REPRODUCTIVE BARRIERS BETWEEN THE CEDAR GLADE ENDEMICS LEAVENWORTHIA ALABAMICA AND L. CRASSA

by

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(Under the Direction of Rodney Mauricio)

ABSTRACT

Speciation, the process by which new species form, is an important evolutionary process that generates biodiversity. The evolution of barriers to reproduction between populations is considered an integral part of the speciation process. Possible barriers can include genetic incompatibilities, habitat isolation, and mating system isolation. In the present work, I describe studies aimed at elucidating the presence of these barriers between *Leavenworthia alabamica* and *L. crassa*, two species in the family Brassicaceae that have been the subject of studies of the evolution of self-fertilization from self-incompatibility, but not previously studied with respect to speciation. To study reproductive barriers in this system, I performed the following: 1) crosses in the greenhouse between *L. alabamica* and *L. crassa* to test for genetic incompatibilities at the level of seeds produced, 2) common garden analyses of hybrid versus parent species floral morphology to test for genetic incompatibilities manifest at the phenotypic level, 3) a reciprocal transplant using both parent species and hybrid individuals to test for habitat isolation and genetic incompatibilities in the field, and 4) an analysis of genetic structure, diversity, and mating system of populations to test for mating system isolation. From the crosses, I found no evidence for genetic incompatibilities, other than the phenomenon known as unilateral

incompatibility, which limits mating between self-incompatible and self-compatible individuals. In addition, hybrids produced in crosses had floral phenotypes within the range of the parent species, although hybrids tended to be intermediate for most traits measured. Hybrids did, however, germinate more quickly than the parent species, which could impact their fitness in the field. The results of the reciprocal transplant yielded no evidence of habitat isolation or of reduced hybrid fitness in the field. *L. alabamica* and *L. crassa* had similarly high levels of genetic structure and diversity, and genetic diversity was lower in self-compatible populations than self-incompatible. I also found that genetic structure was higher between self-incompatible compared to self-compatible populations than between comparisons of self-incompatible populations, indicating that mating system isolation may be present in this system. Thus, unilateral incompatibility and mating system isolation may limit mating between *L. alabamica* and *L. crassa*.

INDEX WORDS: speciation, reproductive isolation, *Leavenworthia*, genetic incompatibilities, unilateral incompatibility, transgressive segregation, habitat isolation, genetic structure, mating system

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DEDICATION

To my parents, who inspired my love of biology

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CHAPTER 1

INTRODUCTION

Speciation, the process by which new species form, is an important evolutionary process that generates biodiversity. Multiple models have been proposed to explain how speciation occurs, but they fall into three main categories: 1) speciation occurs as a by-product of natural selection, 2) speciation occurs as a direct result of natural selection, and 3) speciation occurs as a result of genetic drift (reviewed in Coyne and Orr 2004). In all cases, the result of this natural selection, whether direct or indirect, or genetic drift are barriers to reproduction between lineages. Thus, understanding the types of reproductive barriers present between species, and the genetic basis of those barriers, is an important first step in our understanding of the forces driving this process (Noor and Feder 2006; Rieseberg and Willis 2007).

There are a number of different types of reproductive barriers that can develop between species (reviewed in Coyne and Orr 2004), but only three are relevant to the current work: 1) habitat isolation, 2) intrinsic genetic incompatibilities, and 3) extrinsic genetic incompatibilities. One of the most widely documented forms of differentiation between both animal (e. g. Feder and Bush 1989; Hatfield and Schluter 1999; Filchak et al. 2000; Rundle et al. 2000; Hawthorne and Via 2001; Kocher 2004; Bridle et al. 2006; Fuller et al. 2007) and

plant (e. g. Cruzan and Arnold 1993; Wang et al. 1997; Campbell 2003; Husband and Sabara 2003; Ramsey et al. 2003; Rieseberg et al. 2003; Kay 2006; Savolainen et al. 2006) species is that of adaptation to different ecological niches, yet few studies have explicitly demonstrated habitat isolation (reviewed in Coyne and Orr 2004). One example is in *Rhagoletis* fruit flies, where Feder and Bush (1989) found that, although the species *R. pomonella* and *R. mendax* can produce viable hybrids in the laboratory, hybrids are not found in the field due to host preferences that restrict mating to different host plants (apples/hawthorns and blueberries respectively). An example in plants is found in the classic study of Clausen et al. (1940), in which reciprocal transplants between low-elevation and alpine species of *Horkelia* showed severely reduced survival and reproduction at their non-native elevation, thereby preventing gene flow between the species.

Reproductive barriers in the form of intrinsic genetic incompatibilities can also occur in both plant and animal taxa, and contribute to reproductive isolation between species. These barriers can occur post-mating, but pre-fertilization (Howard 1999), or post-fertilization (Stebbins 1958; Coyne and Orr 2004). Post-fertilization barriers often result from chromosomal (Rieseberg 2001; Brown et al. 2004), genic (Dobzhansky 1936; Muller 1939), or cytonuclear (Michaelis 1954; Levin 2003) incompatibilities. Chromosomal and genic incompatibilities manifest as hybrid inviability or sterility when taxa are crossed, irrespective of the directionality of the cross. Cytonuclear incompatibilities, on the other hand, as

well as barriers that occur post-mating, but pre-fertilization, often result in unidirectional cross success (reviewed in Grant 1975; Tiffin et al. 2001).

In plants, another asymmetrical crossing barrier is possible, termed unilateral incompatibility (Lewis and Crowe 1958), which is related to self-incompatibility. Unilateral incompatibility (UI) occurs when a plant that is self-incompatible (SI) cannot cross, or crosses less successfully, with a self-compatible (SC) plant when the SI plant is the pollen recipient and the SC plant the pollen donor; conversely, the reciprocal cross is successful. UI is widespread in plant families with a genetic self-incompatibility system (Lewis and Crowe 1958; Heslop-Harrison 1982; Hiscock and Dickinson 1993) and has been suggested as a barrier to interspecies mating (Harrison and Darby 1955; Grun and Radlow 1961); however, this remains controversial as it has also been documented within species (Martin 1963; Lloyd 1968; Pandey 1981) and the genetic mechanism remains unknown.

In addition, ecological factors can interact with genetic factors to cause environment-dependent (extrinsic) genetic incompatibilities between species that reduce hybrid fitness in particular environments (reviewed in Coyne and Orr 2004). This type of extrinsic genetic barrier occurs when hybrids are poorly adapted to either niche of the parent species and are less fit in the parent species' habitat. An example of this occurs in big sagebrush (*Artemisia tridentata*), where two subspecies form a hybrid zone (Wang et al. 1997). In a reciprocal transplant experiment, Wang et al. (1997) found that each subspecies was most fit in its habitat of origin, and that hybrids were most fit in the hybrid

zone and outperformed the parent subspecies in that zone. Thus, hybridization between the parent subspecies is limited by environment-dependent selection. A similar study by Hatfield and Schluter (1999) on limnetic and benthic stickleback species of the *Gasterosteus aculeatus* complex showed that F_1 hybrids between the two species did not perform well in either parent species habitat, and that selection likely acts against hybrids in the field.

Genetic incompatibilities can also manifest as phenotypic differences between parent species and their hybrids as the result of hybrid breakdown (due to negative epistasis), hybrid vigor in the F_1 (due to dominance effects resulting from outcrossing), hybrid vigor in later-generation hybrids (due to positive epistasis), and transgressive segregation in F_2, backcross (BC), or later generation hybrids (due to novel additive effects of loci) (reviewed in Burke and Arnold 2001). Which of these phenomena occur in a hybridization event will depend on the genetic architecture of the parent lineages and, often, on the environmental context of hybrids (e.g. Emms and Arnold 1997; Hatfield and Schluter 1999; Rieseberg et al. 2003; Fritz et al. 2006; Wu and Campbell 2006).

Transgressive segregation (or variation) occurs when hybrids achieve trait values not seen in the parent lineages. In general, this is thought to occur when QTL underlying a trait are of mixed effect (i.e. QTL both increase and decrease trait values) and recombination brings together QTL of the same effect that were previously not present in the same genome (Grant 1975; deVicente and Tanksley 1993; Rieseberg et al. 1999) (although inbreeding may also play a role, Rick and Smith, 1953). Transgressive segregation has been documented in both plant and

animal hybridization events (Rieseberg et al. 2003; Albertson and Kocher 2005), and in some cases has allowed hybrid lineages to invade new ecological niches, aiding in hybrid zone stabilization (Schweitzer et al. 2002) or hybrid speciation (Rosenthal et al. 2002; Rieseberg et al. 2003; Bell and Travis 2005). Alternatively, transgressive phenotypes may be selected against in some hybridization events (e.g. Rogers and Bernatchez 2006), possibly inhibiting introgression and selecting for the reinforcement of pre-mating barriers to reproduction (Servedio and Noor 2003). Therefore, transgressive segregation can be an important factor in speciation, and documenting its presence or absence in interspecies hybrids can further our understanding of how species' boundaries are maintained in the face of gene flow.

Population structure is another factor that can influence the rate of gene flow between species. Species with highly structured populations are expected to experience enhanced genetic drift and inbreeding within populations, particularly if populations are small (Hedrick 2005). This can negatively impact population fitness by retarding the effects of current selection or reducing a population's ability to respond to future selection pressures (reviewed in Ellstrand and Elam 1993). The enhancement of genetic drift due to population structure could also create conditions under which reproductive barriers arise via the action of genetic drift alone on neutral genetic variation (Nei et al. 1983). However, to date there are no empirical studies demonstrating speciation through neutral genetic drift. Population structure can also enhance the opportunity for local selection to differentiate populations, thereby leading to speciation (Coyne and Orr 2004).

Another factor that can impact both population genetic structure and, potentially, speciation is mating system evolution, particularly transitions from outcrossing to self-fertilization. Previous studies have demonstrated that selfing reduces genetic diversity within populations and increases the genetic structure among populations compared to outcrossing (e.g. Schoen and Brown 1991; Hamrick and Godt 1996; Charlesworth and Yang 1998; Liu et al. 1998; 1999; Mable and Adam 2007). Thus, gene flow is expected to be reduced between populations when one or more is predominantly self-fertilizing. This has lead to the suggestion (Levin 1978) that the evolution of self-fertilization itself is a reproductive barrier between species. However, this idea has been challenged (Coyne and Orr 2004) on the grounds that selfing individuals within populations would be just as unlikely to mate with one another as with members of another species, and therefore self-fertilization cannot be a reproductive barrier between species. To settle this debate, further empirical work is needed to elucidate how selfing and outcrossing populations are structured and how this impacts gene flow between populations both within and between species.

In the following chapters, I outline my work to elucidate reproductive barriers between the species *Leavenworthia alabamica* and *L. crassa*. These species have been the subject of studies into the evolution of self-fertilization from self-incompatibility due to the observation that, although some populations possess the sporophytic self-incompatibility system common to other members of the Brassicaceae (Bateman 1955; Lloyd 1967), others are capable of autogamous seed production (Rollins 1963; Lloyd 1965). Rollins (1963)

hypothesized that self-fertilization evolved at least three times within this small genus of only eight species (five times if *L. alabamica* and *L. crassa* also represent independent events), and recent phylogenetic evidence has corroborated Rollins' hypothesis of at least three independent events (Beck et al. 2006).

In addition, Lloyd's (1965) work on the striking morphological changes associated with the transition from outcrossing to self-fertilization in these species played an important role in our understanding of the connection between plant breeding system evolution and floral adaptation. In particular, Lloyd (1965) found that more highly autogamous populations of *L. alabamica* and *L. crassa* had smaller flowers, reduced anther-stigma distances, anthers oriented to dehisce toward rather than away from the stigma, smaller pollen to ovule ratios, and little or no floral scent. He hypothesized that these changes were adaptations to a self-fertilizing mating system; a belief now commonly held due to the discovery of these floral trait transitions in a wide variety of plant groups (Holsinger 1996).

The studies of Rollins (1963) and Lloyd (1965) prompted a large body of experimental work to further understand why self-fertilization evolved so many times in *Leavenworthia* and its genetic consequences. Lyons and Antonovics (1991) looked at two populations of *L. crassa* and found that the outcrossing rate in the field was indeed significantly higher in the population believed to be outcrossing based on floral morphology than in the selfing population, confirming the association between morphology and mating system. Furthermore,

inbreeding depression was documented in *L. crassa* (Charlesworth et al. 1994) and *L. alabamica* (Busch 2005a). Busch (2005a) found less inbreeding depression in self-compatible populations compared to self-incompatible populations, and documented heterosis in a small, isolated, and highly self-compatible population (Busch 2006).

In addition, genetic diversity in *Leavenworthia*, including *L. alabamica* and *L. crassa*, was examined by Solbrig (1972) and Solbrig and Rollins (1977). These studies found that self-compatibility throughout the genus is associated with lower genetic diversity (measured using allozymes). Low genetic diversity in the self-compatible compared to the self-incompatible *Leavenworthia* species was also found by Charlesworth and Yang (1998) using allozymes, and by Liu et al. (1998; 1999) using measures of DNA sequence diversity.

The presence of inbreeding depression in *Leavenworthia* (Charlesworth et al. 1994; Busch 2005a), increased vigor following outcrossing (Busch 2006), and lower levels of genetic diversity in selfers (Solbrig and Rollins 1977; Charlesworth and Yang 1998; Liu et al. 1998; 1999) should act to prevent the evolution of selfing in this system. Since that is not the case, studies have looked at the role of other factors, such as reproductive assurance, in the evolution of self-fertilization in *Leavenworthia*. Busch (2005b) examined whether selfers had an advantage over outcrossers in geographically peripheral populations that may receive limited pollinator visitation. He found that selfers outperformed outcrossers in all habitats because all habitats experienced pollen limitation, but that peripheral populations showed reduced genetic variation consistent with a

population bottleneck. Thus, self-compatibility may have evolved as a result of the interplay between population size and the genetic self-incompatibility system following long-distance dispersal. In addition, Anderson and Busch (2006) found evidence of relaxed pollinator-mediated selection in self-compatible *Leavenworthia* taxa, suggesting that lack of pollinator visitation may still play a role in the evolution of self-fertilization in the genus.

Interestingly, floral and mating system evolution in *Leavenworthia* is not strongly associated with speciation. *Leavenworthia* species are primarily distinguished by their strongly divergent fruit shapes (an important species-diagnostic character in the Brassicaceae; Rollins 1993) and by changes in chromosome number (Baldwin 1945). This is particularly true of *L. alabamica* and *L. crassa*, which are solely distinguished by their fruit shapes, overlap in geography, ecology, and floral morphology (Rollins 1963), have the same chromosome number (Baldwin 1945), and can produce viable and fertile F_1 and F_2 hybrids in the greenhouse (Rollins 1963). Importantly, Rollins did not test for the possibility of cytonuclear incompatibility or of unilateral incompatibility. Later work by Lloyd (1968) tested for unilateral incompatibility, but found only weak support for the phenomenon. However, despite this apparent morphological and genetic overlap, *L. alabamica* and *L. crassa* do form weakly monophyletic sister lineages on the basis of cpDNA variation (Beck et al. 2006), suggesting that these taxa are divergent lineages, but may be in the early stages of this divergence. Thus, *L. alabamica* and *L. crassa* are ideal species in which to

identify reproductive barriers resulting from the speciation process, and to investigate the possible role of mating system evolution in speciation.

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CHAPTER 2

INTRINSIC GENETIC BARRIERS TO REPRODUCTION BETWEEN *L. ALABAMICA* AND *L. CRASSA*

INTRODUCTION

Intrinsic genetic barriers to cross-fertilization are found in both plant and animal taxa, and contribute to reproductive isolation between species. These barriers can occur post-mating, but pre-fertilization (Howard 1999), or post-fertilization (Stebbins 1958; Coyne and Orr 2004). Post-fertilization barriers often result from chromosomal (Rieseberg 2001; Brown et al. 2004), genic (Dobzhansky 1936; Muller 1939), or cytonuclear (Michaelis 1954; Levin 2003) incompatibilities. Chromosomal and genic incompatibilities manifest as hybrid inviability or sterility when taxa are crossed, irrespective of the directionality of the cross. Cytonuclear incompatibilities, on the other hand, as well as barriers that occur post-mating, but pre-fertilization, often result in unidirectional cross success (reviewed in Grant 1975; Tiffin et al. 2001).

In plants, another asymmetrical crossing barrier is possible, termed unilateral incompatibility (Lewis and Crowe 1958), which is related to self-incompatibility. Unilateral incompatibility (UI) occurs when a plant that is self-incompatible (SI) cannot cross, or crosses less successfully, with a self-compatible (SC) plant when the SI plant is the pollen recipient and the SC plant

the pollen donor; conversely, the reciprocal cross is successful. UI is widespread in plant families with a genetic self-incompatibility system (Lewis and Crowe 1958; Heslop-Harrison 1982; Hiscock and Dickinson 1993) and has been suggested as a barrier to interspecies mating (Harrison and Darby 1955; Grun and Radlow 1961); however, this remains controversial as it has also been documented within species (Martin 1963; Lloyd 1968; Pandey 1981) and the genetic mechanism remains unknown. Although these types of intrinsic reproductive barriers are known to occur in many organisms, questions remain regarding both their genetic basis (Orr et al. 2004; Orr 2005) and their importance in the speciation process relative to mating barriers that occur pre-fertilization (Levin 1978; Coyne and Orr 2004).

In the present study, I set out to investigate whether or not intrinsic genetic barriers to reproduction occur between the species *Leavenworthia alabamica* and *L. crassa* as part of a larger study aimed at understanding processes promoting speciation between these taxa. These species have been the subject of studies into the evolution of self-fertilization from self-incompatibility due to the observation that, although some populations possess the sporophytic self-incompatibility system common to other members of the Brassicaceae (Bateman 1955; Lloyd 1967), others are capable of autogamous seed production (Rollins 1963; Lloyd 1965). Rollins (1963) hypothesized that self-fertilization evolved at least three times within this small genus of only eight species (five times if *L. alabamica* and *L. crassa* also represent independent events), and recent

phylogenetic evidence has corroborated Rollins' hypothesis of at least three independent events (Beck et al. 2006).

In addition, Lloyd's (1965) work on the striking morphological changes associated with the transition from outcrossing to self-fertilization in these species played an important role in our understanding of the connection between plant breeding system evolution and floral adaptation. In particular, Lloyd (1965) found that more highly autogamous populations of *L. alabamica* and *L. crassa* had smaller flowers, reduced anther-stigma distances, anthers oriented to dehisce toward rather than away from the stigma, smaller pollen to ovule ratios, and little or no floral scent. He hypothesized that these changes were adaptations to a self-fertilizing mating system; a belief now commonly held due to the discovery of these floral trait transitions in a wide variety of plant groups (Holsinger 1996).

The studies of Rollins (1963) and Lloyd (1965) prompted a large body of experimental work to further understand why self-fertilization evolved so many times in *Leavenworthia* and its genetic consequences. Lyons and Antonovics (1991) looked at two populations of *L. crassa* and found that the outcrossing rate in the field was indeed significantly higher in the population believed to be outcrossing based on floral morphology than in the selfing population, confirming the association between morphology and mating system. Furthermore, inbreeding depression was documented in *L. crassa* (Charlesworth et al. 1994) and *L. alabamica* (Busch 2005a). Busch (2005a) found less inbreeding depression in self-compatible populations compared to self-incompatible

populations, and documented heterosis in a small, isolated, and highly self-compatible population (Busch 2006).

In addition, genetic diversity in *Leavenworthia*, including *L. alabamica* and *L. crassa*, was examined by Solbrig (1972) and Solbrig and Rollins (1977). These studies found that self-compatibility throughout the genus is associated with lower genetic diversity (measured using allozymes). Low genetic diversity in the self-compatible compared to the self-incompatible *Leavenworthia* species was also found by Charlesworth and Yang (1998) using allozymes, and by Liu et al. (1998; 1999) using measures of DNA sequence diversity.

The presence of inbreeding depression in *Leavenworthia* (Charlesworth et al. 1994; Busch 2005a), increased vigor following outcrossing (Busch 2006), and lower levels of genetic diversity in selfers (Solbrig and Rollins 1977; Charlesworth and Yang 1998; Liu et al. 1998; 1999) should act to prevent the evolution of selfing in this system. Since that is not the case, studies have looked at the role of other factors, such as reproductive assurance, in the evolution of self-fertilization in *Leavenworthia*. Busch (2005b) examined whether selfers had an advantage over outcrossers in geographically peripheral populations that may receive limited pollinator visitation. He found that selfers outperformed outcrossers in all habitats because all habitats experienced pollen limitation, but that peripheral populations showed reduced genetic variation consistent with a population bottleneck. Thus, self-compatibility may have evolved as a result of the interplay between population size and the genetic self-incompatibility system following long-distance dispersal. In addition, Anderson and Busch (2006) found

evidence of relaxed pollinator-mediated selection in self-compatible *Leavenworthia* taxa, suggesting that lack of pollinator visitation may still play a role in the evolution of self-fertilization in the genus.

Interestingly, floral and mating system evolution in *Leavenworthia* is not strongly associated with speciation. *Leavenworthia* species are primarily distinguished by their strongly divergent fruit shapes (an important species-diagnostic character in the Brassicaceae; Rollins 1993) and by changes in chromosome number (Baldwin 1945). This is particularly true of *L. alabamica* and *L. crassa*, which are solely distinguished by their fruit shapes, overlap in geography, ecology, and floral morphology (Rollins 1963), have the same chromosome number (Baldwin 1945), and can produce viable and fertile F_1 and F_2 hybrids in the greenhouse (Rollins 1963). Importantly, Rollins did not test for the possibility of cytonuclear incompatibility or of unilateral incompatibility. Later work by Lloyd (1968) tested for unilateral incompatibility, but found only weak support for the phenomenon. However, despite this apparent morphological and genetic overlap, *L. alabamica* and *L. crassa* do form weakly monophyletic sister lineages on the basis of cpDNA variation (Beck et al. 2006), suggesting that these taxa are divergent lineages, but may be in the early stages of this divergence. Thus, *L. alabamica* and *L. crassa* are ideal species in which to identify reproductive barriers resulting from the speciation process.

In order to test the hypothesis that intrinsic post-mating barriers to fertilization maintain the distinctness of the *L. alabamica* and *L. crassa* lineages, I conducted crosses in the greenhouse and quantified F_1, F_2, and backcross hybrid

seed set in relation to within-species seed set. I also quantified seed set with respect to which species contributed the plastid and nuclear genomes in a cross, as well as the SI status of the parent plants. This experiment was designed to answer the following questions: 1) are chromosomal or genic incompatibilities present?, 2) are cytonuclear incompatibilities present?, and 3) does unilateral incompatibility occur? From these data, I evaluate the potential importance of post-mating reproductive barriers in the speciation of *L. alabamica* and *L. crassa*.

MATERIALS AND METHODS

Study Organisms—Leavenworthia alabamica and *L. crassa* (Brassicaceae) belong to a small genus of eight species, all of which are winter annuals endemic to limestone glades (Rollins 1963). The limestone glade habitats in which they grow are characterized by thin calcareous soil over a dolomitic limestone base that remains extremely wet during the winter months (Baskin et al. 1995). These species germinate in the fall between late September and early November, flower from early March to mid-April, and fruits mature between mid-April to early May (Rollins 1963). They are pollinated by solitary bee species, as well as honey bees (Lloyd 1965). *L. alabamica* occurs in the Moulton and Tennessee Valleys of northwestern Alabama, where it occupies only four counties. *L. crassa* occurs solely in the Moulton Valley and is found in only one county (Rollins 1963). The ranges of the two species overlap in Morgan Co., Alabama, where potential hybrids have been documented in disturbed habitat

(Rollins 1963; Lloyd 1965). In addition, *L. alabamica* and *L. crassa* are sister species with n=11 chromosomes (Baldwin 1945; Beck et al. 2006).

Experimental Design—To compare the ability of *L. alabamica* and *L. crassa* to produce hybrid and within-species seed, I performed crosses in the greenhouse on plants grown from seed collected from natural populations in the spring of 2004. The experiment was performed in two years, with the first group of plants crossed in the fall of 2004, and the second group crossed in the spring of 2005. The parent generation consisted of 18 plants (nine from each species) grown from three populations of each species. Three individuals, all from different seed parents, were grown from each population. Seeds were planted in Fafard brand potting soil mixed with lime, and, once rosettes were large enough, transplanted to 6" pots for the remainder of the experiment. The first group of parent species' plants were germinated in an 18˚C growth chamber. Due to slow germination and space constraints, all other plants in the experiment were germinated in the greenhouse. All plants were watered daily and fertilized weekly.

F_1 seed from each parent-generation year was then grown, using the methods described above, to examine the ability of F_1 hybrids to produce F_2 and backcross hybrid seed. Plants derived from the first parent generation were crossed in the summer of 2005, and those from the second parent generation were crossed in the spring of 2006. Each group of F_1 crosses consisted of 18 F_1 hybrids crossed to produce F_2 hybrids, and 12 F_1 plants (6 F_1 hybrids and 6 F_1 parentals) crossed to produce backcross hybrids. The F_1 plants used to generate

F_2 hybrids consisted of nine F_1 hybrids of *L. alabamica* x *L. crassa* origin and nine from the reciprocal cross. The F_1 plants used to generate backcross hybrids consisted of three F_1 hybrids of *L. alabamica* x *L. crassa* origin, three from the reciprocal cross, three *L. alabamica* F_1s, and three *L. crassa* F_1s. Since these are annual species, backcrosses could not be performed to the plants from the parent generation, necessitating the use of F_1s derived from within-species crosses (Figure 2.1).

All possible crosses between individuals in each crossing array were performed (including self crosses), and each cross was made five times, except in the first year of parent-generation crosses. In that group, no between-population crosses were performed and each cross was made four times. Fewer crosses were performed in this initial group because, at the time, I did not know how many flowers could be produced or how many fruits matured before an individual plant senesced. After the initial parent generation was crossed, it was clear that additional crosses could be sustained on each plant, and I subsequently increased the number of crosses and performed between-population crosses in the second parent generation. For all crosses, flowers were emasculated one day prior to anthesis to prevent self-fertilization. This was done using a pair of fine forceps to open buds without damaging the pistil and remove all six indehiscent anthers. Crosses were made on the first day of anthesis by brushing an anther from the donor individual on the stigmatic surface of the recipient pistil until it was covered with pollen. Each cross was marked with a

tape label wrapped around the pedicel of the flower. Fruits from crosses were allowed to mature, then collected and the number of seeds per fruit counted.

In addition, I assessed the viability of the pollen of each plant used in crosses. In the first parent generation, pollen quality was determined visually (only well-developed and dehiscent anthers were used in crosses). In all subsequent crossing arrays, I quantified the percentage of viable pollen using Alexander's stain (Kearns and Inouye 1993), which stains viable pollen grains red and inviable grains green. Three flowers from each plant were collected, and an anther from each flower brushed gently on a glass slide to remove the pollen. An appropriate amount of stain was then placed on the pollen, covered with a cover-slip, and heated for a few seconds on a hot plate to fix the stain. From each slide, a sample of 300 pollen grains was counted. Pollen viability was high (between 95-99%) for all plants, including F_1 hybrids. These data will be discussed in more detail in Chapter 3.

I also assessed the mean number of ovules for each plant used in crosses. Three flowers per plant were measured by placing the pistil of a flower between two glass slides, then gently applying pressure on the pistil with the top slide until the ovules were seen under a dissecting scope as dark spots along each half of the ovary. Using this method, I was able to count the number of ovules without dissecting them from the pistil. It was important to know the mean number of ovules per plant due to a difference in mean ovule number between *L. alabamica* and *L. crassa*. *L. alabamica* produces, on average, 12 ovules, whereas *L. crassa* produces 8 (discussed in more detail in Chapter 2). I then

used the ovule number from each crossed plant to correct for the differential seed production between the two species.

Statistical Analyses—I used fixed-effect analysis of variance methods (Quinn and Keough 2002) to test for 1) differences in the amount of F_1 hybrid versus within-species seed produced in crosses of *L. alabamica* with *L. crassa*, and in the amount of F_2 or BC hybrid versus within-species seed produced in crosses of F_1 plants, 2) differences in the direction of cross-compatibility depending on whether or not the individual had *alabamica*- or *crassa*-type plastid and nuclear genomes, and 3) differences in the direction of cross-compatibility depending on the self-incompatibility status of the individual. A Bonferroni-corrected alpha-value of 0.0125 and 0.016 was used as the overall significance level for the parent-generation and F_1-generation analyses, respectively, to control the Type I error rate (Quinn and Keough 2002). For all analyses of variance, the response variable used was the arcsine transformation of the mean proportion of seed produced per cross per plant (the mean number of seeds produced by each plant for each cross divided by the mean number of ovules produced by the plant). The mean proportion of seed produced per cross per plant was used because it accounted for the difference in ovule production between the two species, which otherwise would have resulted in the erroneous conclusion that *L. alabamica* produced more seeds than *L. crassa*. This variable was then arcsine transformed to improve normality. ANOVAs were performed on each generation of plants in each year. All analyses were performed using JMP 6.0 (SAS Institute 2005).

RESULTS

In order to test for F_1 or F_2/BC hybrid-breakdown indicative of chromosomal or genic incompatibilities between *L. alabamica* and *L. crassa*, I analyzed differences in the amount of these seed types produced in crosses relative to within-species seed. I found no significant differences between years, seed types, or in the interaction between seed type and year among the parent-generation plants for the amount of seed produced. The same analysis was performed on F_1 individuals to test for differences in their ability to produce within-species, F_1 hybrid, F_2 hybrid, or BC hybrid seed. A significant year effect was detected ($df=1$, $F=128.8908$, $p<0.0001$), but no significant differences were found among seed types, or for the interaction term. These data indicate that parent- and F_1-generation plants produced all seed types (within-species or hybrid) equally well.

I also tested for the presence of cytonuclear incompatibilities between *L. alabamica* and *L. crassa* based on whether or not crosses yielded different amounts of seed depending on if the crossed individuals had plastid and nuclear genomes from the same species ("*alabamica* ♀ x *alabamica* ♂", "*crassa* ♀ x *crassa* ♂"), or plastid genomes from one species and a portion of the nuclear genome from the other ("*crassa* ♀ x *alabamica* ♂", and "*alabamica* ♀ x *crassa* ♂"). I found no significant differences between the years, between the four cross categories, or for the interaction of the cross type with year on seed set. A linear contrast pooled across years comparing the two categories with opposing plastid and nuclear genomes ("*alabamica* ♀ x *crassa* ♂" and "*crassa* ♀ x *alabamica* ♂")

found no significant difference. With respect to the F_1-generation, I found significant differences in seed production between years (df=1, F=581.7573, p<0.0001), and a marginally significant (after Bonferroni correction) difference among the four cross categories (df= 3, F=3.5694, p=0.0138), as well as a significant cross type by year interaction (df=3, F=8.6149, p<0.0001) on the amount of seed produced. However, a linear contrast pooled across years comparing the "*alabamica* ♀ x *crassa* ♂" and "*crassa* ♀ x *alabamica* ♂" categories was not significant. Nor were contrasts comparing the two categories within year 1 or 2. These data indicate that parent- and F_1-generation plants produce seed equally well whether the cross involves matched ("*alabamica* ♀ x *alabamica* ♂", "*crassa* ♀ x *crassa* ♂") or opposed ("*alabamica* ♀ x *crassa* ♂" and "*crassa* ♀ x *alabamica* ♂") plastid and nuclear genomes.

In addition, I tested the hypothesis that the self-incompatibility (SI) status of the maternal and paternal parents affected the outcome of a cross (SC ♀ x SC ♂, SC ♀ x SI ♂, SI ♀ x SC ♂, and SI ♀ x SI ♂); i.e., for the presence of unilateral incompatibility. For the within-species parent-generation crosses, I found no significant year effect, a significant effect of compatibility type (df=3, F=23.9014, p<0.0001), and no significant effect of the interaction between year and compatibility type (Figure 2.2). Linear contrasts pooled across years comparing the success of cross categories found no significant difference between the SC ♀ x SI ♂ and SI ♀ x SC ♂ compatibility types or between the SI ♀ x SC ♂ and SI ♀ x SI ♂ types. However, there was a significant difference

between the SC ♀ x SC ♂ and SC ♀ x SI ♂ types (t=3.1673, p=0.0018). The results of the between-species crosses among parent-generation plants also showed no significant effect of year, a significant effect of compatibility type (df=3, F=25.8026, p<0.0001), and no significant interaction between the two effects (Figure 2.3). A linear contrast, pooled across years, comparing SC ♀ x SI ♂ with SI ♀ x SC ♂ crosses was significant (t=6.501, p<0.0001). A contrast comparing the SC ♀ x SC and SC ♀ x SI ♂ crosses was not significant, nor was one comparing the SI ♀ x SC ♂ and SI ♀ x SI ♂ categories. In addition, the crosses among F_1-generation plants showed a significant effect of year (df=1, F=480.6881, p<0.001), of compatibility type (df=3, F=37.0747, p<0.0001), and of the compatibility type by year interaction (df=3, F=4.9841, p=0.0020) (Figure 2.4). Comparing the SC ♀ x SI ♂ and SI ♀ x SC ♂ categories with a linear contrast pooled across years, I found a significant difference between the two (t=9.4267, p<0.0001). A contrast between the SC ♀ x SC ♂ and SC ♀ x SI ♂ categories was not significant, nor was the contrast between the SI ♀ x SC ♂ and SI ♀ x SI ♂ categories. Contrasts within each year had the same results for each of the three comparisons. The compatibility type data indicate that, although within-species parent-, between-species parent-, and F_1-generation plants all show a significant effect of compatibility type on seed production, the compatibility types that differ from one another are not the same in each group. The between-species parent-generation (Figure 2.3) and F_1-generation plants (Figure 2.4) show a pattern of

unilateral incompatibility, whereas the within-species parent-generation plants do not (Figure 2.2).

DISCUSSION

A significant difference between years was found when comparing seed production of the F_1-generation plants in all ANOVAs. This difference results from the fact that crosses among the first year of F_1 plants were less successful than those in the second year. This is most likely due to differences in growing conditions between the two years; F_1 plants in the first year were grown during the summer under much higher greenhouse temperatures (sometimes in excess of 35˚C) than those experienced by plants in the second year, and many plants began to wilt and senesce after only a few weeks of flowering. All other crossing groups were grown in the fall/winter months. *Leavenworthia* species are all winter annuals, and it is therefore likely that heat stress not normally experienced by either of these species during their flowering season had an adverse affect on overall seed production.

No significant differences were found between parent- or F_1-generation plants in their ability to produce either within-species or hybrid seed. Nor were any significant differences found in seed production between plants of either generation when comparing crosses involving plants with mismatched plastid and nuclear genomes ("*alabamica* ♀ x *crassa* ♂" or "*crassa* ♀ x *alabamica* ♂"). These results indicate that no intrinsic barriers to species-level hybridization, such as chromosomal, genic, or cytonuclear incompatibilities, are present

between *L. alabamica* and *L. crassa*. This finding agrees with early work on cross-compatibility by Rollins (1963), in which he was able to produce viable and fertile F_1, F_2, and BC hybrids of these species. Given the presumed complexity of genetic incompatibilities and, consequently, the difficulty in losing these traits once evolved (Gould 1970), the absence of genetic incompatibilities between these species makes it unlikely that they were ever present. However, it is possible that purely extrinsic genetic incompatibilities act as barriers to gene flow between these species, a possibility that will be explored in Chapter 4.

In this experiment, I also wanted to test for the presence of a phenomenon described in the literature as unilateral incompatibility (UI). This is a cross-fertilization barrier described as the "SIxSC rule" (Lewis and Crowe 1958), in which crosses where the maternal parent is self-compatible (SC) and the paternal parent self-incompatible (SI) are more successful than those where the maternal parent is self-incompatible (SI) and the paternal parent self-compatible (SC). In other words, crosses between plants with differing SI status are more (or exclusively) successful in one direction.

Unilateral incompatibility has been primarily documented between species of differing SI status (one species is SI and the other is SC) (Anderson and de Winton 1931; Harrison and Darby 1955; Lewis and Crowe 1958; Martin 1961; Martin 1964; Pandey 1962; Sampson 1962; Lloyd 1968; Heslop-Harrison 1982; Onus and Pickersgill 2004). Fewer studies have demonstrated within-species UI (Martin 1963; Lloyd 1968; Pandey 1981), and UI between inter-species hybrids (Anderson and de Winton 1931; Martin 1964; Hardon 1967). Indeed, UI has been

proposed as a barrier to inter-species hybridization (Harrison and Darby 1955; Grun and Radlow 1961), potentially arising from a genetic species-recognition system that is either part of, or similar to, the genetic self-incompatibility system (Sampson 1962; Heslop-Harrison 1982; Hiscock and Dickinson 1993). This hypothesis arose from the widespread observation that UI involves the inhibition of pollen tube germination and/or growth (Hancock et al. 2003; Kemp and Doughty 2003), suggesting a mechanism similar to the genetic self-incompatibility system of a plant group, and therefore genetic explanations have primarily been couched in terms of pollen-pistil interactions (but see Brandvain and Haig 2005 for an alternative hypothesis). Molecular studies in the Solanaceae support the hypothesis that the S-locus is involved in UI in this family (Chetelat and DeVerna 1991; Murfett et al. 1996; Bernacchi and Tanksley 1997; Beecher 1998; Beecher 2001; Cruz-Garcia et al. 2003), but not in the Brassicaceae (Kandasamy et al. 1994; Escobar-Restrepo et al. 2007). There have also been documented cases of UI that do not adhere to the SIxSC rule (Martin 1961; Martin 1963; Pandey 1962; Kuhl et al. 2002; Takada et al. 2005). In these cases, unidirectional cross success is observed irrespective of the SI status of the species, but the crosses that fail still do so because of pollen inhibition rather than postzygotic processes, leading to the hypothesis that UI may be controlled by another mechanism. However, due to the fact that most cases of UI were observed in between-species crosses, it has been difficult to determine if UI is due to an active mechanism of rejection or simply a lack of

recognition of another species pollen due to overall genetic divergence (Hogenboom 1975).

Due to the mating system polymorphism present in both *L. alabamica* and *L. crassa*, and their close evolutionary relationship (Beck et al. 2006), these species offered a rare opportunity to test for unilateral incompatibility within-species, between-species, and between inter-species hybrids, using species not yet highly diverged. Previous work by Lloyd (1968) documented what he termed "partial unilateral incompatibility" within *L. alabamica* and *L. crassa*. However, Lloyd did not self plants used in crosses to determine the SI status of each individual. He estimated the selfing rate of populations based on greenhouse crosses and then categorized those populations as either fully selfing, intermediate, or fully outcrossing. He then crossed individuals from each population type, and found that the "SIxSC rule" did not hold for many of his crosses. It is possible though that some of his crosses were not in fact properly categorized as SC ♀ x SI ♂ or SI ♀ x SC ♂ since he did not know the SI status of the individuals used. In my experiment, in which the SI status of each individual was known, I found evidence of the presence of unilateral incompatibility in between-species crosses of the parent-generation (significantly greater seed production from SC ♀ x SI ♂ crosses v. SI ♀ x SC ♂ crosses; Figure 2.2), as well as in between-hybrid crosses of the F_1-generation (Figure 2.3). The results of the within-species crosses were less clear (Figure 2.1), with a non-significant difference between SC ♀ x SI ♂ and SI ♀ x SC ♂ crosses suggesting that UI is not present within these species. However, the lack of significance may be due to

small sample size (fewer than five crosses were performed in those two categories in the first year of the experiment). This was in part due to the fact that the SI status of the plant was not determined until the experiment was in progress, and because between-population crosses were not performed in the first year. Only one of the populations from which the starting generation was drawn was polymorphic with respect to SI status, and, consequently, without between-population crosses, few SC ♀ x SI ♂ and SI ♀ x SC ♂ crosses were performed. Between-population crosses were performed in the second year, remedying this issue, but pooled across years the sample sizes were not equal. It is therefore possible that this sample size issue affected my ability to detect UI within *L. alabamica* and *L. crassa*. This is supported by the fact that the overall compatibility trend is in the same direction as for the between-species and between-hybrid crosses. The SC ♀ x SI ♂ and SI ♀ x SC ♂ categories do not switch rank in either year with respect to the amount of seed produced, suggesting that unilateral incompatibility could be occurring within these species. It will take further experimental work to confirm the presence or absence of within-species UI in *L. alabamica* and *L. crassa*.

In *L. alabamica* and *L. crassa*, I did not find evidence for crossing barriers other than those related to a plant's SI status. This lends greater weight to the hypothesis that UI is mechanistically related to the genetic self-incompatibility system, and the results of this study suggest a possible mechanism for UI in *Leavenworthia*. Although significant differences in seed production were found between SC ♀ x SI ♂ crosses and SI ♀ x SC ♂ crosses (Figure 2.2 and 2.3), no

significant differences were found between SC ♀ x SI ♂ and SC ♀ x SC ♂ crosses in the between-species parent- and F_1-generation groups, or between SI ♀ x SC ♂ and SI ♀ x SI ♂ crosses in within-species parent-, between-species parent-, or F_1-generation groups. This pattern suggests that the failure of crosses in the SI ♀ x SC ♂ category is due to SI plants recognizing functional S-alleles on the pollen of the SC donor and rejecting it as self-pollen, just as these SI plants recognize functional S-alleles on SI-donor pollen in the SI ♀ x SI ♂ crosses that fail to set seed. On the other hand, the success of crosses in the SC ♀ x SI ♂ and SC ♀ x SC ♂ categories can be attributed to a lack of recognition of functional S-alleles on the pollen of SC or SI donors by the SC plants, and therefore no SI reaction occurs. Although plants in the within-species parent-generation group differed significantly with respect to SC ♀ x SI ♂ and SC ♀ x SC ♂ crosses (Figure 2.1), potentially due to the smaller number of SC ♀ x SI ♂ crosses, the overall pattern is the same as in the other crossing groups, and does not rule out the possibility that SC plants are generally unable to recognize and reject pollen with the same S-alleles as themselves. Consequently, I suggest the possibility that the UI phenomenon in these *Leavenworthia* species may be a direct result of the break-down of the self-incompatibility system rather than due to a functionally related species-recognition system. However, detailed molecular genetic analyses are needed before the mechanism of UI can be determined in *Leavenworthia*, as well as other plant groups. It seems likely that plant groups

will differ in their mechanism(s) of UI, just as they differ in genetic mechanisms of self-incompatibility (Richards 1997).

Although posited as a species-barrier (Harrison and Darby 1955; Grun and Radlow 1961), if UI is indeed occurring both within- and between-species in *Leavenworthia*, then it is not a species-barrier per se. Rather, UI will impact the degree to which SC and SI individuals mate, likely increasing population structure in each species, thereby indirectly impacting the speciation process. However, if UI does occur solely between these species and their hybrids, then it would function as a post-mating reproductive barrier in this system, and may have had an important role in the divergence of *L. alabamica* and *L. crassa*.

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FIGURE LEGENDS

Figure 2.1: Cross design used to produce within-species and hybrid seed of *L. alabamica* and *L. crassa*. Plants of the same and of different species were reciprocally crossed to produce first-generation within-species and hybrid seed. Next, one group of first-generation hybrids were reciprocally crossed to produce second-generation hybrids. Another group of first-generation hybrids and first-generation pure species plants were reciprocally crossed to produce primarily backcross and second-generation hybrids. The experiment was performed twice using different *L. alabamica* and *L. crassa* individuals for each starting generation.

Figure 2.2: The proportion of seed produced from within-species parent-generation crosses involving plants of either the same or differing SI status. SC ♀ x SC ♂ =self-compatible x self-compatible; SC ♀ x SI ♂ =self-compatible x self-incompatible; SI ♀ x SC ♂ =self-incompatible x self-compatible; Si ♀ x SI ♂ =self-incompatible x self-incompatible. A linear contrast comparing the SC ♀ x SC ♂ and SC ♀ x SI ♂ categories was significant (t=3.1673, p=0.0018); marked with an "*". Means are shown with standard error bars.

Figure 2.3: The proportion of seed produced from between-species parent-generation crosses involving plants of either the same or differing SI status. SC ♀ x SC ♂ =self-compatible x self-compatible; SC ♀ x SI ♂ =self-compatible x self-incompatible; SI ♀ x SC ♂ =self-incompatible x self-compatible; Si ♀ x SI ♂ =self-incompatible x self-incompatible. A linear contrast comparing the SC ♀ x SI ♂ and SI ♀ x SC ♂ categories was significant (t=6.501, p<0.0001); marked with an "*". Means are shown with standard error bars.

Figure 2.4: The proportion of seed produced from F_1-generation crosses involving plants of either the same or differing SI status. SC ♀ x SC ♂ =self-compatible x self-compatible; SC ♀ x SI ♂ =self-compatible x self-incompatible; SI ♀ x SC ♂ =self-incompatible x self-compatible; Si ♀ x SI ♂ =self-incompatible x self-incompatible. A linear contrast comparing the SC ♀ x SI ♂ and SI ♀ x SC ♂ categories was significant (t=9.4267, p<0.0001); marked with an "*". Means are shown with standard error bars.

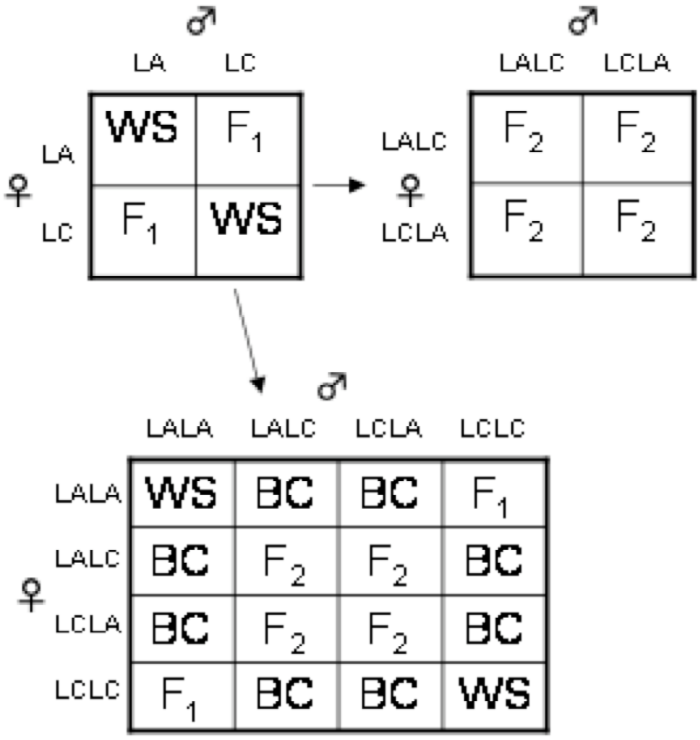
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Figure 2.1

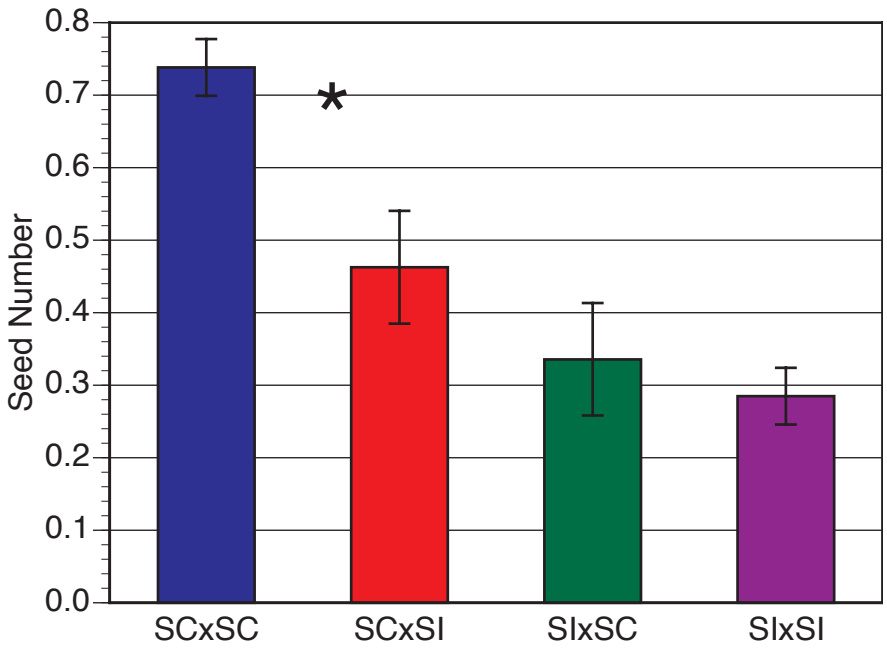

Figure 2.2

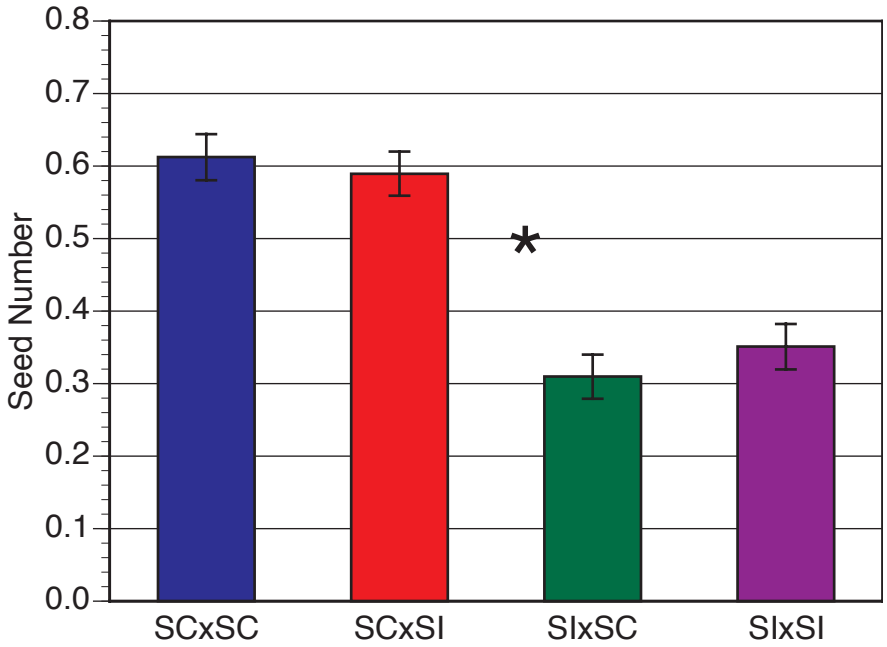

Figure 2.3

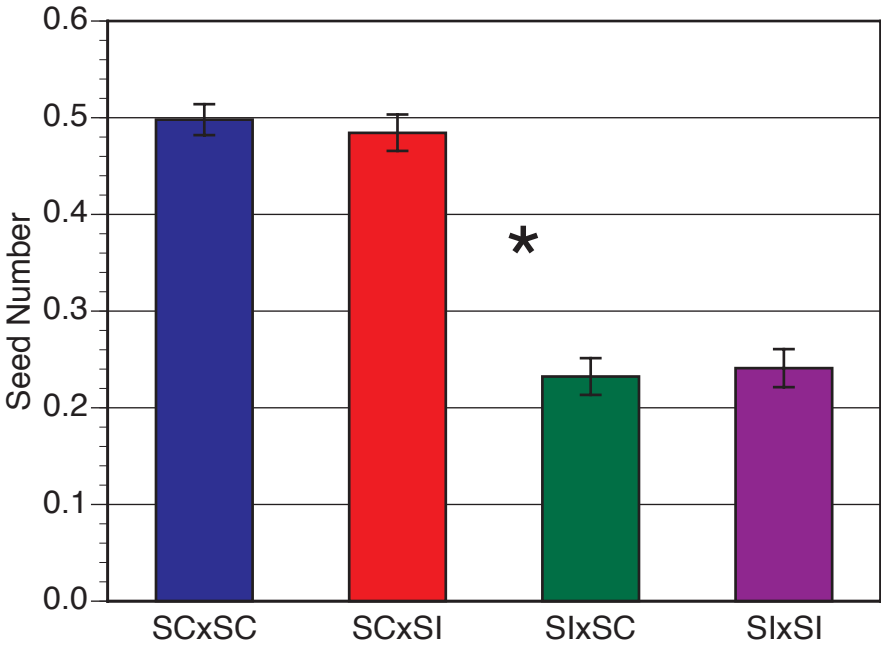

Figure 2.4

CHAPTER 3

PHENOTYPIC DIVERSITY AND TRANSGRESSIVE SEGREGATION IN HYBRIDS OF *LEAVENWORTHIA ALABAMICA* AND *L. CRASSA*

INTRODUCTION

Hybridization is a widespread phenomenon occurring in all taxonomic groups (Arnold 2006), yet for most organisms little is known about the genetic and phenotypic results of this process and their fitness consequences. The potential genetic outcomes of hybridization (reviewed in Burke and Arnold 2001) are hybrid breakdown (due to negative epistasis), hybrid vigor in the F_1 (due to dominance effects resulting from outcrossing), hybrid vigor in later-generation hybrids (due to positive epistasis), and transgressive segregation in F_2, backcross (BC), or later generation hybrids (due to novel additive effects of loci). Which of these phenomena occur in a hybridization event will depend on the genetic architecture of the parent lineages and, often, on the environmental context of hybrids (e.g. Emms and Arnold 1997; Hatfield and Schluter 1999; Rieseberg et al. 2003; Fritz et al. 2006; Wu and Campbell 2006).

Transgressive segregation (or variation) occurs when hybrids achieve trait values not seen in the parent lineages. In general, this is thought to occur when QTL underlying a trait are of mixed effect (i.e. QTL both increase and decrease trait values) and recombination brings together QTL of the same effect that were

previously not present in the same genome (Grant 1975; deVicente and Tanksley 1993; Rieseberg et al. 1999) (although inbreeding may also play a role, Rick and Smith, 1953). Transgressive segregation has been documented in both plant and animal hybridization events (Rieseberg et al. 2003; Albertson and Kocher 2005), and in some cases has allowed hybrid lineages to invade new ecological niches, aiding in hybrid zone stabilization (Schweitzer et al. 2002) or hybrid speciation (Rosenthal et al. 2002; Rieseberg et al. 2003; Bell and Travis 2005). Alternatively, transgressive phenotypes may be selected against in some hybridization events (e.g. Rogers and Bernatchez 2006), possibly inhibiting introgression and selecting for the reinforcement of pre-mating barriers to reproduction (Servedio and Noor 2003). Therefore, transgressive segregation can be an important factor in speciation, and documenting its presence or absence in interspecies hybrids can further our understanding of how species' boundaries are maintained in the face of gene flow.

To this end, I set out to document the presence or absence of hybrid breakdown, hybrid vigor, and/or transgressive segregation in F_1, F_2, and BC hybrids of the species *Leavenworthia alabamica* and *L. crassa*. These species have been the subject of studies into the evolution of self-fertilization from self-incompatibility due to the observation that, although some populations possess the sporophytic self-incompatibility system common to other members of the Brassicaceae (Bateman 1955; Lloyd 1967), others are capable of autogamous seed production (Rollins 1963; Lloyd 1965). Rollins (1963) hypothesized that self-fertilization evolved at least three times within this small genus of only eight

species (five times if *L. alabamica* and *L. crassa* also represent independent events), and recent phylogenetic evidence has corroborated Rollins' hypothesis of at least three independent events (Beck et al. 2006).

In addition, Lloyd's (1965) work on the striking morphological changes associated with the transition from outcrossing to self-fertilization in these species played an important role in our understanding of the connection between plant breeding system evolution and floral adaptation. In particular, Lloyd (1965) found that more highly autogamous populations of *L. alabamica* and *L. crassa* had smaller flowers, reduced anther-stigma distances, anthers oriented to dehisce toward rather than away from the stigma, smaller pollen to ovule ratios, and little or no floral scent. He hypothesized that these changes were adaptations to a self-fertilizing mating system; a belief now commonly held due to the discovery of these floral trait transitions in a wide variety of plant groups (Holsinger 1996).

The studies of Rollins (1963) and Lloyd (1965) prompted a large body of experimental work to further understand why self-fertilization evolved so many times in *Leavenworthia* and its genetic consequences. Lyons and Antonovics (1991) looked at two populations of *L. crassa* and found that the outcrossing rate in the field was indeed significantly higher in the population believed to be outcrossing based on floral morphology than in the selfing population, confirming the association between morphology and mating system. Furthermore, inbreeding depression was documented in *L. crassa* (Charlesworth et al. 1994) and *L. alabamica* (Busch 2005a). Busch (2005a) found less inbreeding

depression in self-compatible populations compared to self-incompatible populations, and documented heterosis in a small, isolated, and highly self-compatible population (Busch 2006).

In addition, genetic diversity in *Leavenworthia*, including *L. alabamica* and *L. crassa*, was examined by Solbrig (1972) and Solbrig and Rollins (1977). These studies found that self-compatibility throughout the genus is associated with lower genetic diversity (measured using allozymes). Low genetic diversity in the self-compatible compared to the self-incompatible *Leavenworthia* species was also found by Charlesworth and Yang (1998) using allozymes, and by Liu et al. (1998; 1999) using measures of DNA sequence diversity.

The presence of inbreeding depression in *Leavenworthia* (Charlesworth et al. 1994; Busch 2005a), increased vigor following outcrossing (Busch 2006), and lower levels of genetic diversity in selfers (Solbrig and Rollins 1977; Charlesworth and Yang 1998; Liu et al. 1998; 1999) should act to prevent the evolution of selfing in this system. Since that is not the case, studies have looked at the role of other factors, such as reproductive assurance, in the evolution of self-fertilization in *Leavenworthia*. Busch (2005b) examined whether selfers had an advantage over outcrossers in geographically peripheral populations that may receive limited pollinator visitation. He found that selfers outperformed outcrossers in all habitats because all habitats experienced pollen limitation, but that peripheral populations showed reduced genetic variation consistent with a population bottleneck. Thus, self-compatibility may have evolved as a result of the interplay between population size and the genetic self-incompatibility system

following long-distance dispersal. In addition, Anderson and Busch (2006) found evidence of relaxed pollinator-mediated selection in self-compatible *Leavenworthia* taxa, suggesting that lack of pollinator visitation may still play a role in the evolution of self-fertilization in the genus.

Interestingly, floral and mating system evolution in *Leavenworthia* is not strongly associated with speciation. *Leavenworthia* species are primarily distinguished by their strongly divergent fruit shapes (an important species-diagnostic character in the Brassicaceae; Rollins 1993) and by changes in chromosome number (Baldwin 1945). This is particularly true of *L. alabamica* and *L. crassa*, which are solely distinguished by their fruit shapes, overlap in geography, ecology, and floral morphology (Rollins 1963), have the same chromosome number (Baldwin 1945), and can produce viable and fertile F_1 and F_2 hybrids in the greenhouse (Rollins 1963). However, despite this apparent morphological and genetic overlap, *L. alabamica* and *L. crassa* do form weakly monophyletic sister lineages on the basis of cpDNA variation (Beck et al. 2006), suggesting that these taxa are divergent lineages, but may be in the early stages of this divergence. Thus, *L. alabamica* and *L. crassa* are ideal species in which to identify reproductive barriers resulting from the speciation process.

To examine the possibility that hybridization between *L. alabamica* and *L. crassa* may be prevented in nature due to the generation of phenotypes with reduced fitness compared to the parent species, I phenotyped *L. alabamica*, *L. crassa*, F_1, F_2, and BC hybrid individuals for a number of characters related to their survival and reproductive biology. I then determined the extent to which the

parent species differed from hybrids for these traits. From these data, I discuss the potential role of transgressive segregation in the speciation of *L. alabamica* and *L. crassa*.

MATERIALS AND METHODS

Study Organisms—Leavenworthia alabamica and *L. crassa* (Brassicaceae) are winter annuals endemic to limestone glades that occur in the Moulton Valley of northwest Alabama (Rollins 1963). A few *L. alabamica* populations also occur in Colbert Co., Alabama, which is located in the Tennessee Valley. These species germinate in the fall, between late September and early November, flower from early March to mid-April, and fruit from mid-April to early May. Seeds of these species remain dormant through the hot, dry summer months (Caudle and Baskin 1968; Baskin and Baskin 1971).

Both *L. alabamica* and *L. crassa* are pollinated by generalist solitary bee species (Lloyd 1965), and have flowers typical of crucifers (Rollins 1963). This means they possess four petals arranged in a cross-like formation, and six stamens, four long and two short (Figure 3.1). Crucifer petals are reflexed, forming a limb (the part attached to the flower base) and a claw (the part that forms the platform of the petal). With respect to floral morphology, *L. alabamica* and *L. crassa* are not distinguishable, and species' taxonomy relies on the large difference in fruit morphology between the two species (Figure 3.2; Rollins 1963).

In addition, they are sister species with the same chromosome number (n=11) (Baldwin 1945) and form monophyletic lineages (Beck et al. 2006),

although these lineages are distinguished by few genetic differences. Hybrids are possible between these species (see Chapter 2), and may occur in nature (Rollins 1963; Lloyd 1965). Thus, *L. alabamica* and *L. crassa* overlap in geography, ecology, and morphology, but maintain some genetic and morphological (fruit shape) differences.

Experimental Design—In order to examine the degree to which hybrids were phenotypically distinct from the parent species, I first conducted crosses to create F_1, F_2, and BC hybrid plants. Crosses were performed on two separate groups of parent species' individuals (years 1 and 2). See Chapter 2: Materials and Methods for a description of the crossing design, methods, and growth conditions of both the parent species and hybrids.

Phenotypic measurements were taken on plants from each generation (parent, F_1, F_2, and BC), and on three flowers per plant. In most cases, all three flowers were measured within a plant's first week of flowering. The total number of plants measured in each generation was 18 *L. alabamica*, 18 *L. crassa*, 98 F_1's, 152 F_2's, and 63 BC hybrids.

The traits measured included the number of days from planting to germination, the number of days from planting to flowering, and the following floral characters: petal width, petal length, corolla tube length, long filament length, short filament length, pistil length, ovule number, the proportion of viable pollen, and anther orientation. Petal width and petal length were, respectively, the width and length of the petal claw (Figure 3.1). Since flowers of *Leavenworthia* do not have fused corolla tubes, corolla tube length was

measured as the length of the petal limb. I measured the length of the filament of a long and short stamen in order to calculate anther exsertion from the corolla tube (length of long filament – length of corolla tube) and anther-stigma distance (length of long filament – pistil length, and length of short filament – pistil length). Given that the short filament lengths were always much shorter than the pistil lengths, such that short anthers were never near the stigmas, this short anther-stigma distance was excluded from analyses.

All length measurements were made to the nearest 0.01 mm using an ocular ruler in the eyepiece of a dissecting microscope. At anthesis, flowers were dissected using a pair of fine forceps, and the flower parts were then pressed between two glass slides to ensure measurement standardization. Only one petal and one pair of long and short filaments were measured, and these were chosen randomly. For a description of how ovule number and the proportion of viable pollen were measured, see Chapter 2: Materials and Methods.

Anther orientation (extrorse or introrse) was scored visually. Although Lloyd (1965) divided anther orientation into multiple angle categories, I placed flowers into the two broader categories of extrorse or introrse based on the general angle and whether or not pollen was present on a flower's stigma. This was done to decrease the time it took to measure each flower, and also because hybrids only exhibited the two angles (towards the stigma or away from the stigma).

Statistical Analyses— Before analyzing differences among plant types (*L. alabamica*, *L. crassa*, and hybrids) for the floral traits measured in this study, I

first determined the extent to which these traits were correlated. I calculated Pearson's correlation coefficient for petal width, petal length, corolla tube length, pistil length, ovule number, anther exsertion, and anther-stigma distance. Anther orientation could not be included in the analysis as it was a categorical variable. I found that petal length was highly correlated with both petal width (ρ=0.8251) and pistil length (ρ=0.8373). Due to these high correlations, petal length was not used in subsequent analyses.

I then performed a MANOVA analysis using the fixed effects of "year", "plant type", and "plant type by year" with the following response variables: petal width, corolla tube length, pistil length, ovule number, anther exsertion, anther-stigma distance, the $\log_{10}$-transformed number of days from planting to germination, and the number of days from planting to flowering. Traits not included in the MANOVA analysis were anther orientation (a categorical variable) and the proportion of viable pollen. The pollen viability data could not be included due to missing values.

Individual ANOVAs were also performed on all continuous response variables once overall significance was established using MANOVA (Tabachnik and Fidell 2001). Bonferroni-correction was used to control for Type I error (Quinn and Keough 2002), and the alpha-value set for each analysis was 0.005. Each univariate ANOVA tested the fixed effects of "year", "plant type", and "plant type by year". Differences between the plant types in anther orientation were examined using a contingency table analysis. This analysis calculates observed

versus expected cell counts and uses the chi-squared test statistic to determine significant differences.

I also used principal components analysis to determine whether or not significant differences were present in the overall floral morphology of the parent species and hybrids. To describe where individual plants fit in multidimensional space with respect to petal width, corolla tube length, pistil length, ovule number, anther exsertion, anther-stigma distance, and anther orientation, principal components were calculated using a correlation matrix. Using the "eigenvalues greater than one" rule (Quinn and Keough 2002), two principal components were retained and rotated using the varimax method to simplify the structure of the data. The loading of each trait on each rotated component was also calculated.

To test for hybrid vigor, hybrid breakdown, and transgressive segregation, I used Dunnett's test to compare the means of each hybrid line to each parent species' population (after the methods of Johansen-Morris and Latta 2006). Dunnett's test is a modified t-test in which all means are compared to a control group (Quinn and Keough 2002). Each parent species' population was designated the control group in a separate analysis, such that six analyses were performed for each trait in each year. The traits analyzed were petal width, corolla tube length, pistil length, ovule number, anther exsertion, anther-stigma distance, the proportion of viable pollen, the number of days from planting to germination, and the number of days from planting to flowering. Analyses were performed using line means rather than individual plant means to control for

random environmental or developmental effects. All statistical analyses were performed using JMP 6.0 (SAS Institute 2005).

RESULTS

To determine if F_1, F_2, or BC hybrids exhibited hybrid breakdown, hybrid vigor, and/or transgressive segregation relative to the parent species, I first examined whether these plant types differed in the traits measured. From the MANOVA, I found significant differences at the $p<0.0001$ level between years, plant types, and in the plant type by year interaction. Subsequent univariate ANOVAs revealed the effect of plant type was significant for petal width ($df=4$, $F=10.2569$, $p<0.0001$), corolla tube length ($df=4$, $F=12.6077$, $p<0.0001$), ovule number ($df=4$, $F=16.3168$, $p<0.0001$), anther exsertion ($df=4$, $F=10.9775$, $p<0.0001$), the proportion of viable pollen ($df=4$, $F=6.7752$, $p<0.0001$), the number of days from planting to germination ($df=4$, $F=91.1824$, $p<0.0001$), and the number of days from planting to flowering ($df=4$, $F=7.2368$, $p<0.0001$). No significant effect of plant type was found for pistil length or anther-stigma-distance. Significant differences between years were only found for petal width ($df=1$, $F=18.4497$, $p<0.0001$), corolla tube length ($df=1$, $F=72.6411$, $p<0.0001$), pistil length ($df=1$, $F=37.4203$, $p<0.0001$), and the number of days from planting to germination ($df=1$, $F=49.6533$, $p<0.0001$). In addition, a significant plant type by year interaction was observed for pistil length ($df=4$, $F=4.0589$, $p=0.0032$), the number of days from planting to germination ($df=4$, $F=29.6141$, $p<0.0001$), and the number of days from planting to flowering ($df=4$, $F=9.8076$, $p<0.0001$). The

means for each trait are listed by plant type in Table 3.1, along with the results of Tukey's HSD test for all pair-wise comparisons of means. The only traits for which the parent species differ significantly from the three hybrid types are ovule number and the number of days from planting to germination. For ovule number, the two parent species differ significantly as well.

Differences between the parent species and hybrid types in the number of individuals with extrorse or introrse anthers were analyzed using a contingency table. I found significant differences between the plant types (df=4, χ^2=22.816, p=0.0001), due to a larger percentage of individuals possessing extrorse anthers in each of the hybrid categories compared to the parent species (Table 3.1).

In order to more simply describe the overall phenotypic appearance of hybrids in relation to *L. alabamica* and *L. crassa*, I performed a principal components analysis using the following floral traits: petal width, corolla tube length, pistil length, ovule number, anther exsertion, anther-stigma distance, and anther orientation score. The data on pollen viability could not be included in the analysis due to missing values. From this analysis, seven principal components were obtained. Only two of these components had eigenvalues greater than one, and so these two components were rotated using the varimax method to simplify the structure of the data (Tabachnik and Fidell 2001; Quinn and Keough 2002). These two rotated components combined explained 61.5% of the variance in these traits. Petal width, corolla tube length, and pistil length loaded positively and strongly on the first rotated principal component, whereas anther-stigma distance had a strong negative loading on this component. Ovule number and

anther exsertion had a strong positive loading on the second rotated component. Anther orientation loaded negatively and more weakly to both components. The relationship of *L. alabamica* and *L. crassa* individuals to one another with respect to the floral traits described by the two rotated principal components can be seen in Figure 3.3. The phenotypic space occupied by the F_1, F_2, and BC hybrids compared to the parent species is shown in Figure 3.4. From these data, it can be seen that the majority of hybrids are phenotypically intermediate with respect to *L. alabamica* and *L. crassa*, and that no hybrid individuals fall far from the parent phenotypes.

I also examined whether hybrid line means differed from the line means of the parent species in order to test for hybrid vigor and/or hybrid breakdown in F_1's, and transgressive segregation in F_2's and BC's. To do this, I performed a Dunnett's test on each trait (except the categorical anther orientation), using the mean trait value of each parent species' population as a control. Each year was analyzed separately, and this meant that six tests were performed on each trait in each year. In order to be considered transgressive, a line's mean trait value would need to be significant ($p \leq 0.05$) in all six analyses. For all floral traits, and for the year 2 pollen viability data, not a single hybrid line was significantly different from all six of the parent species' populations. For the number of days from planting to germination (year 1), most F_1, and all F_2 and BC hybrid lines differed significantly from the parent species' populations (Figure 3.5). Of these lines, most took fewer days to germinate from the day of planting. Only the F_1 line "3x11" was significantly slower to germinate than the parent species'

populations. In year 2, fewer lines differed significantly, but they included the three hybrid types (Figure 3.6). Of these lines, all had lower means for the number of days from planting to germination, except F_1 "1x2" and F_1 "3x15", which had higher means than the parent species' populations. When looking at the number of days from planting to flowering, only one line differed significantly. In year 1, F_1 "16x5" flowered significantly earlier than the parent species' populations.

DISCUSSION

The purpose of this study was to examine how first-, second-generation, and backcross hybrids of *L. alabamica* and *L. crassa* compared phenotypically to the parent species for a suite of floral characters, as well as the timing of germination and flowering. Mean differences for these plant type categories were found for all traits other than anther-stigma distance (Table 3.1), but only ovule number and the log-transformed number of days from planting to germination showed a consistent difference between the three types of hybrids and the parent species. For some traits there were also differences between years and in the plant type by year interaction. However, these differences were small and are most likely due to the fact that different plants were measured in each year.

For the floral trait of ovule number, *L. alabamica* has a mean of 12.44 ± 0.404 whereas *L. crassa* has a mean of 8.08 ± 0.404. *L. alabamica* and *L. crassa* differ significantly in this character, and it is likely related to the striking difference in fruit shape exhibited by these species (Figure 3.2). The mean ovule number of

each hybrid category is around ten, which is intermediate to the parent species' values. The other floral traits did not differ consistently between the parent species and hybrid categories. When all floral traits were examined in a principal components analysis, *L. alabamica*, *L. crassa*, and the F_1, F_2, and BC hybrids overlapped in phenotypic space for floral characters (Figures 3.3 and 3.4). I also found no significant differences between the line means of hybrids and those of the parent species' populations. Thus, I found no evidence for hybrid vigor, hybrid breakdown, or transgressive segregation with respect to hybrid floral morphology. Consequently, if fitness differences exist between the parent species and hybrids (addressed in Chapter 4), they are unlikely to be caused by differences in floral morphology.

When I examined how long it took hybrid lines to flower compared to parent species' populations, I found that only one F_1 hybrid line in one year flowered significantly later. However, in both years, F_1, F_2, and BC hybrid line means differed from the parent species' populations in germination timing (Figures 3.5 and 3.6), indicating either hybrid vigor or hybrid breakdown in the F_1 lines and transgressive segregation in the F_2 and BC lines. I also found that more lines differed in year 1 than in year 2. This year difference was most likely due to the fact that in year 1, the parent species' plants were germinated in an 18˚C growth chamber, which appeared to slow germination, rather than in the warmer greenhouse as for the year 2 plants. All of the hybrids were germinated under the warmer greenhouse conditions, and it is therefore likely that the difference between parent species' populations and their hybrids was inflated in year 1

because of this difference in germination conditions. Of the F_1 hybrid lines that differed significantly, most germinated earlier than the parent species, but a few germinated later. All of the F_2 and BC lines that were significantly different germinated earlier than the parent species. With respect to the F_1 lines, whether this is considered hybrid vigor or hybrid breakdown is dependent on the effect of faster germination on fitness. In addition, I do not know what the impact of the transgressive segregation observed in F_2 and BC lines is on plant fitness. In *Leavenworthia*, seeds are dormant during the summer months when limestone glades are extremely hot and dry (Caudle and Baskin 1968; Baskin and Baskin 1971). In the fall, when temperatures are cooler and substantial rain has occurred, seeds begin to germinate. If faster germination in hybrid lines is a result of a lack of seed dormancy such that hybrids germinate out of season in the field, then it is possible that hybrid fitness would be less than the parent species (Baskin and Baskin 1972; Donohue et al. 2005a,b). However, if faster germination is not due to a lack of dormancy, but simply a quicker response to favorable germination conditions, then hybrids could have a fitness advantage over the parent species. Further experimentation is needed to determine the fitness impact of transgressive segregation in germination timing in this system.

The results of this study demonstrate that, at least for floral traits, the genetic architecture necessary to produce transgressive segregation may not be present in *L. alabamica* and *L. crassa*. Transgressive segregation requires recombination to bring together quantitative trait loci (QTL) with directional effects that were never before combined in the same genome (Rieseberg et al. 1999). If,

in a hybridization event, the QTL combinations controlling a trait are not novel due to few or no genetic differences between the parent genomes, then transgressive segregation is unlikely to occur (Kuczynska et al. 2007). If *L. alabamica* and *L. crassa* simply do not differ enough in the genetics of their floral traits (perhaps due to stabilizing selection exerted by their use of the same generalist pollinators), then that would explain why I did not find transgressive variation for floral characters as has been found in other systems (Bouck et al. 2007). However, in this study, I found evidence for transgressive segregation in the length of time it takes for a seed to germinate, which indicates that, at least for this one trait, *L. alabamica* and *L. crassa* may differ in the direction of the effects of the underlying QTL.

It is also possible that additional unmeasured traits could show transgressive variation in this system. Other studies have shown transgressive segregation in plant ecophysiological (e. g. Rosenthal et al. 2002), growth and fecundity (Johansen-Morris 2006; Latta et al. 2007), and non-floral aspects of morphology (deVicente and Tanksley 1993), which I did not measure in this study. *L. alabamica* and *L. crassa* may differ in these types of traits due to differential habitat adaptation (a possibility addressed in Chapter 4). Surveying additional kinds of traits in these species would help to determine if genetic divergence between *L. alabamica* and *L. crassa* is extensive enough to commonly produce the genetic architecture required for transgressive segregation, as well as its impact on hybrid fitness.

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FIGURE LEGENDS

Figure 3.1: Floral structure of *Leavenworthia* showing the whole flower (marked 9a), stamen and pistil arrangement (marked 9c) and petal (marked 9d). The drawing was made by Ruth Hsu Chen and modified from Rollins 1963

Figure 3.2: Fruit shape of *L. alabamica* and *L. crassa*. *L. alabamica* fruits are shown on the left (marked 1) and *L. crassa* fruits are shown on the right (marked 3 and 4). *L. alabamica* has longer, thinner siliques compared to those of *L. crassa*. Modified from Rollins 1963.

Figure 3.3: The floral phenotype space occupied by *L. alabamica* and *L. crassa*. Floral traits of the two species were examined using principal components analysis. Components 1 and 2 were obtained through varimax rotation of the first two principal components.

Figure 3.4: The floral phenotype space occupied by *L. alabamica*, *L. crassa*, and their F_1, F_2, and BC hybrids. Floral traits of the two species were examined using principal components analysis. Components 1 and 2 were obtained through varimax rotation of the first two principal components.

Figure 3.5: Transgressive segregation for germination timing in F_1, F_2, and BC hybrids of *L. alabamica* and *L. crassa* from the first year of crosses. Hybrid lines were compared to each parent species' population using Dunnett's test. Line means that significantly differ from the parent species' populations at $p<0.05$ are marked with an "*".

Figure 3.6: Transgressive segregation for germination timing in F_1, F_2, and BC hybrids of *L. alabamica* and *L. crassa* from the second year of crosses. Hybrid lines were compared to each parent species' population using Dunnett's test. Line means that significantly differ from the parent species' populations at $p<0.05$ are marked with an "*".

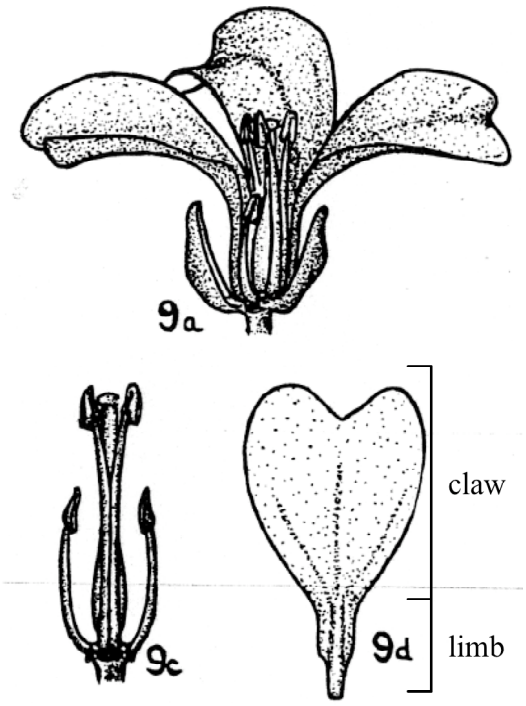


Figure 3.1

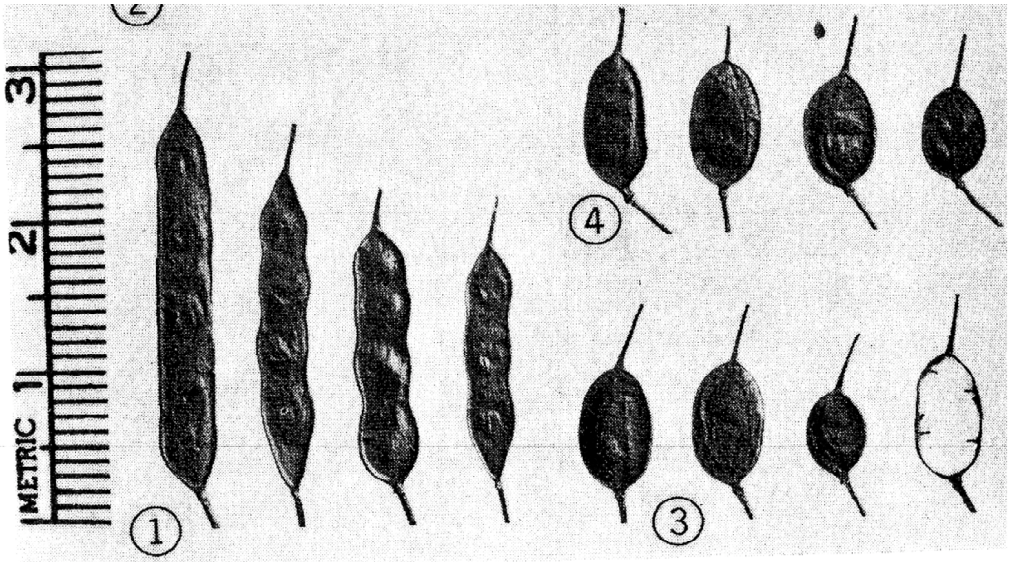


Figure 3.2

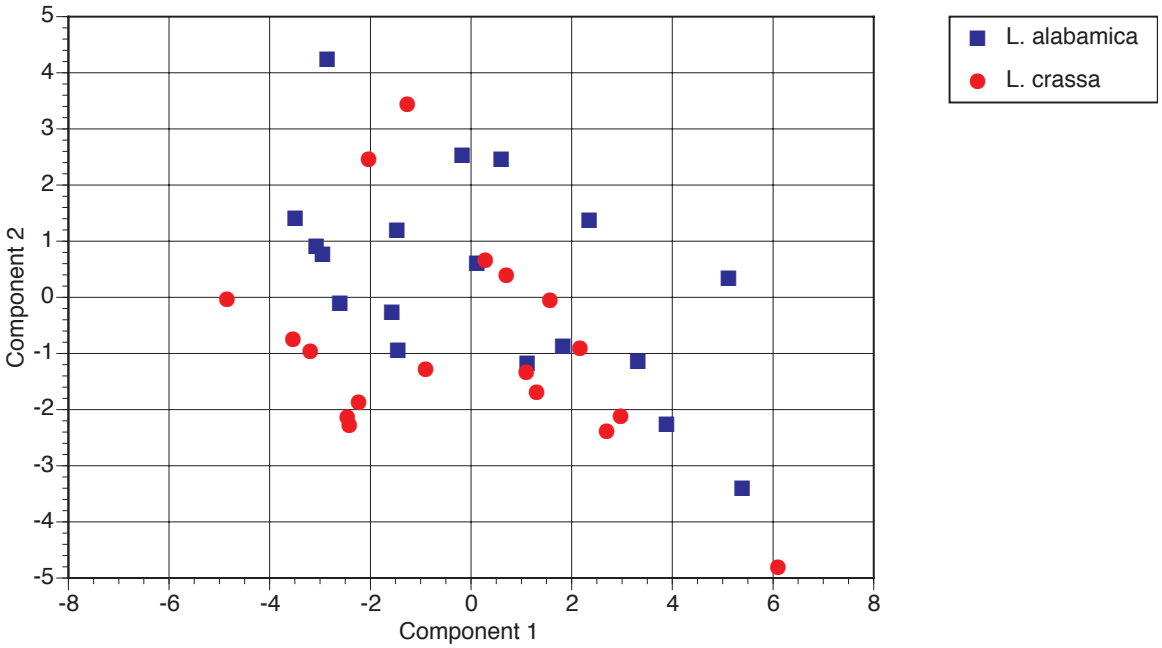
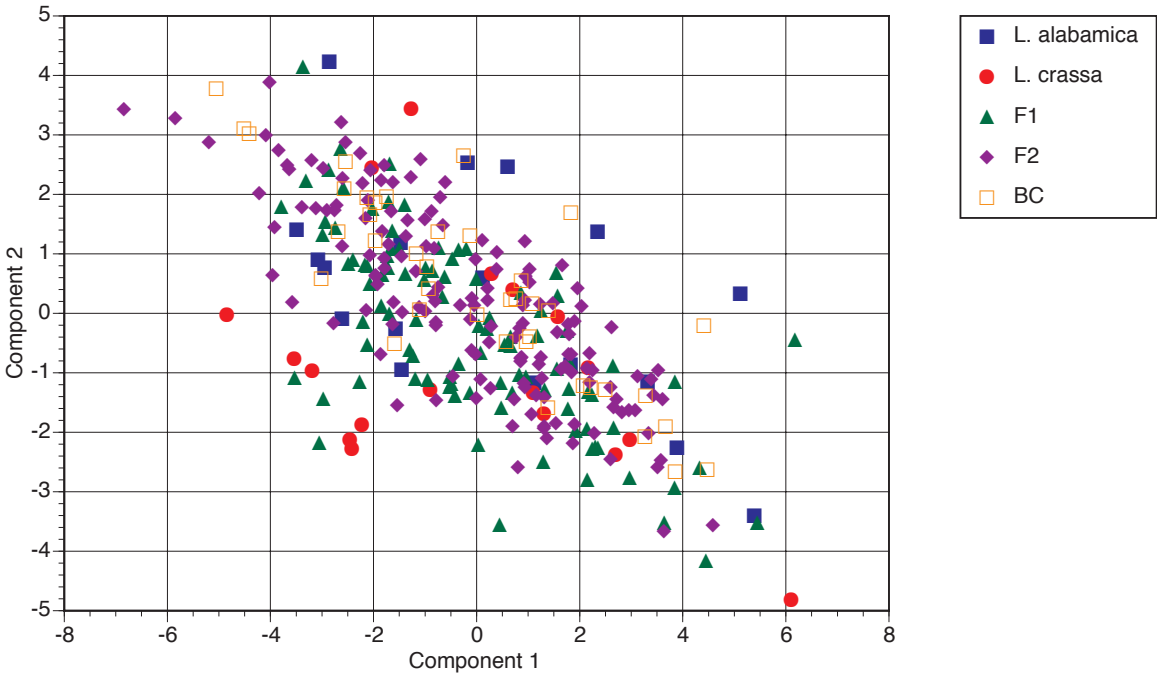

L. alabamica
L. crassa
Component 2
Component 1
5
4
3
2
1
0
-1
-2
-3
-4
-5
-8
-6
-4
-2
0
2
4
6
8

Figure 3.3


L. alabamica
L. crassa
F1
F2
BC
Component 2
Component 1
5
4
3
2
1
0
-1
-2
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-5
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-4
-2
0
2
4
6
8

Figure 3.4

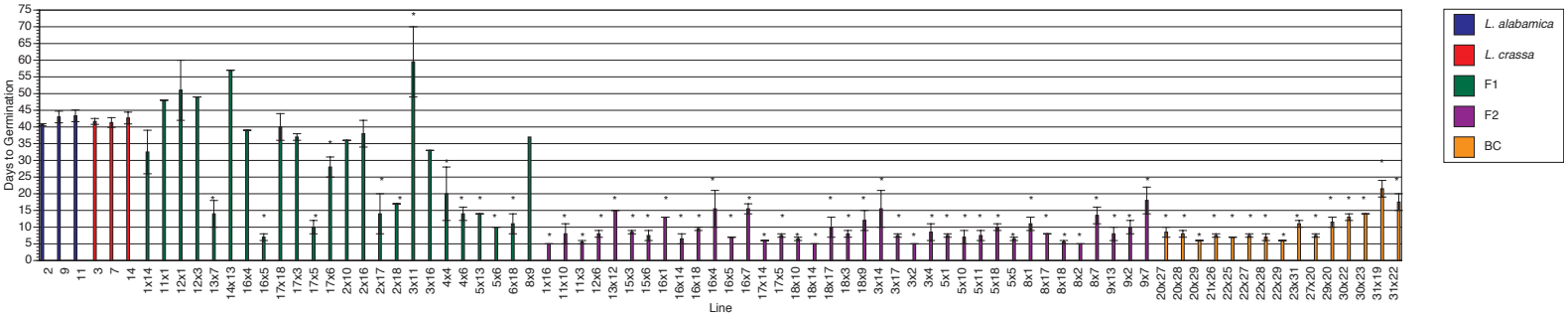
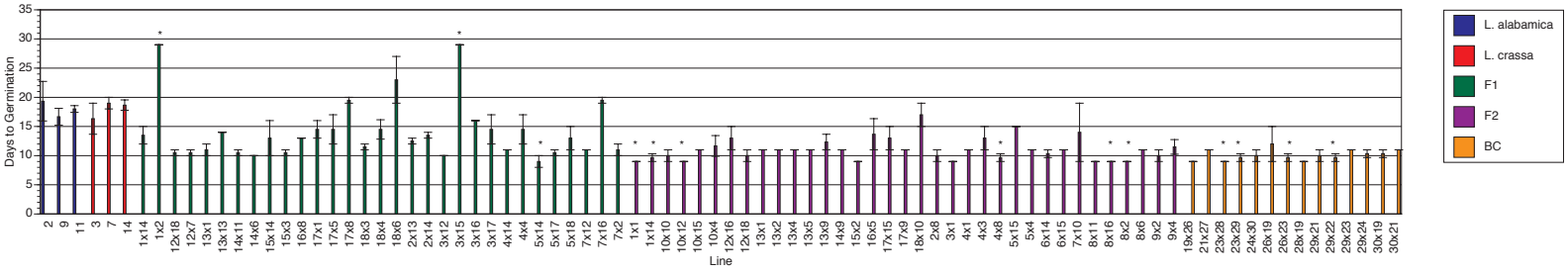


Figure 3.5


Days to Germination
0
5
10
15
20
25
30
35
Line
2
9
11
3
7
14
1x14
1x2
12x18
12x7
13x1
13x13
14x11
14x6
15x14
15x3
16x8
17x1
17x5
17x8
18x3
18x4
18x6
2x13
2x14
3x12
3x15
3x16
3x17
4x14
4x4
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5x17
5x18
7x12
7x16
7x2
1x1
1x14
10x10
10x12
10x15
10x4
12x16
12x18
13x1
13x2
13x4
13x5
13x9
14x9
15x2
16x5
17x15
17x9
18x10
2x8
3x1
4x1
4x3
4x8
5x15
5x4
6x14
6x15
7x10
8x11
8x16
8x2
8x6
9x2
9x4
19x26
21x27
23x28
23x29
24x30
26x19
26x23
28x19
29x21
29x22
29x23
29x24
30x19
30x21
L. alabamica
L. crassa
F1
F2
BC

Figure 3.6

Table 3.1: Comparisons of *L. alabamica*, *L. crassa*, and hybrids for floral, germination time, and flowering traits

	N	Petal Width	Corolla Tube Length	Pistil Length	Ovule Number	Anther Exsertion	Anther-Stigma Distance	% Viable Pollen*,§	% Anthers Extrorse‡	Days to Germination	Days to Flowering
L. alabamica	18	$7.84^{a} \pm 0.266$	$5.02^{a} \pm 0.095$	$6.76^{a,b} \pm 0.187$	$12.44^{a} \pm 0.404$	$1.69^{b,c} \pm 0.086$	$-0.05^{a} \pm 0.139$	$0.98^{a} \pm 0.007$	0.66	$30.17^{a} \pm 1.573$	$97.44^{a,b} \pm 2.870$
L. crassa	18	$7.26^{a,b} \pm 0.266$	$4.89^{a} \pm 0.095$	$6.30^{a,b} \pm 0.187$	$8.08^{c} \pm 0.404$	$1.41^{c} \pm 0.086$	$0.01^{a} \pm 0.139$	$0.97^{a,b} \pm 0.012$	0.66	$29.94^{a} \pm 1.573$	$105.61^{a} \pm 2.870$
F1	98	$6.70^{b} \pm 0.117$	$4.47^{b} \pm 0.042$	$6.42^{a,b} \pm 0.082$	$9.75^{b} \pm 0.177$	$1.75^{b} \pm 0.038$	$-0.23^{a} \pm 0.061$	$0.95^{b} \pm 0.005$	0.87	$20.82^{b} \pm 0.686$	$94.83^{b} \pm 1.261$
F2	152	$7.33^{a} \pm 0.092$	$4.46^{b} \pm 0.033$	$6.35^{b} \pm 0.064$	$10.11^{b} \pm 0.139$	$1.82^{b} \pm 0.030$	$-0.08^{a} \pm 0.048$	$0.97^{a} \pm 0.004$	0.91	$10.03^{c} \pm 0.549$	$91.26^{b} \pm 0.995$
BC	63	$7.76^{a} \pm 0.143$	$4.46^{b} \pm 0.051$	$6.73^{a} \pm 0.100$	$10.45^{b} \pm 0.216$	$2.01^{a} \pm 0.046$	$-0.26^{a} \pm 0.074$	$0.96^{a} \pm 0.006$	0.97	$10.19^{c} \pm 0.870$	$90.69^{b} \pm 1.550$

Least squares means are reported along with the standard error. Pairwise comparisons of means were made using Tukey's HSD test. Means that significantly differ from one another at $p<0.05$ are not connected by the same letter. The first column shows the sample size (N) of each plant type.

* Calculations of the percent viable pollen were based on the following sample sizes: *L. alabamica* N=9, *L. crassa* N=9, F1 N=96, F2 N=79, BC N=42.

§ Mean comparisons were made using the arcsin square root transformed variable.

‡ Percent values derived from contingency table analysis of cell counts. Individual comparisons of means could not be made.

CHAPTER 4

HABITAT ISOLATION AND ENVIRONMENT-DEPENDENT HYBRID INCOMPATIBILITIES AS REPRODUCTIVE BARRIERS BETWEEN *LEAVENWORTHIA ALABAMICA* AND *L. CRASSA*

INTRODUCTION

One of the most widely documented forms of differentiation between both animal (e. g. Feder and Bush 1989; Hatfield and Schluter 1999; Filchak et al. 2000; Rundle et al. 2000; Hawthorne and Via 2001; Kocher 2004; Bridle et al. 2006; Fuller et al. 2007) and plant (e. g. Cruzan and Arnold 1993; Wang et al. 1997; Campbell 2003; Husband and Sabara 2003; Ramsey et al. 2003; Rieseberg et al. 2003; Kay 2006; Savolainen et al. 2006) species is that of adaptation to different ecological niches. If species cannot mate with one another because individuals do not survive or reproduce in the same habitat, then habitat acts as a reproductive barrier between them. However, few studies have shown that adaptation to different habitats prevents or restricts mating between species (reviewed in Coyne and Orr 2004). One example is in *Rhagoletis* fruit flies, where Feder and Bush (1989) found that, although the species *R. pomonella* and *R. mendax* can produce viable hybrids in the laboratory, hybrids are not found in the field due to host preferences that restrict mating to different host plants (apples/hawthorns and blueberries respectively). An example in plants is found in

the classic study of Clausen et al. (1940), in which reciprocal transplants between low-elevation and alpine species of *Horkelia* showed severely reduced survival and reproduction at their non-native elevation, thereby preventing gene flow between the species.

In addition, ecological factors can interact with genetic factors to cause environment-dependent incompatibilities between species that reduce hybrid fitness in particular environments (reviewed in Coyne and Orr 2004). This type of extrinsic genetic barrier occurs when hybrids are poorly adapted to either niche of the parent species and are less fit in the parent species' habitat. An example of this occurs in big sagebrush (*Artemisia tridentata*), where two subspecies form a hybrid zone (Wang et al. 1997). In a reciprocal transplant experiment, Wang et al. (1997) found that each subspecies was most fit in its habitat of origin, and that hybrids were most fit in the hybrid zone and outperformed the parent subspecies in that zone. Thus, hybridization between the parent subspecies is limited by environment-dependent selection. A similar study by Hatfield and Schluter (1999) on limnetic and benthic stickleback species of the *Gasterosteus aculeatus* complex showed that F_1 hybrids between the two species did not perform well in either parent species habitat, and that selection likely acts against hybrids in the field.

Despite documented cases of both habitat isolation and environment-dependent hybrid incompatibilities in the animal and plant kingdoms, the frequency with which these barriers act separately or in concert to initiate speciation and the types of genes underlying them are largely unknown (Orr et

al. 2004; Orr 2005). In plants, only a handful of studies have documented and/or quantified the contribution of all possible reproductive barriers to speciation between closely related taxa (Morrison et al. 1994; Schemske and Goodwillie 1996; Ramsey et al. 2003; Husband and Sabara 2003; Kay 2006). To my knowledge, no such studies have been done in animals. Thus, further studies of the types of reproductive barriers present between species are needed to understand the degree to which each facilitates speciation.

The aim of the present study was to determine the role of adaptation to habitat in promoting the speciation of *Leavenworthia alabamica* and *L. crassa*. These species have been the subject of studies into the evolution of self-fertilization from self-incompatibility due to the observation that, although some populations possess the sporophytic self-incompatibility system common to other members of the Brassicaceae (Bateman 1955; Lloyd 1967), others are capable of autogamous seed production (Rollins 1963; Lloyd 1965). Rollins (1963) hypothesized that self-fertilization evolved at least three times within this small genus of only eight species (five times if *L. alabamica* and *L. crassa* also represent independent events), and recent phylogenetic evidence has corroborated Rollins' hypothesis of at least three independent events (Beck et al. 2006).

In addition, Lloyd's (1965) work on the striking morphological changes associated with the transition from outcrossing to self-fertilization in these species played an important role in our understanding of the connection between plant breeding system evolution and floral adaptation. In particular, Lloyd (1965)

found that more highly autogamous populations of *L. alabamica* and *L. crassa* had smaller flowers, reduced anther-stigma distances, anthers oriented to dehisce toward rather than away from the stigma, smaller pollen to ovule ratios, and little or no floral scent. He hypothesized that these changes were adaptations to a self-fertilizing mating system; a belief now commonly held due to the discovery of these floral trait transitions in a wide variety of plant groups (Holsinger 1996).

The studies of Rollins (1963) and Lloyd (1965) prompted a large body of experimental work to further understand why self-fertilization evolved so many times in *Leavenworthia* and its genetic consequences. Lyons and Antonovics (1991) looked at two populations of *L. crassa* and found that the outcrossing rate in the field was indeed significantly higher in the population believed to be outcrossing based on floral morphology than in the selfing population, confirming the association between morphology and mating system. Furthermore, inbreeding depression was documented in *L. crassa* (Charlesworth et al. 1994) and *L. alabamica* (Busch 2005a). Busch (2005a) found less inbreeding depression in self-compatible populations compared to self-incompatible populations, and documented heterosis in a small, isolated, and highly self-compatible population (Busch 2006).

In addition, genetic diversity in *Leavenworthia*, including *L. alabamica* and *L. crassa*, was examined by Solbrig (1972) and Solbrig and Rollins (1977). These studies found that self-compatibility throughout the genus is associated with lower genetic diversity (measured using allozymes). Low genetic diversity in the

self-compatible compared to the self-incompatible *Leavenworthia* species was also found by Charlesworth and Yang (1998) using allozymes, and by Liu et al. (1998; 1999) using measures of DNA sequence diversity.

The presence of inbreeding depression in *Leavenworthia* (Charlesworth et al. 1994; Busch 2005a), increased vigor following outcrossing (Busch 2006), and lower levels of genetic diversity in selfers (Solbrig and Rollins 1977; Charlesworth and Yang 1998; Liu et al. 1998; 1999) should act to prevent the evolution of selfing in this system. Since that is not the case, studies have looked at the role of other factors, such as reproductive assurance, in the evolution of self-fertilization in *Leavenworthia*. Busch (2005b) examined whether selfers had an advantage over outcrossers in geographically peripheral populations that may receive limited pollinator visitation. He found that selfers outperformed outcrossers in all habitats because all habitats experienced pollen limitation, but that peripheral populations showed reduced genetic variation consistent with a population bottleneck. Thus, self-compatibility may have evolved as a result of the interplay between population size and the genetic self-incompatibility system following long-distance dispersal. In addition, Anderson and Busch (2006) found evidence of relaxed pollinator-mediated selection in self-compatible *Leavenworthia* taxa, suggesting that lack of pollinator visitation may still play a role in the evolution of self-fertilization in the genus.

Interestingly, floral and mating system evolution in *Leavenworthia* is not strongly associated with speciation. *Leavenworthia* species are primarily distinguished by their strongly divergent fruit shapes (an important species-

diagnostic character in the Brassicaceae; Rollins 1993) and by changes in chromosome number (Baldwin 1945). This is particularly true of *L. alabamica* and *L. crassa*, which are solely distinguished by their fruit shapes, overlap in geography, ecology, and floral morphology (Rollins 1963), have the same chromosome number (Baldwin 1945), and can produce viable and fertile F_1 and F_2 hybrids in the greenhouse (Rollins 1963). However, despite this apparent morphological and genetic overlap, *L. alabamica* and *L. crassa* do form weakly monophyletic sister lineages on the basis of cpDNA variation (Beck et al. 2006), suggesting that these taxa are divergent lineages, but may be in the early stages of this divergence.

Given the cryptic nature of these species, what, then, reproductively isolates these taxa? As part of a larger study of reproductive barriers between these species, I performed a reciprocal transplant experiment to answer the following two questions: 1) are *L. alabamica* and *L. crassa* adapted to different limestone glade habitats?, and 2) are hybrids less fit than either of the parent species in the field? I then address the implications of my findings for the speciation of *L. alabamica* and *L. crassa*.

MATERIALS AND METHODS

Study Organisms and Sites—Leavenworthia alabamica and *L. crassa* are sister species within the mustard genus *Leavenworthia* (Rollins 1963; Beck et al. 2006). Species within this genus possess a sporophytic self-incompatibility system (Bateman 1955; Rollins 1963; Lloyd 1967), but both *L. alabamica* and *L.*

crassa also contain self-compatible populations (Rollins 1963; Lloyd 1965). These species also overlap in flowering time and are pollinated by generalist bee species (Lloyd 1965).

In addition, *L. alabamica* and *L. crassa* occur in the Moulton Valley of northwestern Alabama, USA, with some range overlap in the eastern part of the valley leading to potential hybridization events (Rollins 1963; Lloyd 1965). *L. alabamica* also has some populations in the Tennessee Valley, which are located in Colbert Co., Alabama. All species in the genus are endemic to a habitat type commonly called a “cedar glade” that is characterized by a dolomitic limestone outcrop covered with a thin layer of calcareous soil (Baskin et al. 1995). *L. alabamica* and *L. crassa* are winter annuals that begin their life cycle in the fall months (germination occurs from late September to early November) and complete it in the spring (flowering peaks in late March and fruiting peaks in late April) (Rollins 1963; Lloyd 1965). During these months, the soil of these cedar glades remains saturated with water, and evidence suggests that *Leavenworthia* species may be adapted to grow in anaerobic conditions (Baskin and Baskin 1976). The sites chosen for the reciprocal transplant experiment described below were typical of cedar glades throughout the range of both species and were selected due to their central location within each species’ range. The *L. alabamica* home site was located in Lawrence Co. (34˚31’03.97”N and 87˚30’19.18” W) on land owned by The Nature Conservancy of Alabama. The *L. crassa* home site was located in Morgan Co. (34˚21’20.02”N and 86˚59’59.19”W).

Experimental Design—I conducted a reciprocal transplant experiment using *L. alabamica*, *L. crassa*, and their F_1, F_2, and backcross (BC) hybrids to determine if either parental species was locally adapted to its respective home glade site, as well as to compare the fitness of experimentally-produced hybrids to the parent species in the field. This involved planting individuals of each plant type in each of the two planting sites (one within the range of *L. alabamica* and one within the range of *L. crassa*). Plants were first germinated from seed in a greenhouse at the University of Georgia and then planted in the field at the beginning of November 2006. The seed used in the experiment came from two sources: 1) field collections from four populations of *L. alabamica* and three of *L. crassa*, and 2) crosses performed in the greenhouse to produce parental species and F_1, F_2, and BC hybrid seed (for cross details see Chapter 2).

Seeds were planted in Fafard brand potting soil, watered daily, and fertilized weekly. They were planted one to a cell in seedling flats in a randomized block design (Cochran and Cox1992), with two blocks per site, and then planted in that design in the field. Each block contained approximately 250 individuals for a total of around 1,000 individuals in the experiment. Within each block, there were approximately 75 field-derived *L. alabamica* and *L. crassa*, 20 cross-derived *L. alabamica* and *L. crassa*, and 20 F_1, F_2, and BC hybrids.

Once sufficient germination was achieved, the seedlings were planted directly into soil at each site. Since germination was staggered, the rosette diameter of each plant was measured on the day the experiment was planted so that the initial size of each plant was known. Rosette diameter was measured

using digital calipers. Once planted, each block was then covered in remay cloth and the cloth hand-sprayed with water until soaked to aid in transplantation. After one month, the remay cloth was removed and plants were allowed to grow uncovered for the remainder of the experiment. Plants were also hand-watered at each site three times (twice in late March and once in early April). Plant mortality was measured throughout the experiment but was too low to analyze.

In mid-April, each individual plant was collected in its entirety just prior to silique dehiscence. Plants were then dried for approximately one week in a drying oven set to 30˚C to prevent the plant material from rotting. Once sufficiently dried, I counted the number of seeds produced by each plant.

Statistical Analyses—I used mixed model nested analysis of covariance methods (Quinn and Keough 2002) to determine if seed production was greater for each species in its home site relative to its non-home site and to examine seed production in hybrids relative to the parent species. I performed one analysis on data from the plants of field-collected origin and one on the data from cross-derived plants. This is because it would not have been appropriate to compare the fitness of cross-derived hybrids to parent species' individuals of field-collected origin due to potential differences in maternal effects.

The effects tested in the analysis of field-collected plants were "site", "block within site", "species", "species by site", and "block by species within site". Since blocks were randomly placed within a site, "block within site" and "block by species within site" were random effects. All other effects tested were fixed. The same analysis was performed on cross-derived plants. However, the "plant type"

effect replaced “species” since data from F_1, F_2, and BC hybrids, as well as the parent species, were included in this analysis. In each analysis, significance was tested using the expected mean square method of analysis of variance (Quinn and Keough 2002). Both analyses included an individual’s initial rosette diameter as a covariate, and used the square root of seed number as a response variable. The square root transformation was used to meet ANOVA assumptions. The covariate was included to control for the effect of plant size on seed production. All analyses were performed using JMP 6.0 (SAS Institute 2005).

RESULTS

To test for the presence or absence of local adaptation between *L. alabamica* and *L. crassa*, I performed a mixed model nested ANCOVA. I found no significant difference in seed production between sites, species, species by sites (Figure 4.1), or block by species within sites. There was a significant difference between blocks within sites (df=2, F=55.8702, p=0.0174), and a significant effect of the initial rosette diameter of a plant on the seed number produced (df=1, F=284.5877, p<0.0001). These data show that *L. alabamica* and *L. crassa* produced equivalent amounts of seed irrespective of the site in which they were planted, but that seed production differences occurred within a site. However, the within-site differences were regardless of plant type. The significance of the covariate in this analysis was due to the fact that larger plants tended to produce more seed than smaller plants.

I also looked for evidence of differential seed production of hybrid plants with respect to parental plants that would suggest the presence of extrinsic genetic barriers to hybridization, and for evidence of local adaptation in these cross-derived plants. An ANCOVA revealed no significant difference between sites, plant types (Figure 4.2), or plant type by sites (Figure 4.3). There was a significant difference between blocks within sites ($df=2$, $F=30.4762$, $p=0.0002$), block by plant type within sites ($df=8$, $F=2.3391$, $p=0.0185$), and a significant effect of a plant's initial rosette diameter on seed production ($df=1$, $F=226.9380$, $p<0.0001$). These data demonstrate that *L. alabamica*, *L. crassa*, and their hybrids produce the same number of seeds in either site, but that there are differences within a site, and some of the within-site differences are due to differences in seed production among the plant types. Also, larger plants tended to produce more seed than smaller plants.

DISCUSSION

If *L. alabamica* and *L. crassa* had locally adapted to their respective glade sites, then I would have found that *L. alabamica* produced significantly more seed than *L. crassa* in the *L. alabamica* glade site and *L. crassa* produced significantly more seed than *L. alabamica* in the *L. crassa* glade site. This would have been indicated by a significant species by site interaction for the plants of field-collected origin, and a significant plant type by site interaction in the analysis of cross-derived plants. However, no significant species by site or plant type by site interaction was observed. Instead, *L. alabamica* and *L. crassa* individuals, of

both field- and cross-derived origin, produced seed equally in each site (Figure 4.1 and 4.3). In addition, the two species did not significantly differ in seed production, nor did seed production significantly differ between the two sites. This same pattern was also found in the cross-derived plants, where no significant differences in seed production between plant types were observed, nor between sites. The lack of significant differences in seed production between plant types (*L. alabamica*, *L. crassa*, F_1, F_2, and BC hybrids; Figure 4.2) indicates that fitness, as determined by counting seed, did not differ between the parent species and their hybrids. It is possible that seed viability differences were present, but since the seed from the field was not germinated, I do not know if it was viable or not. However, it seems likely that the parent species and hybrids did not differ in seed viability given the overall appearance of the seeds (V. Koelling, personal observation).

I also found a significant effect of blocks within sites in both the field-derived (df=2, F=55.8702, p=0.0174) and cross-derived (df=2, F=30.4762, p=0.0002) datasets due to the fact that seed production was lower in one of the blocks in each site. At the *L. crassa* site, one of the blocks experienced higher mortality from water stress because it was located in an area of particularly shallow soil within the glade, therefore lowering seed production in that block. However, it is unknown why seed production differed between blocks at the *L. alabamica* site. Neither block experienced significant mortality or apparent water stress during the experiment. One possible explanation is that one of the blocks received fewer pollinator visits than the other. Another possibility is that the

blocks differed in the number of self-incompatible and self-compatible plants. If one of the blocks had a higher number of self-incompatible individuals and these individuals did not differ in their S-alleles, then incompatible matings within a block could have lowered seed set. In addition, microsite variation in nutrient content could have impacted seed production between blocks. Whatever the cause, it affected field-derived *L. alabamica* and *L. crassa* equally within a block as indicated by the non-significant interaction of block by species within sites. Cross-derived individuals were not affected equally within a block as indicated by a significant block by plant type within sites interaction (df=8, F=2.3391, p=0.0185). Although no plant type was consistently more successful within a block than any other, which may have resulted from any of the potential genetic and environmental factors within a block.

In this experiment, I found no evidence for local adaptation to glade sites in *L. alabamica* or *L. crassa* plants of either field- or cross-derived origin. This suggests that these species have not adapted to cryptic niches available within limestone glade habitat, and are therefore not reproductively isolated from one another due to an inability to survive or reproduce in the same habitat. This result is also consistent with the findings of Busch (2005b), in which a reciprocal transplant experiment between populations of *L. alabamica* did not find support for local adaptation. However, only two glade sites were used in this study, and it is possible that these sites did not differ in environmental factors important for delineating the species' respective niches (Baskin and Baskin 1988). Reciprocal

transplants using additional glade sites may reveal a pattern of local adaptation not detected in this study.

In addition, I found no evidence for fitness differences between these species and their F_1, F_2, and BC hybrids in the field. This suggests that no environment-dependent genetic factors are present in hybrids that would reduce fitness in the field, and, consequently, that extrinsic genetic barriers do not prevent mating between *L. alabamica* and *L. crassa*. This finding is congruent with the lack of evidence for intrinsic genetic barriers between these species (Chapter 1) and the small phenotypic differences seen between the parent species and their hybrids (Chapter 3). In addition, this finding is not unusual in that there are many cases of fit hybrids in nature (reviewed in Arnold 2006). It is possible, however, that hybrid genotypes untested in this experiment may display reduced fitness relative to the parent species. I feel this is unlikely due to the level of starting genetic variation used to create hybrid lines for this study (for details see Chapter 2: Materials and Methods). Multiple populations from the entire range of each species were used as parents in order to maximize the potential for genetic incompatibilities. Given that no environment-dependent hybrid incompatibilities were found despite the incorporation of genetic variation from distant populations, it seems likely that no such genetic incompatibilities exist between *L. alabamica* and *L. crassa*.

One additional caveat is that I did not test all life-stages of the hybrids and parent species in this experiment since seeds were germinated in the greenhouse before transplantation to the field. In Chapter 3, I showed evidence

for transgressive segregation in the length of time it took some F_2 and BC hybrid lines to germinate, and possible hybrid vigor or hybrid breakdown in some F_1 lines for this trait. What impact the mostly shorter germination times might have had on the fitness of these hybrid lines in the field is unknown. If the fitness impact is negative, it would be evidence of an environment-dependent barrier to hybridization between these species. However, shorter germination times would probably result in greater fitness for hybrids, which would then facilitate reproduction between *L. alabamica* and *L. crassa* rather than restrict it.

The apparent absence of ecological or extrinsic genetic barriers to reproduction between these species is unusual given the number of studies that have found these types of barriers between other species (reviewed in Coyne and Orr 2004). This includes many studies documenting adaptation to different ecological niches in closely related plant species (e. g. Cruzan and Arnold 1993; Campbell 2003; Ramsey et al. 2003; Rieseberg et al. 2003; Kay 2006; Savolainen et al. 2006). Local adaptation within and between populations from different parts of a species' range has been found in numerous studies as well (e.g. Clausen et al. 1940; Bradshaw 1960; Schemske 1984; Linhart and Grant 1996). In other words, niche differentiation and/or local adaptation is usually the rule, not the exception (although there are exceptions, e.g. Galloway and Fenster 1999; Baack and Stanton 2005). Exactly why local adaptation was not detected between *L. alabamica* and *L. crassa* is unknown. It is possible that limestone glades are relatively uniform ecologically, and therefore selection has not favored local adaptation. Another possibility is that extensive gene flow occurs, or has

occurred in the past, between these species, preventing the development of local adaptation and genetic incompatibilities (Holt 1996; Holt 2003; Kirkpatrick and Barton 1997). This possibility will be examined in Chapter 5.

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FIGURE LEGENDS

Figure 4.1: The mean square root-transformed number of seeds produced by *L. alabamica* and *L. crassa* plants grown in a limestone glade naturally occupied by *L. alabamica* (“La Site”) and in one naturally occupied by *L. crassa* (“Lc Site”). Plants were germinated in the greenhouse from seed collected in the field during the spring of 2006. Means are shown with standard error bars.

Figure 4.2: The mean square root-transformed number of seeds produced by *L. alabamica*, *L. crassa*, and their F_1, F_2, and BC hybrids in the field. Plants were germinated in the greenhouse from seed produced by one generation of within- and between-species crosses. Means are shown with standard error bars.

Figure 4.3: The mean square root-transformed number of seeds produced by *L. alabamica*, *L. crassa*, and their F_1, F_2, and BC hybrids grown in a limestone glade naturally occupied by *L. alabamica* ("La Site") and in one naturally occupied by *L. crassa* ("Lc Site"). Plants were germinated in the greenhouse from seed produced by one generation of within- and between-species crosses. Means are shown with standard error bars.

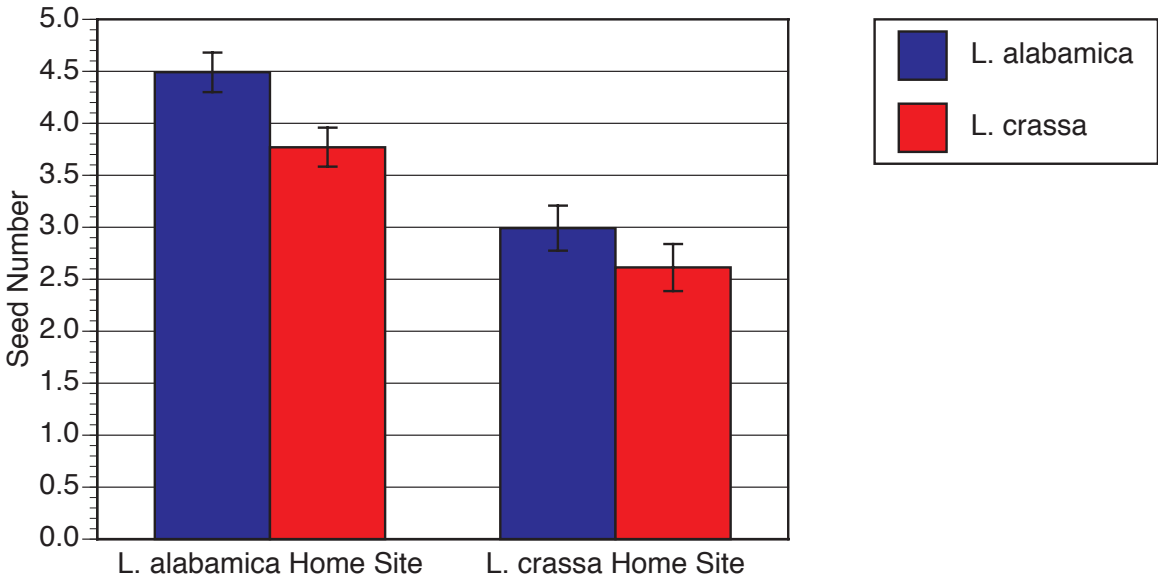
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Seed Number
L. alabamica Home Site
L. crassa Home Site
L. alabamica
L. crassa

Figure 4.1

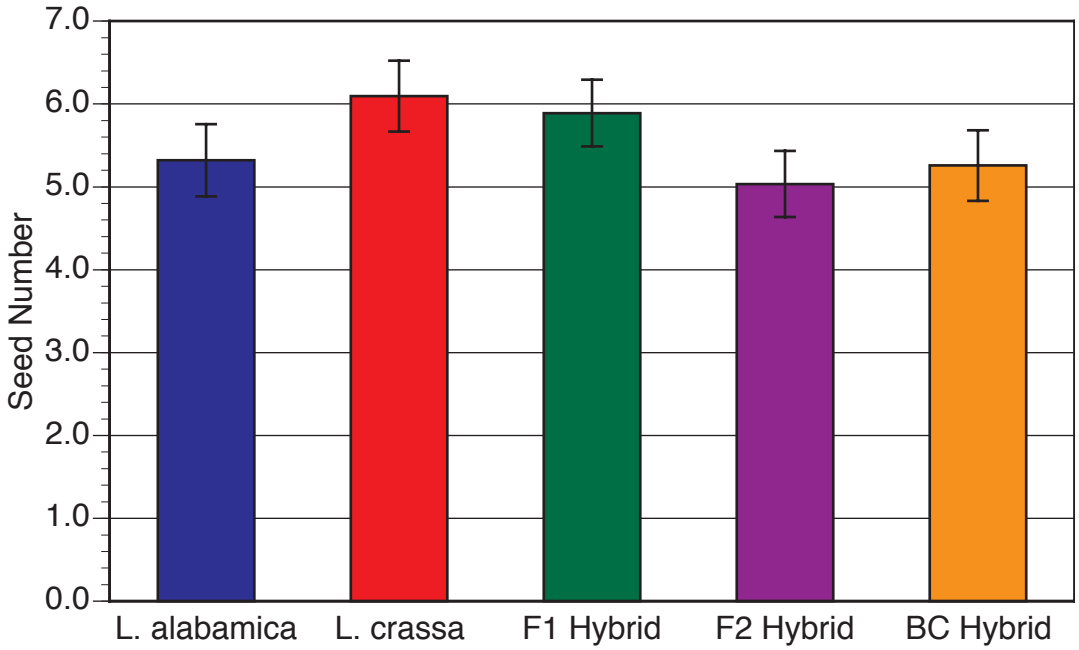
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Seed Number
L. alabamica
L. crassa
F1 Hybrid
F2 Hybrid
BC Hybrid

Figure 4.2

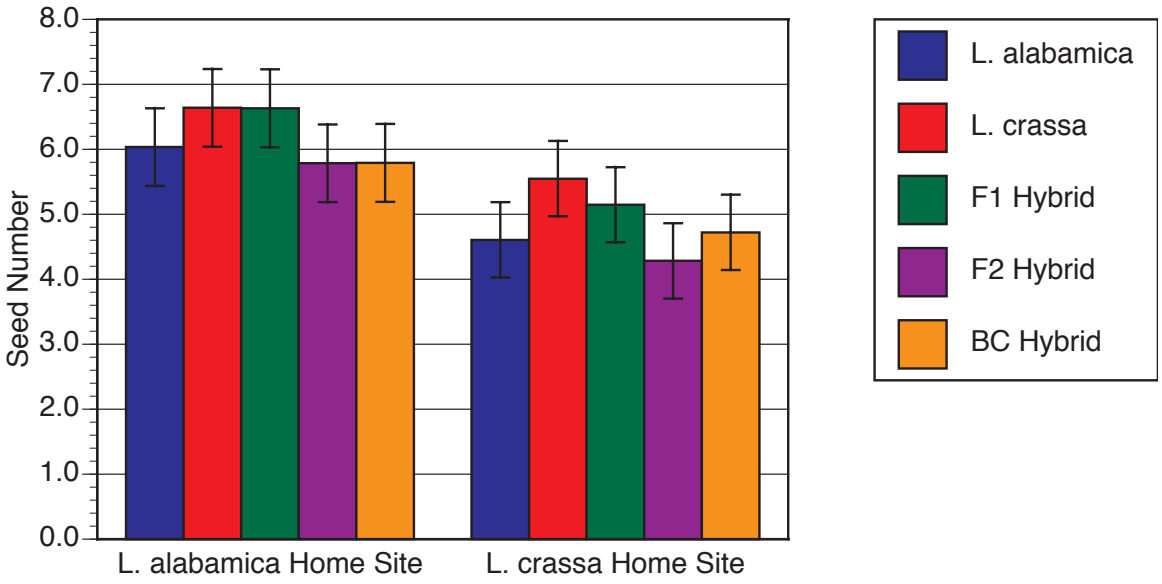
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Seed Number
L. alabamica Home Site
L. crassa Home Site
L. alabamica
L. crassa
F1 Hybrid
F2 Hybrid
BC Hybrid

Figure 4.3

CHAPTER 5

GENETIC DIVERSITY, GENETIC STRUCTURE, AND GENE FLOW BETWEEN *LEAVENWORTHIA ALABAMICA* AND *L. CRASSA*

INTRODUCTION

The structuring of populations within species due to historical (Avise 2000) and life-history factors (Hamrick and Godt 1996) can have profound impacts on levels of genetic diversity, the degree to which genetic variation is shared among populations, and the rate of gene flow between populations. High population genetic structure within a species results from enhanced genetic drift and inbreeding within populations, particularly if populations are small (Hedrick 2005). This can negatively impact population fitness by retarding the effects of current selection or reducing a population's ability to respond to future selection pressures (reviewed in Ellstrand and Elam 1993).

The enhancement of genetic drift due to population structure could also impact the potential for gene flow between species in that models have shown reproductive barriers can arise via the action of genetic drift alone on neutral genetic variation (Nei et al. 1983). However, to date there are no empirical studies demonstrating speciation through neutral genetic drift. It is also possible for population structure within species to enhance the opportunity for local selection to differentiate populations, thereby bringing about speciation as a by-

product of selection. Whether or not speciation is more often the result of natural selection or genetic drift is unknown (Coyne and Orr 2004), and further studies to determine the relative roles of these evolutionary forces are needed.

Another factor that can impact both population genetic structure and, potentially, speciation is mating system evolution, particularly transitions from outcrossing to self-fertilization. Previous studies have demonstrated that selfing reduces genetic diversity within populations and within species, and increases the genetic structure among populations compared to outcrossing (e.g. Schoen and Brown 1991; Hamrick and Godt 1996; Charlesworth and Yang 1998; Liu et al. 1998; 1999; Mable and Adam 2007). Thus, gene flow is expected to be reduced between populations when one or more is predominantly self-fertilizing. This has lead to the suggestion (Levin 1978) that the evolution of self-fertilization itself is a reproductive barrier between species. However, this idea has been challenged (Coyne and Orr 2004) on the grounds that selfing individuals within populations would be just as unlikely to mate with one another as with members of another species, and therefore self-fertilization cannot be a reproductive barrier between species. To settle this debate, further empirical work is needed to elucidate how selfing and outcrossing populations are structured and how this impacts gene flow between both populations within and between species.

In the present study, I set out to examine patterns of genetic diversity, genetic structure, and gene flow in *L. alabamica* and *L. crassa*. These species have been the subject of studies into the evolution of self-fertilization from self-incompatibility due to the observation that, although some populations possess

the sporophytic self-incompatibility system common to other members of the Brassicaceae (Bateman 1955; Lloyd 1967), others are capable of autogamous seed production (Rollins 1963; Lloyd 1965). Rollins (1963) hypothesized that self-fertilization evolved at least three times within this small genus of only eight species (five times if *L. alabamica* and *L. crassa* also represent independent events), and recent phylogenetic evidence has corroborated Rollins' hypothesis of at least three independent events (Beck et al. 2006).

In addition, Lloyd's (1965) work on the striking morphological changes associated with the transition from outcrossing to self-fertilization in these species played an important role in our understanding of the connection between plant breeding system evolution and floral adaptation. In particular, Lloyd (1965) found that more highly autogamous populations of *L. alabamica* and *L. crassa* had smaller flowers, reduced anther-stigma distances, anthers oriented to dehisce toward rather than away from the stigma, smaller pollen to ovule ratios, and little or no floral scent. He hypothesized that these changes were adaptations to a self-fertilizing mating system; a belief now commonly held due to the discovery of these floral trait transitions in a wide variety of plant groups (Holsinger 1996).

The studies of Rollins (1963) and Lloyd (1965) prompted a large body of experimental work to further understand why self-fertilization evolved so many times in *Leavenworthia* and its genetic consequences. Lyons and Antonovics (1991) looked at two populations of *L. crassa* and found that the outcrossing rate in the field was indeed significantly higher in the population believed to be

outcrossing based on floral morphology than in the selfing population, confirming the association between morphology and mating system. Furthermore, inbreeding depression was documented in *L. crassa* (Charlesworth et al. 1994) and *L. alabamica* (Busch 2005a). Busch (2005a) found less inbreeding depression in self-compatible populations compared to self-incompatible populations, and documented heterosis in a small, isolated, and highly self-compatible population (Busch 2006).

In addition, genetic diversity in *Leavenworthia*, including *L. alabamica* and *L. crassa*, was examined by Solbrig (1972) and Solbrig and Rollins (1977). These studies found that self-compatibility throughout the genus is associated with lower genetic diversity (measured using allozymes). Low genetic diversity in the self-compatible compared to the self-incompatible *Leavenworthia* species was also found by Charlesworth and Yang (1998) using allozymes, and by Liu et al. (1998; 1999) using measures of DNA sequence diversity.

The presence of inbreeding depression in *Leavenworthia* (Charlesworth et al. 1994; Busch 2005a), increased vigor following outcrossing (Busch 2006), and lower levels of genetic diversity in selfers (Solbrig and Rollins 1977; Charlesworth and Yang 1998; Liu et al. 1998; 1999) should act to prevent the evolution of selfing in this system. Since that is not the case, studies have looked at the role of other factors, such as reproductive assurance, in the evolution of self-fertilization in *Leavenworthia*. Busch (2005b) examined whether selfers had an advantage over outcrossers in geographically peripheral populations that may receive limited pollinator visitation. He found that selfers outperformed

outcrossers in all habitats because all habitats experienced pollen limitation, but that peripheral populations showed reduced genetic variation consistent with a population bottleneck. Thus, self-compatibility may have evolved as a result of the interplay between population size and the genetic self-incompatibility system following long-distance dispersal. In addition, Anderson and Busch (2006) found evidence of relaxed pollinator-mediated selection in self-compatible *Leavenworthia* taxa, suggesting that lack of pollinator visitation may still play a role in the evolution of self-fertilization in the genus.

Interestingly, floral and mating system evolution in *Leavenworthia* is not strongly associated with speciation. *Leavenworthia* species are primarily distinguished by their strongly divergent fruit shapes (an important species-diagnostic character in the Brassicaceae; Rollins 1993) and by changes in chromosome number (Baldwin 1945). This is particularly true of *L. alabamica* and *L. crassa*, which are solely distinguished by their fruit shapes, overlap in geography, ecology, and floral morphology (Rollins 1963), have the same chromosome number (Baldwin 1945), and can produce viable and fertile F_1 and F_2 hybrids in the greenhouse (Rollins 1963). However, despite this apparent morphological and genetic overlap, *L. alabamica* and *L. crassa* do form weakly monophyletic sister lineages on the basis of cpDNA variation (Beck et al. 2006), suggesting that these taxa are divergent lineages, but may be in the early stages of this divergence. Thus, *L. alabamica* and *L. crassa* are ideal species in which to investigate the possible role of mating system evolution and population genetic structure on gene flow.

As part of a larger study of reproductive barriers between these species, I investigated the degree to which *Leavenworthia alabamica* and *L. crassa* could be genetically differentiated from one another using allozyme marker variation, as well as the impact of mating system evolution on the genetic diversity, genetic structure, and amount of gene flow between populations. I asked the following questions: 1) do the species differ in amounts of genetic diversity and genetic structure, or possess alleles distinct from one another?, 2) can the species be differentiated from one another based on measures of genetic distance?, 3) is there evidence of extensive admixture of alleles between species?, and finally, 4) what is the impact of the evolution of selfing on genetic diversity, genetic structure, and gene flow in these species?

MATERIALS AND METHODS

Study Organisms and Population Sampling—The sister species *Leavenworthia alabamica* and *L. crassa* are limestone glade endemics belonging to a small genus (eight species) of the Brassicaceae (Rollins 1963; Beck et al. 2006). Species within this genus possess a sporophytic self-incompatibility system (Bateman 1955; Rollins 1963; Lloyd 1967), but both *L. alabamica* and *L. crassa* also contain self-compatible populations (Rollins 1963; Lloyd 1965). The limestone glades in which *Leavenworthia* species grow are characterized by thin calcareous soil over a dolomitic limestone base (Baskin et al. 1995). Species of *Leavenworthia* are winter annuals, and grow in the glades during the winter months when the soil is extremely wet. *L. alabamica* and *L. crassa* germinate in

the fall (September-November), and flower (mid-March to mid-April) and fruit (mid-April to early May) in the spring (Rollins 1963; Lloyd 1965). Both are pollinated by generalist bee species (Lloyd 1965).

The glades of *L. alabamica* and *L. crassa* are patchily distributed within the Moulton and Tennessee Valleys of northwestern Alabama (Rollins 1963). *L. crassa* occurs solely within one county (Morgan Co.) in the eastern part of the Moulton Valley, whereas *L. alabamica* occurs in four counties, one of which (Colbert Co.) is located in the Tennessee Valley. The ranges of the two species overlap in Morgan Co., where possible hybrids have been observed (Lloyd 1965). In this study, I collected seed from 10 populations of *L. alabamica* and 5 of *L. crassa*. Populations were located throughout the range of each species (Figure 5.1). An attempt was made to sample each population thoroughly, with seed collected every few meters along transects spanning each population, and therefore the number of individuals sampled varied with population size (estimated visually). Populations tended to be either large (>1,000 individuals) or small (<100 individuals). GPS coordinates were also recorded and geographic distances calculated between populations using the software GenAlEx 6.1 (Peakall and Smouse 2006).

Furthermore, I used information from Lloyd (1965) to determine the self-incompatibility (SI) status and race of each population sampled. Lloyd's racial designations were dependent on both morphological traits and hand self-pollinations performed in the greenhouse. For *L. alabamica*, the races sampled were: a1=strongly SI; a2=weakly SI; a3=SC; Tuscumbia=SC; and

Russellville=SC. For *L. crassa*, I sampled four races: c1=strongly SI; c3=strongly SI; c5=SC; c15=SC. My designation of population CR55 as race c5 is based on Lloyd's (1965) map of racial locations; however, he noted that race c5 was in close geographic proximity to race c3 and that the two overlapped in some areas. Thus, it is possible that CR55 is actually race c3 since self-pollinations were not performed on plants grown from this population.

Genetic Analyses—Field-collected seed from each of the 15 populations was grown in the greenhouse at The University of Georgia to obtain individuals for analyses of genetic structure, diversity, and mating system. The *L. alabamica* and *L. crassa* populations sampled (1-10 and 11-15 respectively) were named as follows (with the number of individuals sampled in parentheses): 1-Isbell (30), 2-Tuscumbia (46), 3-Speck (23), 4-Hatton (112), 5-Winchell (24), 6-Russell (32), 7-Landers (177), 8-StCross (90), 9-CPCC (104), 10-CR46 (65), 11-CR203 (57), 12-CR55 (148), 13-Quarry (116), 14-Bramlett (19), 15-NCP (255). For each population, individuals were separated by their maternal family.

Seeds were planted in Fafard brand potting soil mixed with lime, and grown in 4" pots. Plants were watered daily and fertilized weekly. Once rosettes were large enough, leaf tissue was collected and snap frozen in liquid nitrogen. Tissue samples were then stored in a -80˚C freezer until extraction of allozyme proteins. To extract allozymes, each sample was crushed with a mortar and pestle, and an extraction buffer (Wendel and Parks 1982) added to solubilize and stabilize the enzymes. The enzyme extract was then absorbed onto chromatography paper wicks and stored at -80˚C until starch gel electrophoresis.

I resolved the following enzymes for *L. crassa*: diaphorase (DIA), fluorescent esterase (FE), leucine-amino peptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME), 6-phosphogluconate dehydrogenase (6-PGDH), phosphoglucoisomerase (PGI), shikimate dehydrogenase (SKDH), triose-phosphate isomerase (TPI), and UTP-glucose-1-phosphate (UGPP). These enzymes were also resolved for *L. alabamica*, including one additional enzyme: colorimetric esterase (CE). For *L. alabamica*, I scored 19 loci on 11% starch gels using the following gel-electrode buffer combinations: Buffer 34: (CE-1, CE-2, DIA-1, DIA-2, LAP-1, LAP-2, PGI-1, PGI-2, TPI-1, TPI-2, and UGPP); Buffer 8-: FE-1, FE-3, FE-4, and ME); Buffer 11: MDH, 6-PGDH-1, SKDH-2, and UGPP). For *L. crassa*, I used four gel-electrode buffer combinations to score these 16 loci: Buffer 34: (DIA-1, DIA-2, LAP-1, LAP-2, PGI-1, PGI-2, TPI-1, TPI-2); Buffer 4: (6-PGDH-1 and SKDH-2); Buffer 8-: (FE-1, FE-3, FE-4, and ME); Buffer 11: (MDH and UGPP). All loci were numbered sequentially, with the most anodal locus given the number one. The stain recipe for DIA is given in Cheliak and Pitel (1984), and for UGPP is given in Manchenko (1994). The recipes for all other stains and buffers are found in Soltis et al. (1983).

Statistical Analyses—Measures of genetic diversity were calculated for *L. alabamica* and *L. crassa* (following Hamrick and Godt 1989), and for each population (as in Hedrick 2005) using the program GenAlEx 6.1 (Peakall and Smouse 2005). I calculated the following measures: the percentage of polymorphic loci (P), the mean number of alleles per locus (A), the mean number of alleles per polymorphic locus (AP), the effective number of alleles (A_e), and the

observed (H_o) and expected (H_e) heterozygosity. Population values (shown in Tables 5.1 and 5.2) are given the subscript "p". I report the unbiased estimator of expected heterozygosity (Nei and Chesser 1983) in all tables. Following Schoen and Brown (1991), I also calculated the coefficient of variation for the mean expected heterozygosity of each species, and for the mean expected heterozygosity of self-incompatible and self-compatible populations of each species. I compared the mean population values of *L. alabamica* and *L. crassa* for each genetic diversity measure using t-tests performed in JMP 6.0 (SAS Institute 2005). I also performed t-tests to examine mean differences in measures of genetic diversity between self-incompatible (SI) and self-compatible (SC) populations of each species. Population size was almost entirely confounded with SI status (only one large population was of a self-compatible race and only one small population of a self-incompatible race), so its affect on genetic diversity was not examined separately. In addition, I tested for deviations from Hardy-Weinberg equilibrium at each polymorphic locus, using GenAlEx 6.1 (Peakall and Smouse 2005) to calculate the fixation index (F; Wright 1922) and conduct a χ^2 test of significance (Li and Horvitz 1953).

To estimate the proportion of total genetic diversity found among populations at each polymorphic locus, I calculated the among-species and among-population genetic structure (F_{ST}) of *L. alabamica* and *L. crassa* using AMOVA (Excoffier et al. 1992; Michalakis and Excoffier 1996) in GenAlEx 6.1 (Peakall and Smouse 2005). The among-species calculations were based on 14 polymorphic loci and the among-population calculations were based on 16 and

12 polymorphic loci for *L. alabamica* and *L. crassa*, respectively. F_{ST} values were tested for significance against 9999 random permutations of the data. I also used this software to calculate pairwise F_{ST} values (from 9999 random permutations) for each population (both within and between species). These measures give insight into the degree of genetic differentiation between populations and species, and the amount of historical gene flow. I then compared pairwise population F_{ST} values between self-incompatible (SI) by self-incompatible (SI), self-incompatible (SI) by self-compatible (SC), and self-compatible (SC) by self-compatible (SC) populations within each species via an ANOVA performed in JMP 6.0 (SAS Institute 2005). Means comparisons were made using contrasts.

I also computed Nei's genetic identity (I) and distance (D) values for each pair of populations (Nei 1972) both within and between species using GenAlEx 6.1 (Peakall and Smouse 2005). Nei's genetic distance between populations was then used to construct a UPGMA phenogram in PHYLIP (Felsenstein 2005) to look at the genetic relationships between populations of both *L. alabamica* and *L. crassa*. In addition, I tested for isolation by distance (a significant positive correlation between genetic and geographic distance) using a Mantel test (Smouse et al. 1986).

I also examined the genetic structure of *L. alabamica* and *L. crassa* populations using the clustering method of Pritchard et al. (2000) implemented in the program Structure 2.2. This program probabilistically assigns each individual to K clusters or populations, and individuals can be assigned to one or more populations as an indication of admixture. I ran the program using the admixture

and independent allele frequencies models from K=1 to 30, with 10 replicates at each K of 100,000 iterations after a burn-in period of 10,000 iterations. Log-likelihood values are assigned to each K, and I employed a likelihood ratio test (Sokal and Rohlf 1995) to determine the most likely number of genetically differentiated populations. For the most likely K, I then used the program CLUMPP (Jakobsson and Rosenberg 2007) to permute cluster matches from the set of 10 replicates at that K. The program Distruct (Rosenberg 2004) was used to graphically display the cluster matching results from CLUMPP.

Finally, I estimated the multilocus outcrossing rate (t_m) of six populations (three of *L. alabamica* and three of *L. crassa*) using the methods developed by Ritland and Jain (1981) and Ritland (2002). The populations (with number of families used in analyses in parentheses) are as follows: Hatton (7), Landers (8), CPCC (8), CR55 (11), Quarry (11), and NCP (19). Each family analyzed consisted of at least eight progeny. The analyses were performed using the program MLTR (Ritland 2002).

RESULTS

Genetic diversity within L. alabamica and L. crassa—For *L. alabamica*, 16 of 19 loci were polymorphic (84.2%) in at least one population, whereas *L. crassa* had a slightly lower number, 12 of 16 loci (75.0%; Table 5.3). *L. alabamica* had 3.38 alleles per polymorphic locus, and *L. crassa* had 3.17 alleles per polymorphic locus. There were also differences in the total number of alleles per locus (3.00 v. 2.63), effective number of alleles (1.47 v. 1.32), and expected

heterozygosity (0.229 v. 0.183) respectively. In addition, the species did not differ significantly in their mean population values for all of the calculated measures of genetic diversity. The range of population values was larger in *L. crassa* for the percent of polymorphic loci, the total number of alleles per locus, the mean number of alleles per polymorphic locus, but smaller for the effective number of alleles and expected heterozygosity. The range differences are likely due to the difference in the number of populations sampled for each species. For all species-level measures, *L. alabamica* had higher levels of genetic diversity than *L. crassa*.

Genetic diversity and mating system within populations of L. alabamica and L. crassa—At the population-level, both species show associations of genetic diversity with self-incompatibility status (Tables 5.1 and 5.2). For *L. alabamica*, populations designated by Lloyd (1965) as self-compatible have a significantly lower ($p<0.05$) percentage of polymorphic loci (42.10 v. 63.17), total number of alleles per locus (27.67 v. 39.28), mean number of alleles per polymorphic locus (2.07 v. 2.69), effective number of alleles (1.08 v. 1.35), and lower expected heterozygosity (0.045 v. 0.195) (Table 5.4). In addition, the coefficient of variation of mean expected heterozygosity was slightly lower for self-compatible populations compared to self-incompatible populations (22.53 v. 23.34). Although self-incompatible and self-compatible populations did not significantly differ in genetic diversity measures for *L. crassa*, the mean value of all measures was lower in self-compatible populations compared to self-incompatible populations (Table 5.4), in keeping with the pattern of lower genetic

diversity in self-compatible populations. It seems likely that these measures would have been significant had more *L. crassa* populations been sampled.

Within *L. alabamica*, observed genotype frequencies conformed to the expectations of Hardy-Weinberg equilibrium in all populations for only 12.5% of polymorphic loci. There were 68 cases (62.9%) of fixation indices significantly different from zero ($p<0.05$); 62 were cases of heterozygote deficiency and six were cases of heterozygote excess. Two of the six cases of heterozygote excess were at locus DIA-1, and four were at locus DIA-2. The mean fixation index across all polymorphic loci and populations was 0.360 ± 0.037. The observed genotype frequencies of *L. crassa* conformed to Hardy-Weinberg expectations in all populations for only 16.7% of polymorphic loci. Fixation indices significantly differed from zero ($p<0.05$) in 28 cases (71.8%), with 25 cases of heterozygote deficiency and three cases of heterozygote excess. Of the three cases of heterozygote excess, two were at locus DIA-2 and one at locus UGPP-1. The mean fixation index across all polymorphic loci and populations was 0.378 ± 0.055. These data indicate that the majority of loci exhibit evidence of inbreeding in all populations of both species, whereas only a few loci may be under heterotic selection or show evidence of negative assortative mating.

I also determined the multilocus outcrossing rate (t_m) of three populations of each species. For *L. alabamica*, I found that the self-incompatible Hatton (Pop. 6; race a1) and the self-compatible CPCC (Pop. 9; race a3) were both highly outcrossing, with the more weakly self-incompatible Landers (Pop. 7; race a2) outcrossing at a more intermediate level (Table 5.1). With respect to *L. crassa*,

the outcrossing rates of populations NCP (Pop. 15; self-incompatible race c3) and CR55 (Pop. 12; self-compatible race c5) were intermediate (similar to Landers), and the outcrossing rate of the Quarry population (Pop. 13; self-compatible race c15) was low (Table 5.2). In four of the six populations, the estimate of outcrossing rate is in keeping with the self-incompatibility status designated by Lloyd (1965).

Genetic structure and gene flow among populations of L. alabamica and L. crassa—The proportion of total genetic variation explained by differences among populations (F_{ST}) of *L. alabamica* was 0.450 (Table 5.3) and this value was significantly different from zero. The F_{IS} and F_{IT} values were 0.290 and 0.609, respectively. The mean genetic identity among populations of *L. alabamica* was 0.9134 ± 0.0100, with a range from 0.8309 to 0.9432 (Table 5.1). The weakly SI population belonging to the a2 race (Landers) had the lowest mean genetic identity with other *L. alabamica* populations. The pairwise population estimates of F_{ST} ranged from 0.042-0.714. The Speck and Winchell populations had the lowest F_{ST} value (0.042), whereas the Landers and CPCC populations had the highest F_{ST} (0.714) among *L. alabamica* populations. A significant correlation between genetic and geographic distance was detected for this species (r=0.532, p=0.000), which suggests that the high population genetic structure may in part be due to isolation by distance.

For *L. crassa*, F_{ST} was 0.358 (Table 5.3), which was also significantly different from zero. The F_{IS} and F_{IT} values were 0.364 and 0.591, respectively. The mean genetic identity among populations of *L. crassa* was 0.9060 ± 0.0157,

and ranged from 0.8628-0.9366 (Table 5.2). The highly self-compatible c15 race populations (Quarry and Bramlett) had the lowest mean genetic identity with other populations. In addition, pairwise population F_{ST} values ranged from 0.046-0.758. The *L. crassa* populations in closest geographic proximity, CR55 and NCP, had the lowest F_{ST} value (0.046), possibly due to current gene flow between these populations. The populations with the highest F_{ST} value (0.758), Quarry and Bramlett, are both of the highly self-compatible race c15. I found a significant correlation between genetic and geographic distance in *L. crassa* as well (r=0.112, p=0.000), suggesting isolation by distance. However, the correlation is weaker in *L. crassa*, most likely due to the small geographic distances between most of the sampled *L. crassa* populations.

Furthermore, there were nine (mean frequency=0.120) and six (mean frequency=0.193) private alleles partitioned among five *L. alabamica* and four *L. crassa* populations, respectively. In *L. alabamica*, Hatton had the most private alleles (four of nine), followed by Landers (two of nine), with Speck, StCross, and CPCC each having one private allele. The *L. crassa* populations with private alleles were CR55 (two of six), NCP (two of six), CR203 (one of six) and Bramlett (one of six). Interestingly, one of the private alleles in CR55 is the same allele only found in CPCC in *L. alabamica*. CPCC (Pop. 9) is an isolated population of *L. alabamica* within close geographic proximity to populations of *L. crassa* (Figure 5.1), and the presence of this allele in only one *L. alabamica* and one *L. crassa* population suggests gene flow may have occurred between them.

In addition, I calculated the among-species genetic structure (F_{ST}) and obtained an estimate of 0.025, which was significantly different from zero ($p<0.01$), indicating that genetic differentiation is low between *L. alabamica* and *L. crassa*. I then examined the relationship of populations of both species to one another in a combined analysis using Nei's identity and genetic distance between populations, and using the genetic distance values to construct a UPGMA phenogram (Figure 5.2). The mean I of *L. alabamica* populations to one another was 0.9241 ± 0.0088. For *L. crassa* populations, the mean I was 0.9217 ± 0.0207. Comparisons of populations between species yielded a mean I of 0.8797 ± 0.0109. From the UPGMA phenogram (Figure 5.2), it can be seen that the species did not cluster separately in this analysis.

Admixture across species' boundaries was also seen in the clustering analysis performed by Structure (Figure 5.3). The same alleles were found in both *L. alabamica* and *L. crassa* (indicated by bands of the same color), suggesting shared ancestry or current gene flow. However, it can also be seen that, although populations share alleles, different alleles predominate in many populations. The likelihood ratio test indicated that K=12 was the most likely number of clusters to fit these data. From the color-banding in Figure 5.3, Speck (Pop. 3) and Winchell (Pop. 5), StCross (Pop. 8) and CR46 (Pop. 10), and CR55 (Pop. 12) and NCP (Pop. 15) have similar allelic patterns. This result indicates these are not highly genetically differentiated populations, and is in agreement with the small genetic distance calculated between these populations (Figure 5.2).

I also investigated the possibility that genetic structure was higher (and potentially gene flow lowest) between populations of self-compatible races, and between populations of self-incompatible and self-compatible races, than between populations of self-incompatible races. For *L. alabamica*, I found a significant effect of the SI status of population pairs on pairwise F_{ST} values (df=2, F=12.6516, p<0.0001), with SCxSC population comparisons having a mean F_{ST} value of 0.437 ± 0.081, SIxSC population comparisons having a mean of 0.462 ± 0.031, and SIxSI population comparisons showing a mean F_{ST} value of 0.248 ± 0.031. Tests of significance of each pair of means revealed that the SCxSC and SIxSC groups did not differ from one another, but that the SIxSI group was significantly different (p<0.05) from both. For *L. crassa*, the effect of the SI status of population pairs on pairwise F_{ST} values was not significant, most likely due to the small number of populations sampled. However, the trend was similar to *L. alabamica* in that the SIxSI group had the lowest mean (0.235 ± 0.160), and the means of the SIxSC and SCxSC groups (0.338 ± 0.065 and 0.606 ± 0.092, respectively) were higher. Contrasts between all pairs of means were not significant.

However, two of the populations (Landers and CR55) used in the analysis were of questionable SI status: Landers is of the a2 race classified by Lloyd as weakly SI, and CR55 was geographically classified as the self-compatible c5 race, but may instead be the self-incompatible c3 race. I therefore re-ran the analyses with Landers classified as a self-compatible population and CR55 as a self-incompatible population. For *L. alabamica*, there was again a significant

effect of the SI status of population pairs on pairwise F_{ST} values (df=2, F=32.6271, p<0.0001). In this analysis, SCxSC populations had the highest mean (0.536 ± 0.050) and SIxSC populations were somewhat lower (0.447 ± 0.023), with SIxSI populations exhibiting the lowest genetic structure between populations (0.192 ± 0.027). Again, the SIxSI group was significantly different (p<0.05) from both the SIxSC and SCxSC groups. The SIxSC and SCxSC groups did not significantly differ. For *L. crassa*, there was still no significant effect of SI status on pairwise F_{ST} values. However, the contrast comparing SIxSI to SCxSC was significant (p<0.05). The above analyses indicate that gene flow may be reduced between self-incompatible and self-compatible populations due to the difference in mating system.

DISCUSSION

L. alabamica and *L. crassa* exhibited similarly high levels of genetic diversity for all measures, as well as comparable levels of among-population genetic structure (Table 5.3). Population genetic structure was high, suggesting that there may be significant levels of genetic drift and inbreeding, and potentially little current gene flow between populations. This is not surprising given that the habitats in which they grow are geographically disjunct (Figure 5.1), and seeds of these species are gravity-dispersed. These genetic structure data also fit well with the patterns found in other annual species with mixed-mating systems (Hamrick and Godt 1996). However, the levels of genetic diversity for both *L. alabamica* and *L. crassa* are higher than for most plant taxa as a whole, most

endemic plants, and most plants with gravity-dispersed seeds (Hamrick and Godt 1989). They are also higher than in most animal-pollinated outcrossing annuals. It seems likely that the high levels of genetic diversity observed are due to self-incompatibility in these taxa, which, as an obligate outcrossing mechanism, has maintained allelic variation in those populations. This variation is then spread through pollen or seed dispersal to other populations, including those that engage in selfing.

L. alabamica and *L. crassa* also had low among-species genetic structure (F_{ST}=0.025), small genetic distances between populations and clustering across species' boundaries (Figure 5.2), as well as some admixture of alleles between species (Figure 5.3). Shared alleles could be due to either common ancestry or a history of hybridization; however, an allele private to both an *L. alabamica* and an *L. crassa* population indicates that occasional hybridization events have occurred, or may still occur, where the species' ranges overlap in Morgan Co. This finding of high genetic similarity between *L. alabamica* and *L. crassa* is supported by prior phylogenetic work on these species by Beck et al. (2006), in which the authors confirmed that *L. alabamica* and *L. crassa* were sister species, but found only a few distinguishing cpDNA marker differences between them.

Although I found few genetic differences between *L. alabamica* and *L. crassa* in this study, there were private alleles present in each species. However, these alleles were private to one or a subset of populations in each species, rather than the species as a whole. The average frequency of a private allele within a population was relatively high (0.120 in *L. alabamica* and 0.193 in *L.*

crassa). This result, along with the fact that, in general, alleles tend to predominate in certain populations (Figure 5.3), suggests that substantial genetic drift may occur in populations of both *L. alabamica* and *L. crassa*, and may be the primary cause of genetic differentiation.

In addition, I found evidence that population-level differences in mating system affect genetic diversity, genetic structure, and gene flow between populations of both *L. alabamica* and *L. crassa*. Self-incompatible populations maintain higher levels of genetic diversity than self-compatible populations (Table 5.4). This result is consistent with previous studies of allozyme variation in *Leavenworthia* (Solbrig 1972; Solbrig and Rollins 1977; Charlesworth and Yang 1998), which found selfing species, and selfing populations of *L. alabamica* and *L. crassa*, exhibited lower levels of genetic diversity than outcrossing species or populations. This result is also consistent with studies of DNA sequence diversity in selfing v. outcrossing populations of *L. crassa* (Liu et al. 1998; 1999) and *L. alabamica* (Busch 2005b), which found little to no sequence variation in self-compatible populations compared to outcrossing populations at the ADH-1 (Liu et al. 1998) and PGI-C (Liu et al. 1999; Busch 2005b) loci, respectively. A pattern of reduced genetic diversity in selfing species compared to outcrossing species has also been observed in a variety of plant groups (Schoen and Brown 1991; Hamrick and Godt 1996).

Furthermore, I found that pairwise estimates of F_{ST} are significantly lower between self-incompatible populations than between self-incompatible and self-compatible populations. This suggests that the evolution of selfing in these

species could have reduced gene flow between selfing populations and their outcrossing progenitors, allowing genetic drift or local adaptation to occur. Reduced gene flow between outcrossing and selfing populations also would have allowed for selfing populations to evolve the associated floral adaptations documented by Lloyd (1965).

Although I did not self-pollinate plants from each population to independently confirm its self-incompatibility (SI) status apart from what was documented by Lloyd (1965), I did estimate the multilocus outcrossing rate of six populations (Tables 5.1 and 5.2) to see how whether it fit with the population's SI status. In four of the six cases, the outcrossing rate estimate fit with the designated SI status of the population. The highly self-incompatible a1 race population (Hatton) had a higher t_m (1.085 ± 0.066) than that of the weakly self-incompatible a2 race population (Landers), which had a t_m of 0.794 ± 0.116. In addition, the c3 race population (NCP) had a higher t_m (0.765 ± 0.063) than that of the c15 race population (Quarry), which had a t_m of 0.285 ± 0.162. The exceptions were populations CPCC (self-compatible race a3) and CR55 (self-compatible race c5).

In the case of CR55, it is possible that the racial classification was made incorrectly for that population, and it is in fact race c3. This would make sense given that its estimated t_m (0.767 ± 0.086) is virtually identical to that of NCP, they are in close geographic proximity to one another, and Lloyd (1965) documented areas of overlap between them. It is also possible that, although self-compatible, outcrossing was high in the year this population was measured.

Previous work by Lyons and Antonovics (1991) found low rates of outcrossing in two *L. crassa* populations (one of the c5 and one of the c12 race; both self-compatible), and both estimates (t_m=0.330 and 0.030, respectively) were much lower than those found in this study. This suggests there may be substantial year-to-year variability in outcrossing rates, at least within populations of *L. crassa*.

An additional possibility is that the sampling was biased towards outcrossing events due to the fact that most plants did not produce enough progeny to be analyzed, and plants that did produce enough progeny may have been larger and more likely to receive pollinator service. Bias or a year of greater pollinator service could also explain the high outcrossing rate observed in the CPCC population (1.200 ± 0.034), which is almost certainly self-compatible (no self-incompatible populations of *L. alabamica* are found in Morgan Co.) and is likely to consist of less than 50 individuals. Due to the small and uneven sample sizes used for mating system analysis in this study, the estimates of outcrossing rate cannot be used to make strong inferences about outcrossing in populations of either species.

An additional result of interest was my finding that fixation indices were high in both self-incompatible and self-compatible populations, with most loci exhibiting heterozygote deficiency and only a few cases of heterozygote excess. Charlesworth and Yang (1998) also found high fixation indices in self-incompatible and self-compatible populations of *L. alabamica* and *L. crassa*. These data suggest that even self-incompatible populations may experience

levels of inbreeding significant enough to reduce heterozygosity. This is possible given that low levels of autogamy and pseudo-compatibility were documented in the self-incompatible races of *L. alabamica* and *L. crassa* (Lloyd 1965). Pseudo-compatibility can fluctuate depending on environmental conditions (Levin 1996), and there may be years in which self-incompatible populations produce a significant number of progeny through self-fertilization. It is also possible that there is selection at some of these allozyme loci, which has been previously found to occur in some species, such as *Avena barbata* (Hamrick and Allard 1972).

Thus, in the present study, I find evidence that the genetic similarity between *L. alabamica* and *L. crassa* is high, either due to recent common ancestry (Beck et al. 2006) or hybridization. Although more detailed studies of mating patterns using larger sample sizes and more detailed sampling could shed additional light on whether or not introgression has occurred. The genetic similarity of *L. alabamica* and *L. crassa* suggests that these species may be in the early stages of lineage divergence. Studies of the genetic basis of quantitative traits that distinguish these species, such as fruit shape, could further our understanding of the processes promoting their speciation. In particular, whether species' differences are due to genetic drift or natural selection.

Furthermore, I find evidence that the evolution of self-compatibility has had a significant impact on genetic diversity within populations of both species, and on their population genetic structure; a finding consistent with both previous studies of *Leavenworthia* (Solbrig 1972; Solbrig and Rollins 1977; Charlesworth

and Yang 1998; Liu et al. 1998; 1999), and of other plant groups containing both outcrossing and selfing species (Schoen and Brown 1991; Hamrick and Godt 1996). As a consequence, populations are highly structured, and individual populations serve as repositories of unique genetic variation in these species. Any efforts to conserve *L. alabamica* or *L. crassa* will need to consider these population genetic factors when designing a conservation strategy.

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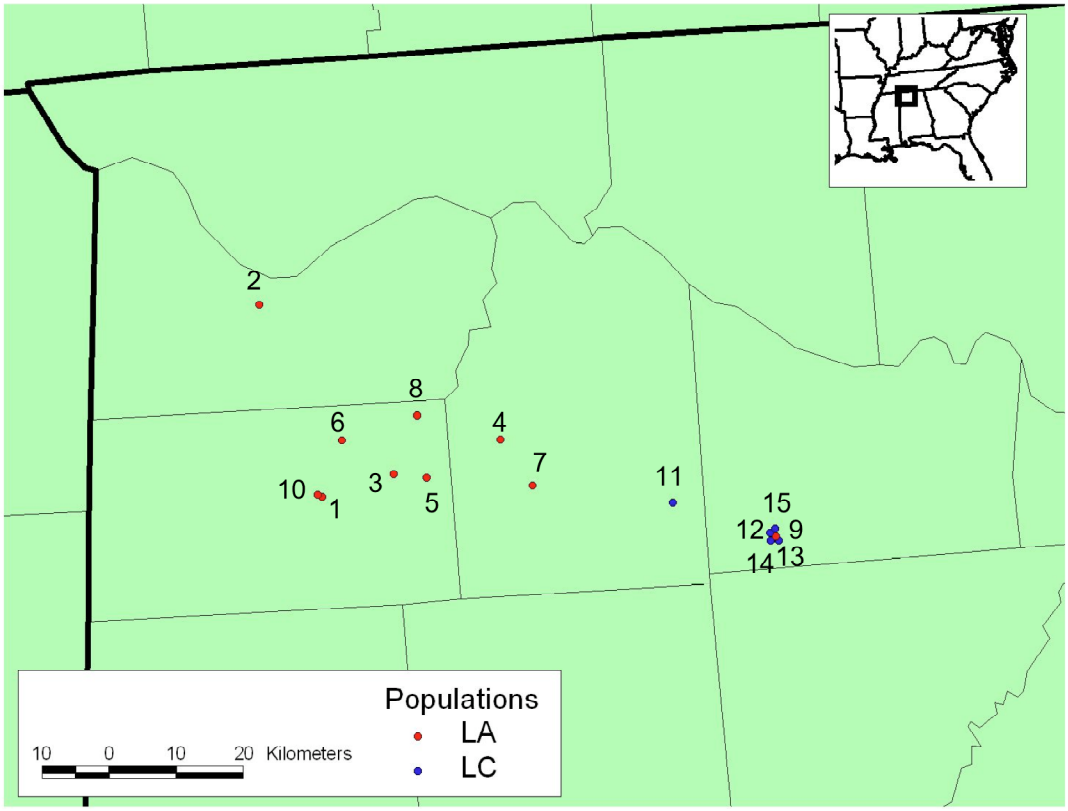
FIGURE LEGENDS

Figure 5.1: The geographic distribution of sampled populations of *L. alabamica* and *L. crassa* within the Moulton and Tennessee Valleys of northwestern Alabama. *L. alabamica* populations (LA) are shown in red and numbered 1-10: Isbell, Tuscumbia, Speck, Hatton, Winchell, Russell, Landers, StCross, CPCC, and CR46, respectively. *L. crassa* populations (LC) are shown in blue and numbered 11-15: CR203, CR55, Quarry, Bramlett, and NCP, respectively.

Figure 5.2: UPGMA phenogram of 10 *L. alabamica* and 5 *L. crassa* populations based on Nei's (1972) genetic distance values. The *L. crassa* populations are NCP, CR203, CR55, Bramlett, and Quarry. All other populations are *L. alabamica*.

Figure 5.3: Allele frequencies and distributions among 15 sampled geographic populations of *L. alabamica* and *L. crassa* as assigned by the program Structure 2.2 (Pritchard et al. 2000). *L. alabamica* populations (LA) are numbered 1-10: Isbell, Tuscumbia, Speck, Hatton, Winchell, Russell, Landers, StCross, CPCC, and CR46, respectively. *L. crassa* populations (LC) are numbered 11-15: CR203, CR55, Quarry, Bramlett, and NCP, respectively. Population width denotes sample size. Each allele is shown using a different colored band, and the width of the band represents the frequency of that allele in the population.


2
8
6
4
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Populations
LA
LC
10 0 10 20 Kilometers

Figure 5.1

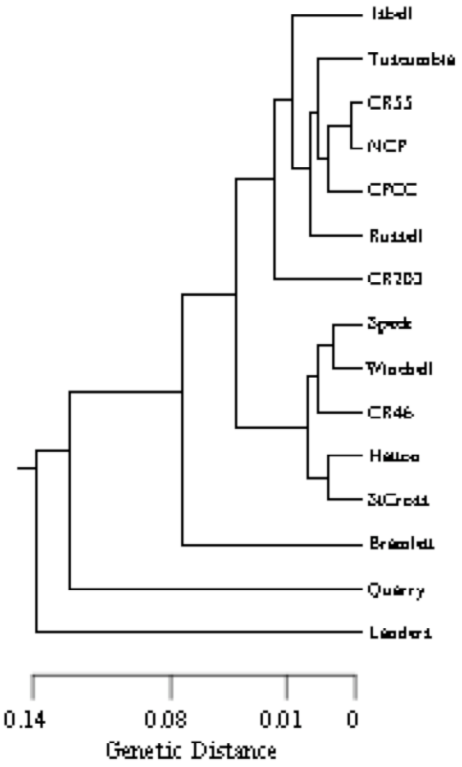
Isbell
Tuscumbia
CR55
NCP
CPCC
Russell
CR203
Speck
Winchell
CR46
Hatton
30Creek
Brimfield
Quarry
Leaders
0.14
0.08
0.01
0
Genetic Distance

Figure 5.2

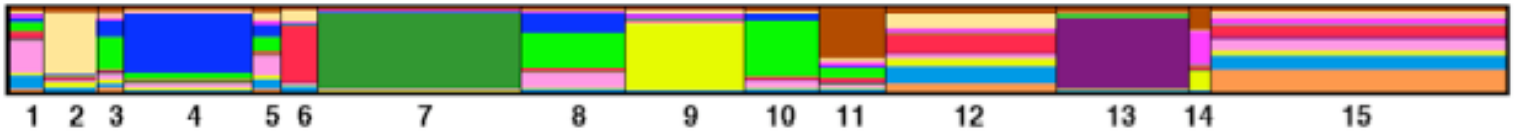

Figure 5.3

Table 5.1: Genetic diversity for *L. alabamica* populations

Population	Pop Size	SI status	Lloyd's race§	P_p(%)	Total A_p	AP_p	A_{ep}	H_{op}	H_{ep}	Mean I	t_m
Isbell	L	SI	a1	57.9	40	2.91	1.45 ± .121	0.182 ± .053	0.234 ± .056	0.9295	-
Speck	L	SI	a1	68.4	40	2.62	1.45 ± .116	0.145 ± .046	0.246 ± .051	0.9380	-
Hatton	L	SI	a1	68.4	42	2.77	1.34 ± .129	0.114 ± .041	0.167 ± .053	0.9191	1.085 ± .066
Winchell	L	SI	a1	57.9	38	2.73	1.42 ± .124	0.137 ± .042	0.222 ± .054	0.9432	-
StCross	L	SI	a1	63.2	37	2.50	1.24 ± .070	0.110 ± .031	0.156 ± .039	0.9206	-
Landers	L	SI	a2	63.2	40	2.75	1.20 ± .078	0.065 ± .022	0.125 ± .039	0.8309	0.794 ± .116
CR46	S	SI	a1	63.2	38	2.58	1.38 ± .048	0.165 ± .050	0.214 ± .051	0.9291	-
CPCC	S	SC	a3	52.6	31	2.20	1.07 ± .056	0.055 ± .050	0.038 ± .027	0.9090	1.200 ± .034
Tuscumbia	S	SC	Tuscumbia	42.1	27	2.00	1.07 ± .052	0.057 ± .051	0.041 ± .026	0.9114	-
Russell	S	SC	Russellville	31.6	25	2.00	1.09 ± .053	0.059 ± .049	0.057 ± .029	0.9032	-

Populations are ordered by population size and self-incompatibility status. Standard errors are shown where available.
P_p = percent polymorphic loci; Total A_p = total number of alleles per population (including monomorphic loci); AP_p = mean number of alleles per polymorphic locus; A_{ep} = mean effective number of alleles per locus; H_{op} = observed heterozygosity; H_{ep} = expected heterozygosity (genetic diversity); I = Nei's genetic identity; t_m = multilocus outcrossing rate.

§ Lloyd, 1965.

Table 5.2: Genetic diversity statistics for *L. crassa* populations

Population	Pop Size	SI status	Lloyd's race§	P_p(%)	Total A_p	AP_p	A_{ep}	H_{op}	H_{ep}	Mean I	t_m
NCP	L	SI	c3	68.7	37	2.91	1.23 ± .090	0.102 ± .035	0.150 ± .047	0.9346	0.765 ± .063
CR203	S	SI	c1	62.5	31	2.50	1.42 ± .114	0.158 ± .058	0.229 ± .058	0.9224	-
CR55	S	SC	c5	62.5	32	2.60	1.16 ± .063	0.075 ± .027	0.109 ± .036	0.9366	0.767 ± .086
Bramlett	S	SC	c15	25.0	21	2.25	1.09 ± .057	0.010 ± .005	0.061 ± .035	0.8736	-
Quarry	S	SC	c15	25.0	22	2.50	1.14 ± .080	0.039 ± .031	0.082 ± .042	0.8628	0.285 ± .162

Populations are ordered by population size and self-incompatibility status. Standard errors are shown where available.
P_p = percent polymorphic loci; Total A_p = total number of alleles per population (including monomorphic loci); AP_p = mean number of alleles per polymorphic locus; A_{ep} = mean effective number of alleles per locus; H_{op} = observed heterozygosity; H_{ep} = expected heterozygosity (genetic diversity); I = Nei's genetic identity; tm = multilocus outcrossing rate.
§ Lloyd, 1965.

Table 5.3: Comparisons of species-level genetic diversity and genetic structure for *L. alabamica* and *L. crassa*

	P (%)	AP	A	A_e	H_e	F_{ST}
L. alabamica	84.20	3.38	3.00	1.47	0.229	0.45
Mean population values	56.8 ± 3.75	2.51 ± .104	1.88 ± .070	1.27 ± .050	0.150 ± .015	-
Range	31.6-68.4	2.00-2.91	1.32-2.21	1.07-1.45	0.038-0.246	-
L. crassa	75.00	3.17	2.63	1.32	0.183	0.36
Mean population values	48.7 ± 9.76	2.55 ± .106	1.79 ±	1.21 ± .057	0.126 ± 0.020	-
Range	25.0-68.7	2.25-2.91	1.31-2.31	1.09-1.42	0.061-0.229	-

Standard errors are shown where available.

P = percent of polymorphic loci; AP = mean number of alleles per polymorphic locus; A = mean number of alleles per locus (including monomorphic loci); A_e = mean effective number of alleles per locus; H_e = mean expected heterozygosity (gene diversity); F_{ST} = among population genetic structure.

Table 5.4: Comparison of mean genetic diversity statistics for self-incompatible (SI) and self-compatible (SC) populations of *L. alabamica* and *L. crassa*

	N	P_p(%)	Total A_p	AP_p	A_{ep}	H_{ep}
L. alabamica						
SI	7	63.17 ± 1.62	39.28 ± .644	2.69 ± .052	1.35 ± .038	0.195 ± .017
SC	3	42.10 ± 6.06	27.67 ± 1.76	2.07 ± .067	1.08 ± .007	0.045 ± .010
L. crassa						
SI	2	65.6 ± 3.10	34.00 ± 3.00	2.70 ± .205	1.32 ± .095	0.189 ± .039
SC	3	37.5 ± 12.50	25.00 ± 3.51	2.45 ± .104	1.13 ± .021	0.084 ± .014

Means are shown with standard errors. The sample size (N) is shown in the first column. P_p = percent polymorphic loci; Total A_p = total number of alleles per population (including monomorphic loci); AP_p = mean number of alleles per polymorphic locus; A_{ep} = mean effective number of alleles per locus; H_{ep} = expected heterozygosity (genetic diversity).

CHAPTER 6

CONCLUDING REMARKS

Speciation is dependent on the evolution of barriers to reproduction between diverging lineages, either through the action of natural selection or genetic drift (Coyne and Orr 2004). Consequently, to investigate the factors promoting speciation between *Leavenworthia alabamica* and *L. crassa*, I tested for the presence of reproductive barriers between these species, and examined the extent to which population structure and mating system may affect gene flow between them. First, I tested for intrinsic genetic incompatibilities, either chromosomal (Rieseberg 2001; Brown et al. 2004), genic (Dobzhansky 1936; Muller 1939), or cytonuclear (Michaelis 1954; Levin 2003), between *L. alabamica* and *L. crassa* that would prevent or reduce hybridization. I found no evidence for these types of reproductive barriers, which agreed with Rollins' (1963) early work on cross-compatibility within the genus.

In addition, I tested for an SI-related phenomenon known as unilateral incompatibility (UI; Lewis and Crowe 1958), in which a self-incompatible (SI) plant cannot cross, or crosses less successfully, with a self-compatible (SC) plant when the SI plant is the pollen recipient and the SC plant the pollen donor; conversely, the reciprocal cross is successful. I found conclusive evidence for this type of reproductive barrier between the species, between their hybrids, and

possibly within each species as well. UI has been posited as a species-barrier (Harrison and Darby 1955; Grun and Radlow 1961), but if UI is indeed occurring both within- and between-species in *Leavenworthia*, then it is not a species-barrier per se. Rather, UI will impact the degree to which SC and SI individuals mate, likely increasing population structure in each species, thereby indirectly impacting the speciation process. However, if UI does occur solely between these species and their hybrids, then it would function as a post-mating reproductive barrier in this system, and may have had an important role in the divergence of *L. alabamica* and *L. crassa*.

Secondly, I examined the possibility that heterosis, hybrid breakdown, or transgressive segregation in F_1, F_2, and backcross hybrids could lead to fitness differences between *L. alabamica*, *L. crassa*, and their hybrids. Transgressive segregation occurs when recombination brings together QTL of the same effect that were previously not present in the same genome (Grant 1975; deVicente and Tanksley 1993; Rieseberg et al. 1999), and results in hybrid trait values that are outside the range of either parent species. In hybrids of *L. alabamica* and *L. crassa*, I did not find heterosis, hybrid breakdown, or transgressive segregation in floral morphological traits or the number of days to first flower, but did find possible heterosis, hybrid breakdown, and transgressive segregation with respect to the number of days from planting to germination. Hybrids did not take as long to germinate as the parent species, although the fitness impact of more rapid hybrid germination in these species is unknown.

I also tested for the presence of extrinsic genetic incompatibilities between the parent species (i.e. fitness differences between parent species and hybrids manifest only in the field), and for adaptation to different habitats that could prevent *L. alabamica* and *L. crassa* from mating. I found no evidence for either type of reproductive barrier, despite the fact that many studies have documented adaptation to different ecological niches in closely related plant species (e. g. Cruzan and Arnold 1993; Campbell 2003; Ramsey et al. 2003; Rieseberg et al. 2003; Kay 2006; Savolainen et al. 2006), as well as local adaptation within and between populations from different parts of a species' range (e.g. Clausen et al. 1940; Bradshaw 1960; Schemske 1984; Linhart and Grant 1996), suggesting that either selection does not favor the evolution of habitat isolation between these species, or gene flow is occurring that counteracts selection.

In order to investigate the possibility that gene flow is extensive between *L. alabamica* and *L. crassa*, and to examine how population structure and mating system impact population genetic differentiation in these species, I used allozyme markers to estimate genetic diversity, genetic structure, and gene flow among populations of both species. I also used genetic distance measures to group populations of *L. alabamica* and *L. crassa* to see if the species clustered separately, as well as the model-based clustering method of Pritchard et al. (2000). I found that the species did not cluster separately due to small genetic distances between populations, and that genetic differentiation between the species was low, which was consistent with prior work by Beck et al. (2006).

However, both species had high among-population genetic structure, and low estimates of historical gene flow between populations.

In addition, selfing populations had significantly lower amounts of genetic diversity and significantly higher pairwise estimates of population genetic structure, indicating that the mating system plays a significant role in the structuring of populations of both *L. alabamica* and *L. crassa*; a finding consistent with both previous studies of *Leavenworthia* (Solbrig 1972; Solbrig and Rollins 1977; Charlesworth and Yang 1998; Liu et al. 1998; 1999), and of other plant groups containing both outcrossing and selfing species (Schoen and Brown 1991; Hamrick and Godt 1996).

Taken together, data from these studies suggests that few, if any, reproductive barriers are present between *L. alabamica* and *L. crassa*. This may be because these species are in the early stages of divergence. It is also possible that, although the species are substantially different in fruit shape (Rollins 1963), they are not separate species, but rather morphotypes of the same species. Additional studies examining the genetic basis of fruit shape differences are needed to understand exactly how *L. alabamica* and *L. crassa* differ, and whether their differences are the result of natural selection or genetic drift.

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