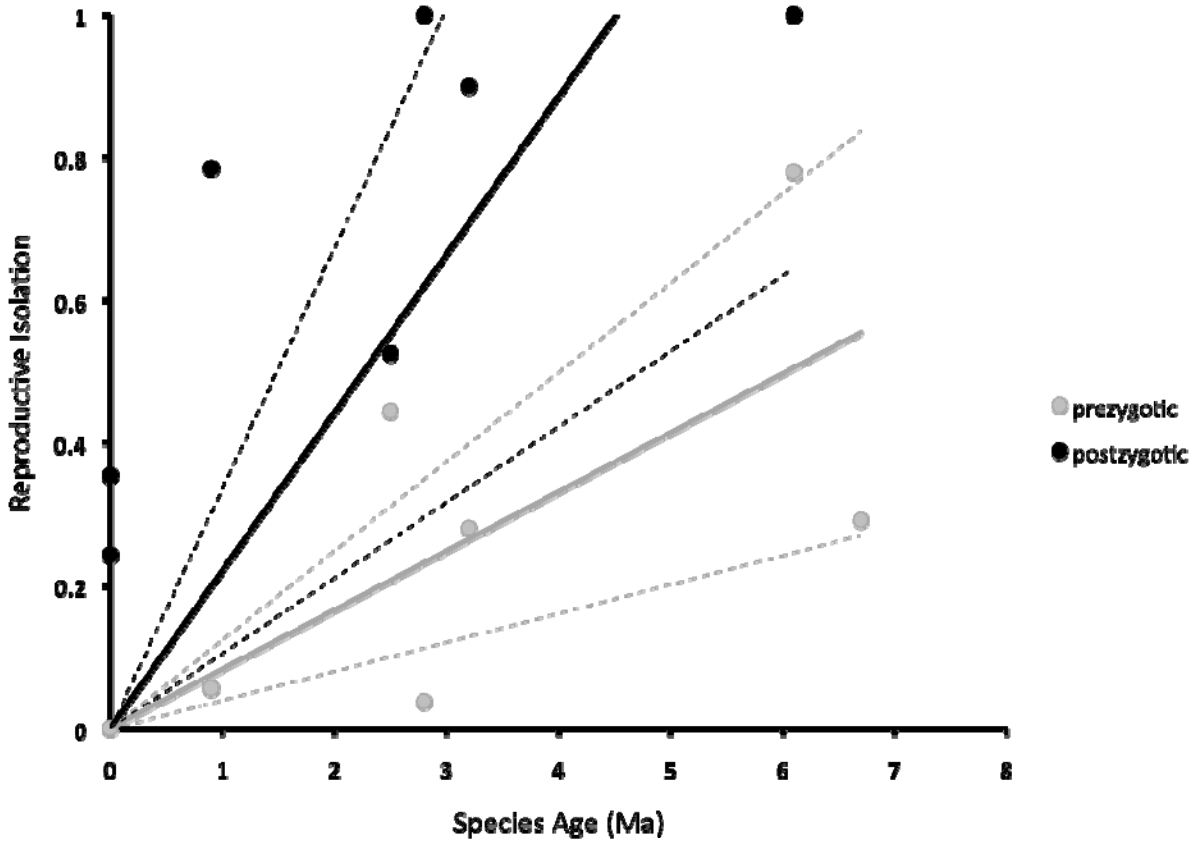

Figure 3.4. Linear regression (+/- 95% CI) of reproductive isolation on divergence time for prezygotic and postzygotic reproductive isolation among strictly independent pairs of reciprocal crosses of *Collinisa* species.

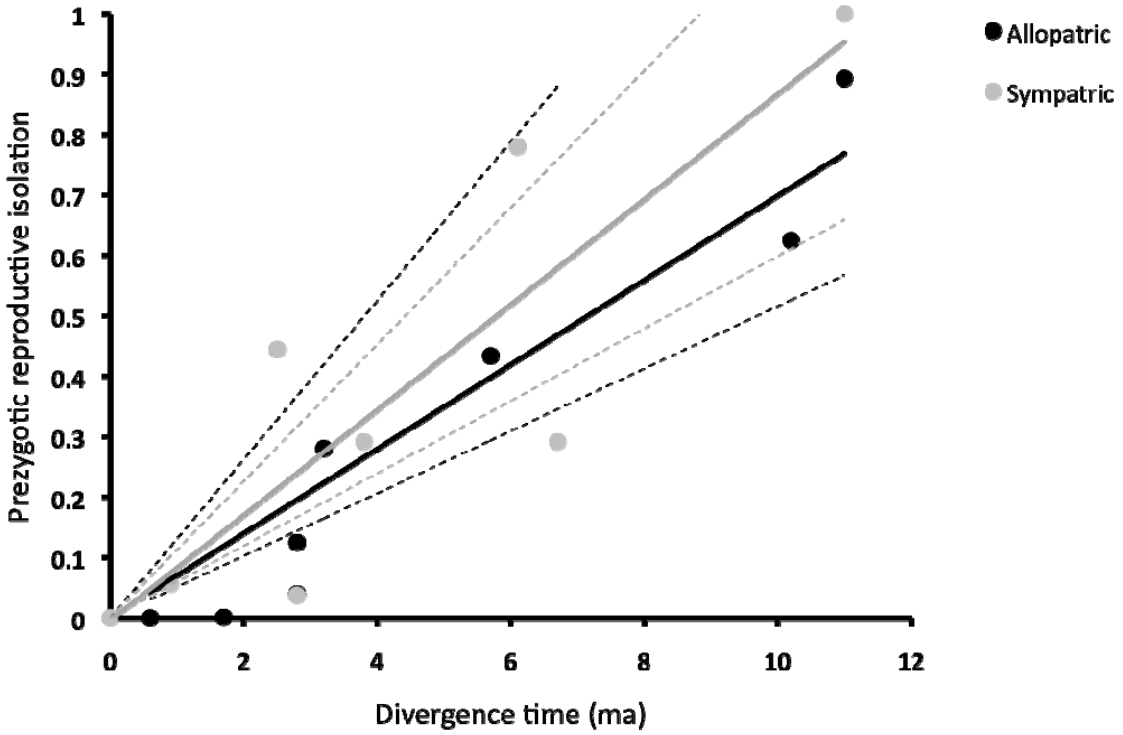

Figure 3.5. Linear regression (+/- 95% CI) of post-mating prezygotic isolation among species pairs that occur in sympatry and allopatry. Species were considered sympatric if they overlapped in any portion of their range (following Coyne and Orr 1989; Moyle et al. 2004).

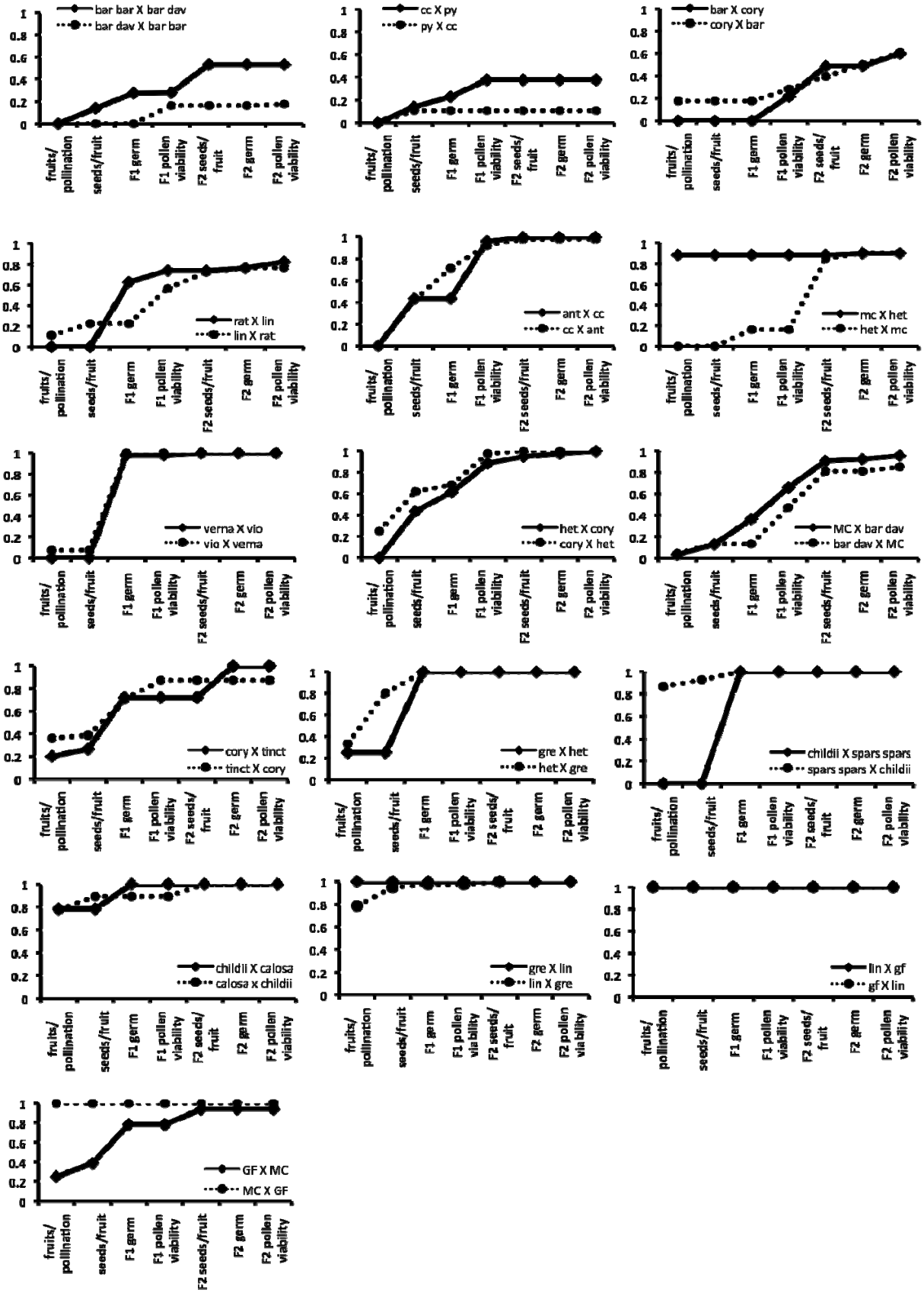
bar bar X bar dav
bar dav X bar bar
cc X py
py X cc
bar X cory
cory X bar
rat X lin
lin X rat
ant X cc
cc X ant
mc X het
het X mc
verna X vio
vio X verna
het X cory
cory X het
MC X bar dav
bar dav X MC
cory X tinct
tinct X cory
gre X het
het X gre
childii X spars spars
spars spars X childii
childii X calosa
calosa x childii
gre X lin
lin X gre
lin X gf
gf X lin
GF X MC
MC X GF
fruits/ pollination
seeds/fruit
F1 germ
F1 pollen viability
F2 seeds/ fruit
F2 germ
F2 pollen viability

Figure 3.6. Accumulation of reproductive isolating barriers in reciprocal hybrid crosses. The value of reproductive isolation at each stage is quantified relative to the conspecific cross and is 0 if isolation is less than or equal to the parental cross at each stage. Because each stage represents a sequential period of the life-cycle, early stages of reproductive isolation have greater effect on total isolation than later stages (Ramsey et al. 2003).

4.0 DOES MATING SYSTEM CONTRIBUTE TO REPRODUCTIVE ISOLATION IN SYMPATRIC POPULATIONS OF *COLLINSIA* SPECIES?

4.1 ABSTRACT

When closely related species co-occur in sympatry, prezygotic isolating barriers are predicted to minimize the opportunity for heterospecific gene flow between species. In *Collinsia,* many species are known to co-occur in sympatry, to co-flower, and to share pollinators. However, few hybrid offspring are observed in natural populations. Thus, I examined the degree to which prezygotic isolating barriers minimize the potential for gene flow between two sympatric sister species of *Collinsia*; *C. linearis* and *C. rattanii*. I examined geographic, phenological, pollinator, and mating-system isolation between *C. linearis* and *C. rattanii.* I quantified the geographic and elevational range overlap across the entire range of both species using herbarium collection data. In four sympatric sites in southern Oregon, I measured the overlap in flowering phenology. Pollinator observations were conducted in four sympatric site and two allopatric - *C. rattanii* sites to look at the extent of pollinator movement between species in sympatry, and to compare the visitation rates of *C. rattanii* in sympatry and allopatry. Finally, across 12 sites, I collected seeds from 17 allopatric and sympatric populations of *C. rattanii* and *C. linearis* to determine if the timing of autonomous selfing differed between sympatric and allopatric populations of each species. I found that species overlapped over large

portions of their range, with the majority of the geographic and elevational range of *C. linearis* shared with *C. rattanii*. There was almost complete overlap in flowering duration and substantial overlap in peak flowering time between *C. linearis* and *C. rattanii* in sympatric sites. Pollinators moved between species 5% of the time when movements were scored on a per flower basis, and 8% of the time when the movements were scored on a per plant basis. In general, pollinators visited the larger flowered *C. linearis* more than the smaller flowered *C. rattanii.* However, in allopatry, a greater proportion of available flowers of *C. rattanii* received pollinator visits, suggesting competition for pollinators in sympatry. When *C. rattanii* was sympatric with *C. linearis* it self-pollinated at a significantly earlier stage, whereas, *C. linearis* self-pollinated later. These results suggest that there may be reinforcement of reproductive isolation in sympatry via shifts in the timing of selfing.

4.2 INTRODUCTION

In many plant communities, the taxa that co-occur include a mixture of both evolutionarily distantly related species and more closely related species. When congeneric species co-occur, there is a potential for interspecific gene flow if reproductive barriers between species are incomplete. Strong isolating barriers can explain how closely related taxa can co-occur in sympatry, yet maintain their unique species' identities. Specifically, pre-zygotic barriers to reproduction can prevent the formation of hybrid offspring, which can have fitness costs (Dobzhansky 1937). A number of different factors can create prezygotic barriers including ecogeographic isolation (e.g., Ramsey et al. 2003), non-overlapping flowering times (Petit et al. 1997, Soliva and Widmer 1999, Martin and Willis 2007), and pollinator specialization (Grant

1992, 1993; Hodges and Arnold 1994, 1995; Schemske and Bradshaw 1999, Jones and Reithel 2001, Campbell 2003, Aldridge and Campbell 2009). When combined, these prezygotic barriers can prevent the formation of unfit hybrid offspring by reducing gene flow between congeners by nearly 100 percent (Ramsey et al. 2003, Martin and Willis 2007). In contrast, some co-occurring species appear to have weak prezygotic barriers, which results in wide hybrid zones and species that introgress freely (Cruzan and Arnold 1993, Rieseberg et al. 1999). A paradox arises when prezygotic isolating barriers appear to be weak (e.g, species overlap in range, co-flower, and share pollinators), but no evidence of hybridization is found in nature. This could be due to post-mating prezygotic or intrinsic post-zygotic isolating barriers (Randle Ch 2 and references therein), or could be due to additional prezygotic barriers that have not been measured.

A reproductive isolating barrier that is not often considered, but that could have significant isolating effects is the timing and ability of a species to self-pollinate. Here I define mating system as differences in the propensity, and/or timing of self-pollination. Differences in the mating system between species can result in barriers to hybridization in a couple of ways. First, the competitive ability of pollen (pollen vigor) may differ between species that are primarily selfing and those that are primarily outcrossing (Brandvain and Haig 2005), resulting in asymmetric reproductive isolation, where the selfing species is more likely to be a hybrid seed parent than a pollen parent. In addition, species that differ in mating system may differ in their relative pollen production (Cruden 1977, Martin and Willis 2007), which can result in a similar pattern to that described above. Finally, selfing can reduce the impact of pollen receipt from non-locally adapted conspecific donors or from heterospecific donors (Antonovics 1968, Fishman and Wyatt 1999). Given this, I expect that for congeners, mating system may differ between sympatric and allopatric populations when other prezygotic barriers in sympatry are not

complete. Specifically, for the species likely to receive heterospecific pollen in sympatry (i.e., the selfer), if the cost of heterospecific pollen receipt is high, and premating barriers are weak, then earlier selfing should be favored in that species.

The genus *Collinsia* provides an excellent opportunity to test for the strength of prezygotic isolating barriers, including mating system, because many *Collinsia* species are known to overlap in range, co-flower, and share generalist bee pollinators. Although all *Collinsia* species are self-compatible, there is substantial variation in the propensity for autonomous selfing ability across the genus (Randle Ch. 1).

Here I examined four potential prezygotic barriers to gene flow between two sister species in the genus *Collinsia*, *C. linearis* and *C. rattanii*. These taxa were chosen because they co-occur over large parts of their geographic range and co-occur sympatrically, and seemingly overlap in the timing flowering and their potential pollinators. I quantified geographic isolation, which includes the geographic range overlap and the elevational range overlap between these species. In the field, I identified 5 allopatric *C. linearis* populations, 2 allopatric *C. rattanii* populations and 5 sympatric *C. linearis* and *C. rattanii* populations. In four of the five sympatric populations, I quantified overlap in flowering time and pollinator visitation within and between *C. linearis* and *C. rattanii* flowers. Finally in all 12 sites (17 sympatric and allopatric populations of *C. linearis* and *C. rattanii*), I compared the developmental stage within a flower when autonomous selfing occurs in a controlled greenhouse experiment. This allowed me to contrast the timing of selfing for sympatric and allopatric populations of each species and test the prediction that mating system may be in important barrier to interspecific gene flow.

4.3 METHODS

4.3.1 Model System

The tribe Collinsieae is a monophyletic group comprised of ~22 *Collinsia* species and 2 *Tonella* species. All species are self-compatible winter and spring annuals (Neese, 1993). Flowers are zygomorphic, with a 5-lobed calyx and a 2-lipped corolla with a constricted tube. The corollas of *Collinsia* have one folded ventral petal that forms a keel and contains the 1 pistil and 4 stamens. All species secrete nectar and are visited by bee pollinators that collect both pollen and nectar. Bee pollinators that visit *Collinisa* include both generalists and specialist bee species (Vogler and Kalisz 2003). The genus *Collinsia* is comprised of several sister-taxa pairs that differ in flower size and in their propensity to autogamously self-pollinate (Randle Ch. 1), both factors that may act as important isolating barriers in areas where sister taxa co-occur.

In Southern Oregon, where this fieldwork was conducted, many *Collinsia* species are often found in the sympatry, including *C. rattanii* (small flowered) and *C. linearis* (large flowered) (Fig. 4.1). *Collinsia rattanii* and *C. linearis* exhibit significant variation in floral developmental traits associated with mating system (Randle Ch. 1) and differ significantly in floral size (Randle Ch. 1), and floral morphology, (Kalisz et al. 1999, Armbruster et al. 2002, Randle Ch. 1), which are expected to influence pollinator attraction. Differences in these floral traits may act as important prezygotic isolation barriers to hybridization in sympatry. Strikingly, these two species readily hybridize when hand-pollinated in the greenhouse (Randle Ch. 2), yet there is little evidence of natural hybrids in the field. Some plants of intermediate flower size have been found (Randle unpubl. data); however, it is not clear if they are actual hybrids.

For this study, I located 12 sites where *C. linearis* and *C. rattanii* occur in sympatry or allopatry (C. linearis allopatric sites=5; C. rattanii allopatric sites= 2; and C. linearis and C. rattanii sympatric sites=5) for a total of 17 populations. In the four sympatric sites I quantified flower phenology and pollinator observations [Applegate (AG), Butte Falls (BF), Lick Gulch (LG), and Lincoln Creek (LC)]. I also conducted pollinator observations in the two allopatric *C. rattanii* sites [Keen Creek (KC) and Burnt Creek-39 (BC-39)]. Two of the sites were located in the Applegate Valley (AG and LG), and four of the sites were located ~ 60 mi south of the Applegate Valley near the California border, in the Green Springs Recreation Area (BF, LG, KC, BC-39). Sites within regions were at least two kilometers apart, and were considered independent. From these and the remaining six sites, I collected seeds from 20-30 plants of both *C. linearis* and/or *C. rattanii* to be used in the timing of selfing experiment described below.

4.3.2 Ecogeographic Isolation – Range Overlap

To determine range overlap of *C. linearis and C. rattanii*, I first calculated the range size of each species using distribution data from herbarium specimens (for methods, see Randle Ch. 1). I then calculated the area of range overlap between *C. linearis* and *C. rattanii* (see methods Ch. 2). To determine the area of sympatry for each species range I divided the area of overlap by the total range size of each species. For each species, I also used the herbarium collections to determine the mean elevation for (see methods Ch. 1). I compared the mean elevation between species with an independent t-test (SPSS).

4.3.3 Floral Isolation – Phenology

To compare the flower phenology of these species, I set up ten 1 m x 1 m plots in each of the four sites (above) in the early spring of 2006, as seedlings were just beginning to emerge. Within each site, the plots were established haphazardly across representative microhabitats, in locations where *Collinsia* seedlings were emerging. Although I attempted to sample each site weekly, there were some weeks where this was not possible. To determine the overlap in flowering time between *C. linearis* and *C. rattanii* and the peak flowering time for each species, I counted the number of stems of each species and the total number of open flowers of each species in each plot at each site, during each sampling period. To account for differences in the relative abundances of each species in a site, I summed the total number of open flowers across all ten plots for all sampling periods at each site. I then calculated the percent of the total number of flowers in my sample that were open on each sampling day for each species at each site. These data were plotted to estimate the percent overlap in flowering time for each species at each site.

4.3.4 Pollinator Isolation

To assess the degree to which pollinators were shared between the two co-flowering species of *Collinsia,* I set up ten 1 m x 2 m plots in each of the four sites (above) where *C. linearis* and *C. rattanii* co-occur. As above, plots were established haphazardly prior to flowering, thus species could not be identified. This resulted in some plots containing both species, while others only contained either *C. linearis* or *C. rattanii*. Single species plots were not used in this analysis. For one site (LCR) I observed pollinators during both the 2005 and

2006 flowering season. In 2006, LC and AG plots were observed on two different days, whereas BC and LG plots were only observed on one day. All pollinator observations were conducted between May 29th and June 4 in 2006, and on June 12 in 2005. For each plot observation, two observers would wait for 1 minute on the edge of the plot prior to the start of pollinator observations; this was done to mitigate any initial disturbance caused to pollinators by our arrival. Observations of all pollinators that entered the plot were recorded for 12 min bouts. The observers recorded the number of flowers visited, the sequence of flowers visited, the species of flower visited, the number of geitonogamous visits, and the type of pollinator visiting. Visits were only counted for those pollinator species that contacted the reproductive parts of the plant. Finally, in each plot I counted the number of stems of each species and the total number of open flowers. I also recorded a suite of environmental variables including elevation, temperature, time, wind, and cloud cover. On days that pollinators were not seen flying, I did not attempt to collect pollinator data.

From these data I calculated the proportion of open *C. rattanii* and *C. linearis* flowers in each plot. For each plot I also calculated the number of pollinator visits each species received. To determine if pollinators were visiting *Collinsia* in proportion to their abundance, I conducted a X^2 test to compare the observed visits to each species with the expected visits. My expectation was that pollinators would visit each species as a function of the relative abundance of its open flowers in each plot. I also quantified the proportion of pollinator movements within and between conspecific and heterospecific flowers and stems to determine to potential for gene flow between species.

4.3.5 Timing of Selfing

To determine the timing of selfing for each species in sympatry and allopatry, I collected seeds from at total of 17 Collinsia populations within five sympatric sites (the four sites above and TRMO, which was not included in phenology and pollinator observations), 5 allopatric *C. linearis* sites, and 2 allopatric *C. rattanii* sites. Seeds were planted and grown to flowering under optimal conditions in the greenhouse facilities at the University of Pittsburgh. Approximately 10 plants from each population were used to determine the timing of selfing. For each plant, I marked 1-2 flowers at each of 1-4 developmental stages with a unique color non-toxic paint on the calyx and subtending leaf. As in Kalisz et al. (1999), I define the developmental stage of a flower relative to the number of mature stamens exhibiting anther dehiscence (e.g. stage 1=1 anther dehisced, etc.). For each flower, at each stage, I carefully removed the stigma and allowed the fruit to mature. If self-pollen had autonomously reached the stigma prior to stigma removal, then fruit development should occur, whereas if pollen did not reach the stigma prior to stigma removal, no fruit will develop. I allowed all fruits to remain on plants until mature. I recorded whether mature fruits were formed and collected all fruits. Fruit production data were averaged for each stage by family and then pooled within species across sympatric or allopatric sites in order to compare the timing of selfing.

I used the SAS protocol 'Mixed' to test for differences in mean fruit production within species using stage and site type (sympatric or allopatric) and the stage*site type interaction and to compare least square means using SAS 9.2 (SAS Institute 2007). Because the interaction term was non-significant for both species, I dropped the interaction term from the model and re-ran the analyses. I also tested for differences in stage-specific effects on mean fruit

production within species between site types by running a analysis [model: mean fruit production = site] for each species and stage.

4.4 RESULTS

4.4.1 Ecogeographic Isolation – Range Overlap

C. linearis and *C. rattani* were found to overlap substantially in their geographic range. More of the total range of *C. linearis* overlapped with C. rattanii than vice versa; 87% of the total range of *C. linearis* is shared with *C. rattanii*. In contrast, only 39% of the range of *C. rattanii* is shared with C. linearis (Fig. 4.2). *C. linearis* and *C. rattanii* overlap in their elevational range (C. linearis range = 60-2045 m, C. rattanii range 24-2045 m), however, the mean elevations differed significantly between species (mean +/- standard error; *C. rattanii* = 920.6 m +/- 44.85; *C. linearis* = 726.81 +/- 26.23; t=-3.899, df=375, p=0.001; Fig. 4.3).

4.4.2 Floral Isolation – Phenology

In sites where *C. linearis* and *C. rattanii* were sympatric, the species overlapped substantially in their flowering times (Fig. 4.4). The peak flowering time for *C. linearis* and *C. rattanii* were the same in three of the four sites (BC, LC, LG). In addition, flowering times completely overlapped for one site (BC) and overlapped by approximately 75% for the remaining 3 sites. Thus, there was ample opportunity for pollinators to move between open flowers of *C. linearis* and *C. rattanii* at each site.

4.4.3 Pollinator Isolation

All insect visitors to *C. linearis* and *C. rattanii* in both the sympatric and allopatric sites were bees (Table 4.1). Bee species that visited *Collinisa* included both generalist species (*Bombus* and *Apis*) and specialist bee species (*Osmia* and *Andrena*). Flies were rarely seen hovering over *Collinisa*, but never contacted reproductive parts.

Observations of pollinator plots were pooled across sites and days, resulting in 39 observation periods in plots where *C. lineairs* and *C. rattanii* co-occurred. In 7.8 h of pollinator observations, 381 pollinator visits were recorded to *C. linearis* and *C. rattanii*. For each plot, we calculated the proportion of open flowers of each species, and used that to calculate an expected visitation rate, if pollinators visited *C. linearis* and *C. rattanii* at random, but scaled to their relative abundances. Using a X^2 goodness of fit test to compare the observed number of visits to each species with the expected, we found that visits were biased towards *C. linearis*, but this was only moderately significant (X^2=53, df=38, $0.05<p<0.10$). I also found that the majority of pollinator movement was between flowers of the same species, with 5% of pollinator movement occurring between *C. linearis* and *C. rattanii* flowers. However, when I remove the geitonogamous pollinator visits, and only look at movement of pollinators between plants, then pollinator movement between species increases to 8% of the total between plant movements. Overall, pollinators visited 4.4% of the available *C. linearis* flowers and 5.5% of the available *C. rattanii* flowers.

In the two allopatric *C. rattanii* sites, I conducted 7.6 h of pollinator observations in 38 plots across sites and days. A total of 361 individual pollinator visitors were recorded, but only 82 visited *C. rattanii*. Pollinators visited a total of 7% of the available *C. rattanii* flowers in allopatric sites.

4.4.4 Timing of Selfing

I compared the timing of selfing, measured as the ability of a flower to produce fruit after stigma removal at each developmental stage, between *C. linearis* and *C. rattanii* in sympatry and allopatry. I found significant differences in the timing of selfing between populations of each species in sympatry and allopatry (Fig. 4.5). The timing of self-pollination in *C. linearis* was significantly later when it occurred in sympatry with *C. rattanii*, and differed significantly from its allopatric populations at floral developmental stage 2 ($F=5.59$, $df=107$, $p=0.0199$). Conversely, the timing of self-pollination in *C. rattanii* was significantly earlier when sympatric with *C. linearis,* and differed significantly from its allopatric populations at floral development stage 3 ($F=6.94$, $df=64$, $p=0.01$).

4.5 DISCUSSION

I found that *C. linearis* and *C. rattanii* overlapped substantially in their geographic and elevational ranges, but that more of the range of *C. linearis* was shared *with C. rattanii* than vice versa. In areas of sympatry, I found that *C. linearis* and *C. rattanii* substantially overlap in flowering time. Although our measure of flowering phenology was coarse, and may not detect the fine-scale differences in flower phenology between *C. linearis* and *C. rattanii*, at other sites where we measured the flower phenology of four co-occurring *Collinsia* species at the same sampling interval, I did detect differences peak flowering time (Randle upublished data). Thus, the time of peak flowering between *C. linearis* and *C. rattanii* is substantially more overlapping

with each other that either species is with other co-occurring *Collinsia* species (Randle unpublished data). Competition for pollinators has been shown to result in divergence in flowering time among sympatric species (Petit et al. 1997, Soliva and Widmer 1999). For *Collinisa linearis* and *C. rattanii*, there is little evidence from these data to suggest that selection for divergent flowering times in these species has occurred.

Bees were found to visit *C. linearis* more than *C. rattanii*, which is expected given that *C. linearis* has significantly larger flowers than *C. rattanii* (Randle Ch. 1), and flower size purported to be the primary pollinator attractor (Bell, 1985). Despite the differences in flower size, a common pool of pollinators visited both *Collinsia* species. Pollinators moved between *C. linearis* and *C. rattanii*, with 5% of the pollinator movements between *C. linearis* and *C. rattanii*, even though C. rattanii made up only 14% of the available flowers on average. When all geitonogamous pollinations were removed and I considered only movement between plants, pollinator movements between *C. linearis* and *C. rattanii* plants increased to 8%. Thus, although the majority of gene flow is within a species, the range and flowering times overlap, and pollinators shared creates non-trivial opportunities for heterospecific pollen transfer. Strong pollinator isolation has been found in a number of studies on prezygotic isolating barriers between species in sympatry, the most well known of which is likely the pollinator isolation demonstrated between sympatric sister taxa *Mimulus lewisii* and *M. cardinalis* (Schemske and Bradshaw 1999, Ramsey et al. 2003). Even though the majority of gene flow was within species, 5-8% of pollinator visits between species may be enough to result in the production of costly hybrids. However, differences in flower size between these two species may be substantial enough to result in differential placement of pollen on the pollinators (Armbruster et al. 1994, Moeller 2004). But my observations of pollinator visits suggest that this is unlikely.

The total percent of open *C. rattanii* flowers visited by pollinators differed between the sympatric and allopatric sites. In sympatry, ~ 4.4% of open *C. rattanii* flowers were visited, whereas in allopatric sites, 7% of open flowers were visited. This implies that there may be competition for pollinator services where *C. linearis* and *C. rattanii* are sympatric. Competition for pollinators between co-occurring species (*Mimulus* and *Lobelia*) has been shown to result in lower outcrossing rates when these species were grown together in experimental arrays (Bell et al. 2005), which implies that self-fertilization may occur when species compete for pollinators, because of reduced outcrossed pollen receipt (i.e. selfing provides reproductive assurance: Lloyd 1979, 1988, 1992; Kalisz and Vogler 2003, Randle Ch. 1 and references therein).

Differences in the timing of selfing between sympatric and allopatric species of *Collinsia*, specifically earlier selfing in *C. rattanii* in sympatric populations, suggests that either competition for pollinator services is limiting pollen receipt or that earlier selfing has evolved to reduce the likelihood of heterospecific pollen receipt (Fishman and Wyatt 1999, Antonovics 1968). My pollinator data suggest that more open flowers of *C. rattanii* are pollinated when in allopatry compared to sympatry, which could mean *C. rattanii* are pollen limited in sympatry. However, all *Collinsia* species can self-pollinate, so why would pollen limitation select for *earlier* selfing? Perhaps the small amount of between species movement is enough to favor earlier selfing as a means to avoid costly hybrid pollen receipt. Thus, if earlier selfing has evolved in response to selection against the production of unfit hybrid offspring, the shift in the timing of selfing in *C. rattanii* in sympatry vs. allopatry may be an example if reinforcement of reproductive isolation (reviewed in Servideo 2004). From our data, we cannot determine the isolating effects of early selfing, but work comparing the fitness of F1 hybrids between these two

species shows that hybridizing can be costly (Randle Ch. 2). Clearly, more work needs to be done on this front.

Table 4.1. All visitors to *Collinsia linearis* and *C. rattanii* were bees. Bee species observed visiting *Collinsia* contacted reproductive parts and thus were likely pollinators. Bee species included representitives of the genera *Osmia*, *Andrena*, *Bombus*, and *Apis*. The maximum number of bee species to visit *Collinisa* in a 12 minute observation = 5 species [mean (+/ SD) = 0.049 (+/- 0.91)].

	C. linearis	***C. rattanii***	**Total**
# of flowers visited by bees (sympatric)	800	129	929
# of flowers visited by bees (sympatric and allopatric)	1482	213	1695

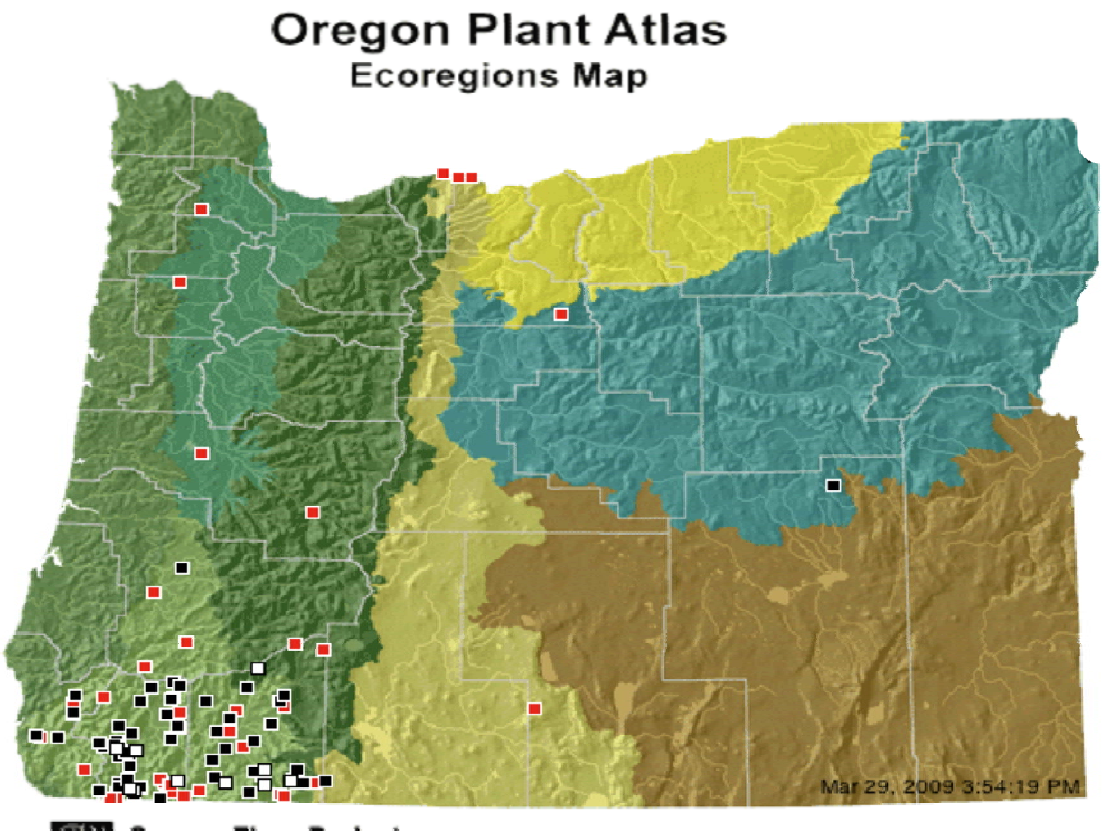

Figure 4.1. Collection sites for vouchered *Collinsia linearis* (black) and *Collinsia rattanii* (red) specimens. This map shows the significant overlap in range in southern Oregon. Oregon Plant Atlas Project.

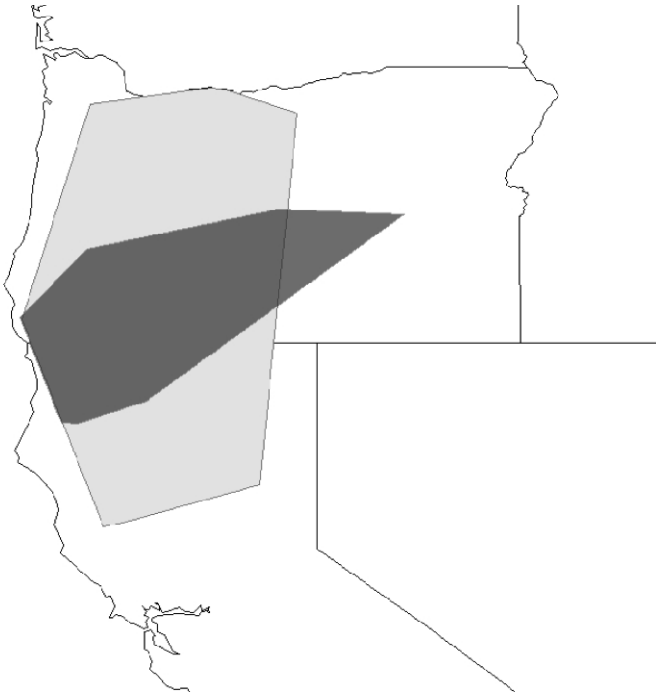

Figure 4.2. The total range of *C. linearis* (black) and *C. rattanii* (gray) and their range overlap (from Randle Ch. 1).

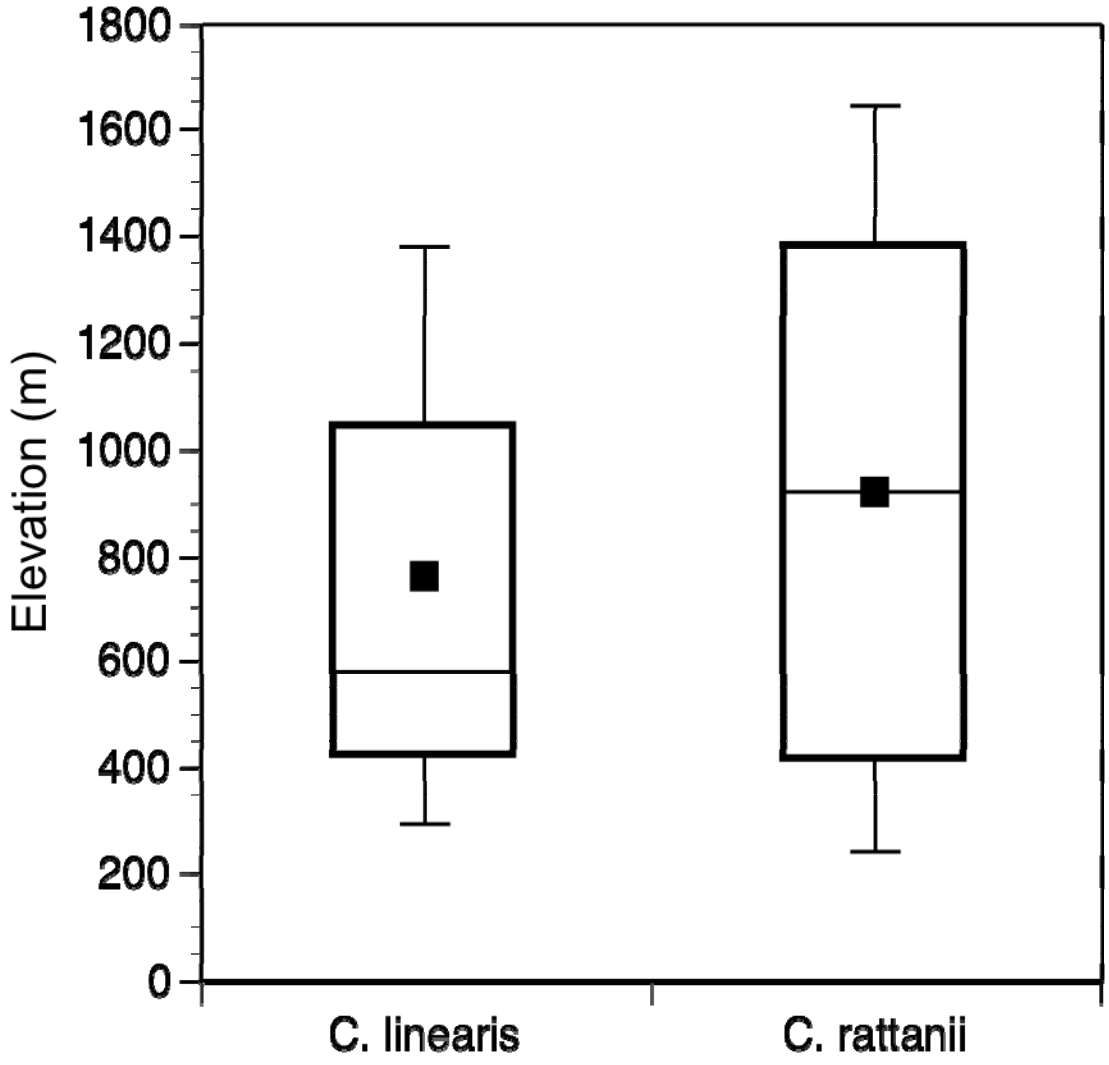

Figure 4.3. Box plots showing elevational range of *C. linearis* and *C. rattanii*.

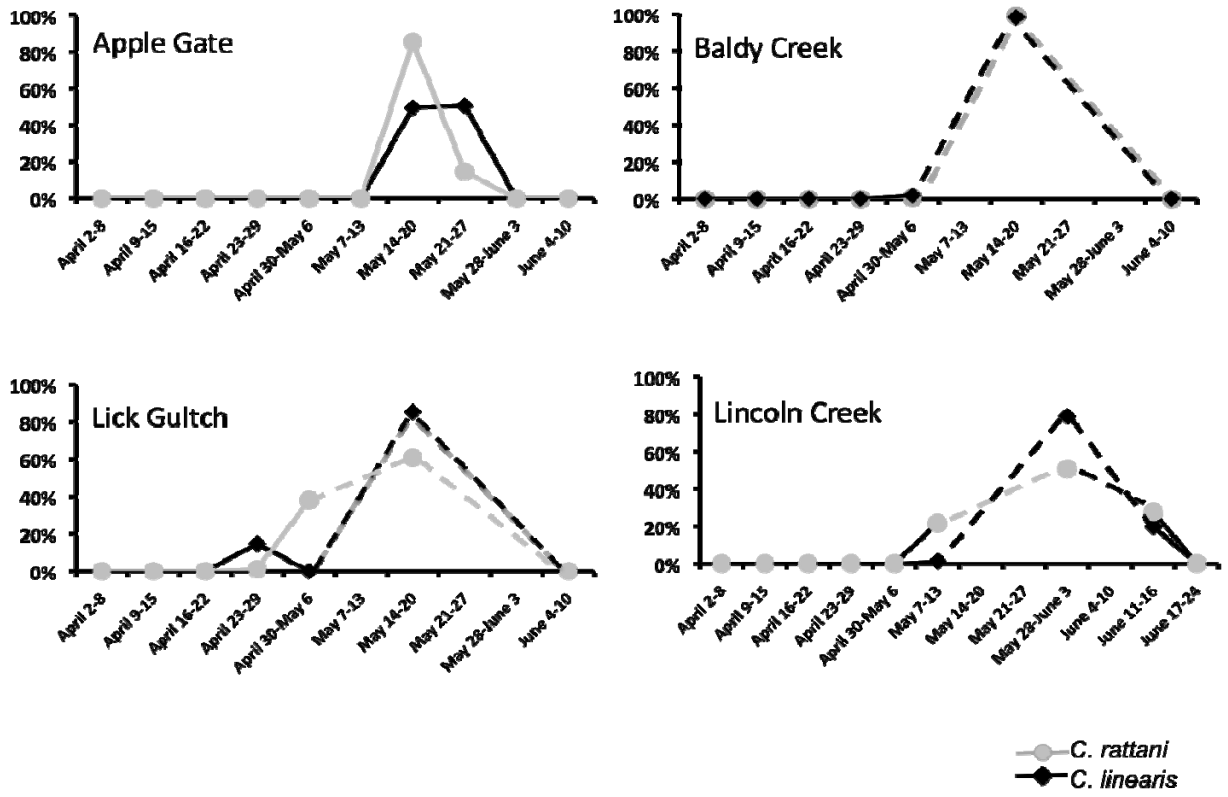

Figure 4.4. Percent of the total number of open flowers across time for *C. linearis* and *C. rattanii* at four sites in southern Oregon.

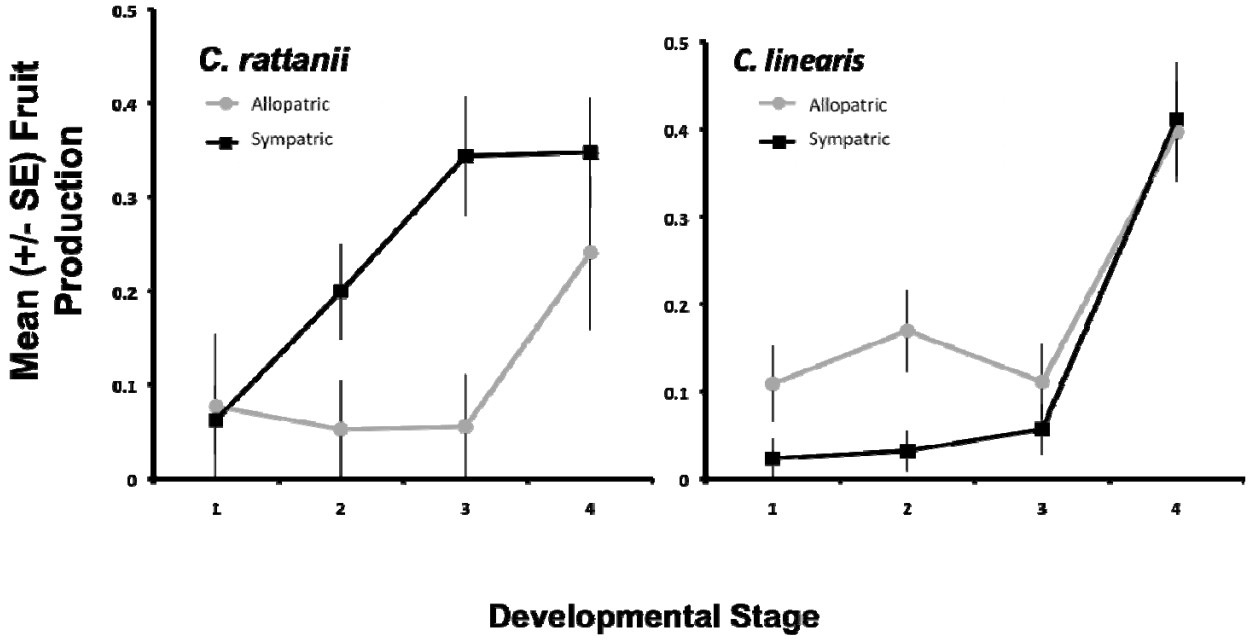

Figure 4.5. The timing of selfing for *C. linearis* and *C. rattanii* in sympatric and allopatric populations.

5.0 CONCLUSIONS

The aim of my thesis research was to examine broad patterns that drive differences in species' distributions and to explore the evolutionary processes that lead to the origin and maintenance of species boundaries. To do this, I chose to work with an emerging model system, the genus *Collinsia*, for which the phylogenetic relatedness and relative species ages was well resolved (Baldwin et al. unpubl). In addition, I made use of extensive herbarium collections, which included nearly one hundred years of data on the distribution of *Collinsia* species. I used these two important pieces of data to test a series of classic question in evolutionary biology. First, I examined how mating system might influence the distribution of species. This question revisited the pioneering work of Herbert G. Baker (1955). Baker noticed that self-compatible species tended to be more common in distant locations such as islands and range margins, and hypothesized that self-compatible species are more likely to colonize and establish populations after long-distance dispersal than species that were not self-compatible (Baker 1975). With this pattern of species distributions relatively well supported, I extended Baker's hypothesis to include any dispersal distance (Barrett and Pannel 1998) and a more nuanced definition of the mating system. I predicted that species better at autonomous self-pollination would be better colonizers, and could thus expand their range size more quickly than species less adept at autonomous self-pollination. In *Collinsia*, I showed that sister taxa pairs of the same age differ in their ability to autonomously self-pollinate. This allowed me to test the prediction that

differences in autonomous selfing ability could lead to differences in range size. I found that small-flowered, autonomously self-pollinating species had larger range sizes than their larger-flowered sister taxa that were less adept at selfing.

In Chapter 3, I tested the prediction of Bateson (1909), Dobzhansky (1937) and Müller (1940) that neutral genetic changes accumulate within allopatric populations overtime. These genetic changes are positive or neutral within a lineage; however, between lineages negative epistatic interactions can occur between loci and result in intrinsic reproductive isolation. The prediction is that, as divergence time between species increases, so should the number of negative epistatic interactions. Thus, postzygtic reproductive isolation is predicted to increase with increasing divergence time. This work was experimentally tested in *Drosophila* (Coyne and Orr 1989, 1997) and led to a number of experimental tests in animal systems as phylogenies became available, most of which supported the predictions of Bateson (1909), Dobzhansky (1937) and Müller (1940). Few studies have been conducted to determine if plants species express a similar pattern of isolation. Two studies have tested the pattern of postzygotic reproductive isolation over time in plants across an entire group of related species (Moyle et al. 2004, Scopece et al. 2008), and the results about whether plants accumulate post-zygotic reproductive barriers in a similar manner to animal species were equivocal. I found that postzygotic reproductive isolation increased with increasing divergence time, as predicted by the BDM model of reproductive isolation (RI). The rate of increase of RI was rapid until total isolation was reached, which is consistent with the "snowball effect" predicted by Orr (1995). Further, I found low levels of asymmetry in reproductive isolation among species pairs, which provides further support for nuclear incompatibilities accumulating between species. My results add to the previous work on this topic in plants, and provide strong support for the Bateson-

Dobzhansky-Müller (BDM) model of post-zygotic isolation in plants. In this chapter, I also compared the relative rates of post-mating, pre- vs. post-zygotic reproductive isolation and looked for evidence of reinforcement of reproductive barriers among species that occur in sympatry. I found no differences in the relative rates of pre-and post-zygotic isolation, nor did I find evidence of reinforcement of reproductive barriers among sympatric populations. These latter two comparisons are likely to be more prominent when examining pre-mating prezygotic isolation rather than post-mating prezygotic isolation. Importantly, unlike in previous work on BDM incompatibilities, I endeavored to test a major assumption of the BDM model: that species diverged in allopatry. Using the phylogenetic hypotheses for species relatedness and age, and the herbarium data of species distributions, I tested for the geographic mode of speciation among species of *Collinsia.* I found strong support for allopatric speciation in the California clade, but little support in the northeastern clade. I suspect that rapid shifts in range size, particularly in *C. parviflora*, obscured any pattern of geographic mode of speciation I might see (Barraclough and Vogler 2000).

Finally, in the fourth chapter, I explore patterns of pre-mating prezygotic isolation among sister species of *Collinisa*: *C. rattanii* and *C. linearis.* These two species are found to co-occur frequently in nature, and can hybridize when hand pollinated in the greenhouse. Based on hybrid crosses between these two species, I found that F1 hybrid offspring were less fit than pure breeding offspring (Chapter 3). Thus, given that *C. linearis* and *C. rattanii* co-occur and that hybrid offspring are less fit than pure breeding offspring, I examined the strength of pre-zygotic isolating barriers between species in sympatry, to determine if prezygotic isolation was sufficient to limit gene flow between species and thus reduce the cost incurred by the production of unfit hybrid offspring. I also examined differences in the timing of selfing among sympatric an

allopatric sites for each species test the hypothesis that earlier self-pollination might evolve as a reproductive isolating barriers if other prezygotic barriers are not complete. I found that *C. linearis* and *C. rattanii* overlapped substantially in range, flowering time, and shared pollinators, and that prezygotic isolation (due mostly to pollinator isolation) was nearly, but not entirely complete. Surprisingly, I did find that *C. rattanii* selfed at a significantly earlier stage when sympatric with *C. linearis,* and that the sympatric *C. linearis* selfed at a significantly later stage. Earlier selfing in *C. rattanii* in sympatry with *C. linearis* is consistent with what I would predict if there was the potential to receive heterospecific pollen, and that heterospecific pollen receipt was more costly than receiving self pollen (Antonovics 1968, and Fishman and Wyatt 1999). Assuming this is also true for this *Collinsia* species pair, then my results are one of only three examples showing that there can be selection for increased selfing as a means to reinforcement reproductive barriers in sympatry. More work in our system needs to be done to show this definitively, however the general pattern is present.

Together, this work tests a series of prominent hypotheses on the evolution of species boundaries and the factors that maintain those boundaries in plants. In addition, it incorporates novel predictions about how mating system may influence the range size of species, and maintain reproductive isolation in sympatry. These broad patterns have created a foundation for future work in this system. In the future, I hope to build upon this work, to further explore the factors that drive divergence in closely related lineages.

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