THE EVOLUTION OF SELF-COMPATIBILITY AND ITS GENETIC
CONSEQUENCES IN *LEAVENWORTHIA ALABAMICA* (BRASSICACEAE)

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For his many contributions to evolutionary biology and his profound insight into

Leavenworthia, I dedicate my thesis to David G. Lloyd.

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IN *LEAVENWORTHIA ALABAMICA* (BRASSICACEAE)

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The evolution of self-fertilization from the outcrossing condition is a common transition in flowering plants which strongly alters the genetic structure of populations. In general, it is thought that self-fertilization may evolve in response to its innate transmission advantage or pollen limitation, and that this mating system may endanger the long-term viability of populations through mutation accumulation. The purpose of this dissertation was to evaluate the costs and benefits of self-fertilization in the species *Leavenworthia alabamica,* which exhibits variation among populations in the presence or absence of self-incompatibility. This variation in mating-system made it possible to directly answer the following questions: 1) what are the agents of selection driving the fixation of self-compatibility alleles in populations?; 2) does inbreeding depression selectively maintain self-incompatibility in nature, and if so, what happens to these deleterious alleles following the transition to self-compatibility?; and 3) does a history of self-fertilization cause populations to accumulate deleterious mutations and potentially experience extinction?

In *L. alabamica*, self-incompatibility predominates in large, stable and geographically central populations. In contrast, self-compatibility and adaptations for self-fertilization evolve in the small, disturbed, and geographically peripheral populations of this species. A field experiment shows that self-compatible genotypes are selectively favored in all environments, but that reductions in mate availability likely favor their spread and fixation in the smallest of populations. This model of mating-system

evolution is supported by the nearly complete or complete loss of sequence diversity in all of the independently derived self-compatible taxa of *Leavenworthia*. Inbreeding depression plays a role in the maintenance of self-incompatibility in *L. alabamica*, and the spread and fixation of self-compatibility alleles purge populations of these strongly deleterious mutations. Nearly all of the populations of this species do not suffer from a substantial local drift load caused by the fixation of mildly deleleterious mutations. That being said, the oldest and most isolated self-fertilizing population showed a dramatic increase in fitness following crosses between populations. Overall, these results suggests that mutation accumulation may eliminate highly inbreeding plant populations as the stochastic fixation of mildly deleterious mutations depresses fitness over many generations.

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CHAPTER 1

LEAVENWORTHIA ALABAMICA AS A MODEL FOR UNDERSTANDING MATING-SYSTEM

EVOLUTION IN FLOWERING PLANTS

Mating systems strongly influence nearly every aspect of the evolutionary process, ranging from the maintenance of genetic variation, natural selection against harmful mutations, and the creation and dissolution of gene combinations. In light of their far-reaching effects, evolutionary biologists have long sought to understand how natural selection may drive the evolution of mating systems in the wild (Darwin 1876; Jain 1976; Lloyd 1979). Consequently, the costs and benefits of inbreeding have been thoroughly evaluated in nearly every imaginable type of organism (Keller and Waller 2002). Of all the species studied, flowering plants possess the greatest amount of variation in the propensity to inbreed, and therefore present many of the most attractive systems for the study of mating systems and their evolution (Barrett 2002). In general, most species of flowering plants produce offspring through outcrossing, though there also a fair number of species that primarily self-fertilize (Vogler and Kalisz 2001). In an attempt to explain this pattern, biologists have suggested that even though self-fertilization evolves readily from the outcrossing condition (Stebbins 1974; Grant 1981), this transition has a variety of deleterious consequences on a population's viability, and is therefore an evolutionary "dead end" (Stebbins 1957; Takebayashi and Morrell 2001).

It is of great interest to understand the evolutionary costs and benefits of self-fertilization in the wild. In natural populations, self-fertilization is thought to be strongly favored by natural selection because it has an innate transmission advantage. This advantage results from the fact that an individual mating with itself passes an extra copy of chromosomes through seeds in comparison to individuals that outcross (Fisher 1941). Apart from its innate genetic advantage, self-fertilization may also be favored by various ecological agents of natural selection. In particular, any potential environmental factor

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reducing pollen availability to such an extent that it limits seed production will favor alleles for self-fertilization because this mode of reproduction provides reproductive assurance (Darwin 1876). In support of this idea, self-fertilization often predominates in environments lacking adequate pollinators, or in isolated, peripheral, marginal, or island habitats, where the availability of mates may limit seed production by outcrossing (Baker 1955, 1967; Stebbins 1957; Lloyd 1980; Barrett and Shore 1987; Inoue et al. 1996; Schueller 2004). Given that the transition to self-fertilization may be driven by its many genetic or ecological advantages over outcrossing, it is a bit surprising that this mode of reproduction is not ubiquitous in nature.

Perhaps the most widely researched barrier to the evolution of self-fertilization is inbreeding depression. Inbreeding depression occurs in offspring generated by self-fertilization because most deleterious alleles are partially recessive, such that their effects are unmasked in the homozygous state (Charlesworth and Charlesworth 1999). As a consequence, inbreeding depression is an extremely general and potentially strong force limiting the evolution of self-fertilization. Many genetic models have examined the conditions in which self-fertilization should be favored by natural selection, and have found the transmission advantage can favor self-fertilization when levels of inbreeding depression are relatively low (Lloyd 1979; Lande and Schemske 1985; Campbell 1986; Charlesworth et al. 1990; Uyenoyama and Waller 1991a, 1991b). Once the alleles causing self-fertilization begin to spread in a population, the deleterious mutations causing inbreeding depression should be purged from the population in the homozygous state (Barrett and Charlesworth 1990; Byers and Waller 1999). Therefore, inbreeding depression may only be an important factor in mating-system evolution during the initial

spread of alleles for self-fertilization (Lande and Schemske 1985; Charlesworth and Charlesworth 1999).

Although inbreeding depression is caused by strongly deleterious alleles, the vast majority of deleterious mutations have more modest effects on fitness (Keightley and Lynch 2003). This class of mutations is thought to endanger the long-term viability of populations because their dynamics may be only weakly influenced by the purifying action of natural selection (Kimura et al. 1963; Lynch and Gabriel 1990; Lande 1994; Higgins and Lynch 1999). Moderately deleterious mutations therefore are likely to reduce the viability of effectively small populations as they may stochastically fix in response to genetic drift (Lynch et al. 1995a, Wang et al. 1999; Bataillon and Kirkpatrick 2000). The transition to self-fertilization will reduce the effective population size and the efficacy of recombination (Ingvarsson 2002; Charlesworth 2003), and may accelerate the accumulation of deleterious mutations (Charlesworth et al. 1993; Lynch et al. 1995b). It is possible, therefore, that long periods of inbreeding cause mutation accumulation, and that the maintenance of outcrossing may be explained by the loss of self-fertilizing populations to extinction (Schultz and Lynch 1997). This hypothesis has not been evaluated in populations of flowering plants with divergent rates of self-fertilization, and must be addressed if we are to further our understanding of the factors maintaining outcrossing in the wild.

The purpose of this dissertation was to understand the evolutionary forces favoring and disfavoring self-fertilization as a reproductive strategy in flowering plants. To do so, it was necessary to study a plant species with genetically based variation within or among populations in the propensity to self-fertilize. There are several well studied

candidates such as species of *Amsinckia*, *Clarkia, Gilia*, *Leavenworthia, Linanthus,* and *Mimulus* (Takebayashi and Morrell 2001). Of all these possibilities, the species *Leavenworthia alabamica* was chosen for several important reasons: 1) *L. alabamica* exhibits clear variation among populations in the presence or absence of sporophytic single locus self-incompatibility (Rollins 1963; Lloyd 1965; Lloyd 1967); 2) work throughout the genus provides some of the best evidence for the effects of self-fertilization on the maintenance of neutral genetic variation in the wild (Lyons and Antonovics 1991; Charlesworth and Yang 1998; Filatov and Charlesworth 1999; Liu et al. 1999); and 3) the self-compatible populations of this species exhibit adaptations for autonomous seed production by self-fertilization, suggesting a relatively long history of inbreeding (Lloyd 1965).

The mating-system variation present in the species *L. alabamica* provides an excellent system for testing hypotheses on the evolutionary costs and benefits of self-fertilization in the wild. Therefore, the experiments conduced in *L. alabamica* are meant to elucidate the evolutionary forces that operate more generally on all species of flowering plants to shape variation in the mating system. The results of the research conducted in this species are presented in three sections. The first portion of research (chapter 2) examines the selective agents favoring self-compatibility in natural populations of *L. alabamica*. The second section (chapter 3) examines how the transition to self-compatibility has altered the genetic load causing inbreeding depression. The third and final portion of research (chapter 4) quantifies the fixed mutation load within populations in order to determine whether the long-term viability of the self-compatible and highly self-fertilizing populations may be compromised by mutational decline.

CHAPTER 2: THE EVOLUTION OF SELF-COMPATIBILITY IN GEOGRAPHICALLY PERIPHERAL

POPULATIONS OF *LEAVENWORTHIA ALABAMICA* (BRASSICACEAE)

The first portion of research aims to understand what agents of natural selection likely favored the evolution of self-compatibility in populations of *L. alabamica*. These selective factors may be either primarily genetic or ecological in nature. From a genetic standpoint, self-fertilization has an innate transmission advantage that should replace outcrossing because selfing genotypes pass on an extra copy of their chromosomes through seeds (Fisher 1941). The fact that this transmission advantage does not cause self-fertilization to be widely found in plants is probably the result of inbreeding depression halting the spread of alleles for self-fertilization (Lloyd 1979; Lande and Schemske 1985). Although the genetic benefits of self-fertilization have been widely researched, there are often strong ecological correlates of this mating system, which suggest that these may play a large role in mating-system transitions (Baker 1955; Lloyd 1980).

Self-fertilization is often found in populations found in marginal, peripheral, isolated, or island environments (Baker 1955, 1967; Stebbins 1957; Lloyd 1980; Barrett and Shore 1987; Inoue et al. 1996; Schueller 2004). This ecological pattern may be the result of an underlying deterioration of the quality of the environment that favors self-fertilization because it provides reproductive assurance in the face of pollen limitation (Burd 1994). One possibility is that pollinators become scarce in certain environments, such that limited seed set is caused by reduced movement of pollen between individuals within populations (Darwin 1876). Another possibility is that the plants themselves become less common in environments of low quality and therefore limited seed set is

caused by reductions in pollen availability *per se*. This latter hypothesis is termed "Baker's Law", and was originally put forth following the observation that the floras of islands contain a preponderance of self-fertilizing species, presumably because colonization favors the establishment of selfing genotypes (Baker 1955, 1967). Results from a battery of laboratory, greenhouse, and field experiments are presented in this section to shed light on how natural selection favors self-compatibility in natural populations of *L. alabamica*.

CHAPTER 3: INBREEDING DEPRESSION IN SELF-INCOMPATIBLE AND SELF-COMPATIBLE
POPULATIONS OF *LEAVENWORTHIA ALABAMICA*

There has been much theoretical research into the relationship between the mating system and deleterious mutations because harmful alleles are an extremely general and potentially strong force limiting the spread of alleles for self-fertilization (Lloyd 1979; Lande and Schemske 1985; Campbell 1986; Charlesworth et al. 1990; Uyenoyama and Waller 1991a, 1991b). Since deleterious mutations are primarily recessive in their phenotypic effects, their effects will be exposed in progeny produced by self-fertilization, causing inbreeding depression (Charlesworth and Charlesworth 1999). However, prolonged self-fertilization can remove many deleterious alleles in the homozygous state and therefore purge populations of the alleles causing inbreeding depression. This theoretical paradigm predicts that there should be a strong negative correlation between inbreeding depression and the rate of self-fertilization in natural plant populations (Lande and Schemske 1985; Johnston and Schoen 1996; Takebayashi and Delph 2001).

In the past 20 years, there has been a great deal of interest in experimentally evaluating the theoretical expectations for inbreeding depression as a force in mating-system evolution. Researchers generally either forcibly self-fertilize naturally outcrossing families of plants for several generations (Barrett and Charlesworth 1990; Carr and Dudash 1997; Dudash et al. 1997; Willis 1999) or compare populations or lineages of plants that have divergent rates of self-fertilization in natural populations (Holtsford and Ellstrand 1990; Carr and Dudash 1996; Johnston and Schoen 1996; Fishman 2001; Takebayashi and Delph 2001). In general, there has been moderate, though not universal support for the idea that prolonged self-fertilization may reduce inbreeding depression (Byers and Waller 1999). Therefore, crosses were conducted within populations to determine whether the self-compatible and highly self-fertilizing populations of *L. alabamica* have reduced inbreeding depression relative to populations that are self-incompatible.

CHAPTER 4: ISOLATION INCREASES THE LOCAL DRIFT LOAD IN A POPULATION OF THE ENDEMIC FLOWERING PLANT *LEAVENWORTHIA ALABAMICA*

All finite populations may potentially suffer from the fixation of mildly deleterious mutations that reduce individual fitness (Crow 1948; Muller 1950; Kimura et al. 1963). As a result, evolutionary biologists have spent a great deal of effort trying to understand the factors that accelerate mutation accumulation and potentially threaten natural populations with extinction (Lynch and Gabriel 1990; Lynch et al. 1995a). Aside from the mutation rate, the two most important factors in limiting mutation accumulation are the size of populations and their connectedness to other populations through

migration (Whitlock et al. 2000; Theodorou and Couvet 2002; Glemin et al. 2003; Rose and Rousset 2004). In general, populations are thought to be protected from extinction by the accumulation of mildly deleterious mutations if their effective size is on the order of several hundred individuals (Lande 1988, 1994; Lynch et al. 1995a; Schultz and Lynch 1997). Given the importance of effective size in the long-term persistence of populations, there has been a great deal of effort put forth in evaluating this hypothesis by measuring the mutation load in natural populations. Recent experiments in many species of plants have found a substantial increase in the fitness of inter-population hybrids caused by the masking of locally common deleterious alleles, especially in the smallest and most isolated populations (Levin 1984; van Treuren et al. 1993; Hauser and Loeschcke 1994; Ouborg and van Treuren 1994; Heschel and Paige 1995; Richards 2000; Paland and Schmid 2003).

In theory, it is possible that the transition to self-fertilization may threaten populations with extinction at the hands of mildly deleterious mutations since inbreeding reduces effective population size and the ability of recombination to generate offspring with fewer deleterious mutations (Heller and Maynard Smith 1979; Charlesworth et al. 1993; Lynch et al. 1995b; Schultz and Lynch 1997). This possibility is attractive because it may provide a mechanism for why self-fertilization may is not ubiquitous among species of flowering plants (Vogler and Kalisz 2001). However, there have been no known empirical attempts to this date that have examined the mutation load within plant populations that have divergent mating systems. Therefore, crosses were conducted between populations to determine whether the self-compatible and highly self-fertilizing populations of *L. alabamica* suffer from the deleterious effects of mutation accumulation.

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CHAPTER 2

THE EVOLUTION OF SELF-COMPATIBILITY IN GEOGRAPHICALLY PERIPHERAL POPULATIONS

OF *LEAVENWORTHIA ALABAMICA* (BRASSICACEAE)

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ABSTRACT

Self-compatibility and adaptations to self-fertilization are often found in plant populations at the periphery of species' ranges or on islands. Self-compatibility may predominate in these environments because it provides reproductive assurance when pollinators or availability of mates limits seed production. This possibility was studied in *Leavenworthia alabamica*, a flowering plant endemic to the southeastern United States. Populations at the center of the species' range retain sporophytic self-incompatibility, but peripheral populations are smaller, self-compatible, and have adaptations for self-fertilization. A reciprocal-transplant experiment was designed to test whether there is pollen limitation of seed set and to examine its strength in central and peripheral populations. Self-compatible genotypes produced more fruit and 17–22% more seed than self-incompatible genotypes in all environments, suggesting that the transition to self-compatibility may be favored by natural selection in all populations inhabited by *L. alabamica*. Sequence analyses demonstrated that two peripheral populations have 90–100% reductions in genetic variation, consistent with the effects of small population size or historical bottlenecks. Although pollen limitation of seed set occurs in all environments, self-compatibility may evolve at the periphery in *L. alabamica* because the benefits of reproductive assurance are influenced by population size or bottlenecks following extinction and colonization.

Key words: Baker's law; Brassicaceae; cedar glades; inbreeding; mating system; pollen limitation; reproductive assurance; self-incompatibility.

The evolution of self-fertilization from outcrossing is one of the most common evolutionary transitions in plants and has occurred in many taxonomic groups (Stebbins, 1974; Barrett and Shore, 1987; Barrett et al., 1989; Barrett et al., 1996; Kohn et al., 1996; Schoen et al., 1997; Takebayashi and Morrell, 2001; Barrett, 2002). One recurring theme is the association of peripheral, isolated, and island environments with self-compatible mating systems (Baker, 1955, 1967; Stebbins, 1957; Lloyd, 1980; Barrett and Shore, 1987; Inoue et al., 1996; Schueller, 2004; Herlihy, and Eckert, 2005). Self-compatibility may be restricted to these portions of species' ranges because of lower environmental quality (Brown, 1984; Sagarin and Gaines, 2002), such that pollinators or conspecific plants become scarce. If reduced pollinator activity or plant density causes pollen limitation of seed set in these peripheral environments, alleles conferring self-compatibility will spread within populations because the capacity to self-fertilize provides reproductive assurance (Moore and Lewis, 1965; Jain, 1976; Lloyd, 1979, 1992).

Darwin (1876) was the first to suggest that inadequate pollinator service would favor the evolution of autonomous seed production by natural selection. This hypothesis for the evolution of self-fertilization has recently been tested in a variety of empirical systems (Barrett et al., 1998; Eckert and Schaefer, 1998; Fausto et al., 2001; Herrera et al., 2001; Herlihy and Eckert, 2002; Elle and Carney, 2003; Kalisz et al., 2004; Schueller, 2004). Investigators have either examined the effects of natural variation in pollinator availability on pollen limitation of seed set (Fausto et al., 2001; Herrera et al., 2001; Kalisz et al., 2004; Schueller, 2004; Moeller and Geber 2005) or have experimentally

manipulated the ability of plants to autonomously self-fertilize (Leclerc-Potvin and Ritland, 1994; Klips and Snow, 1997; Eckert and Schaefer, 1998; Herlihy and Eckert, 2002; Davis and Delph, 2005). Empirical attempts to test pollinator limitation must demonstrate either that (1) patterns of reduced pollinator visitation correlate with selection for self-fertilization in nature (Inoue et al., 1996; Fausto et al., 2001; Elle and Carney, 2003; Kalisz et al., 2004); or (2) individuals lacking the ability to autonomously self-fertilize produce fewer seeds (Leclerc-Potvin and Ritland, 1994; Klips and Snow, 1997; Eckert and Schaefer, 1998; Herrera et al., 2001; Herlihy and Eckert, 2002; Elle and Carney, 2003; Schueller, 2004; Davis and Delph, 2005).

Reproductive assurance and the benefits of self-compatibility may also be strongly influenced by plant density or population size (Baker 1955, 1967). Bottlenecks during the founding of new populations could strongly favor self-compatibility and explain the preponderance of self-compatible species on islands or in isolated regions of the species' range (Stebbins, 1957; Lloyd, 1980; Barrett et al., 1989; Inoue et al., 1996; Schueller, 2004). In species with genetically controlled self-incompatibility systems, reductions in plant density may dramatically limit the reproductive success of individuals, since low S-allele diversity can obviate the production of offspring through outcrossing (Wright, 1964; Byers and Meagher, 1992; Husband and Barrett 1993; Reinartz and Les, 1994; Fischer et al., 2003). In support of this idea, numerous researchers have shown that historical colonization and low plant density may favor the evolution of self-compatibility, although pollinator limitation likely also plays a role (Lloyd, 1980; Barrett et al., 1989; Eckert and Barrett, 1992; Schueller, 2004; Moeller and Geber 2005).

Species of *Leavenworthia* are excellent candidates for the study of the ecological factors favoring the evolution of self-compatibility. A history of work in *Leavenworthia* suggests that selection for reproductive assurance has likely led to the independent evolution of self-compatibility four times among the eight extant species (Rollins, 1963; Lloyd, 1965; Solbrig and Rollins, 1977; Lyons and Antonovics, 1991). Single-locus, sporophytic self-incompatibility is found throughout the Brassicaceae (Bateman, 1955), is the ancestral mating system in the genus *Leavenworthia* (Lloyd, 1967; J. Beck, I. Al-Shehbaz, and B. Schaal, Washington University, unpublished data) and is absent in some populations of *L. alabamica* (Lloyd, 1965; Busch, 2005). This species is endemic to the Moulton Valley, a region in northern Alabama that is approximately 96 km long in an east–west direction and 5–16 km wide (Johnston, 1930). Differentiation among *L. alabamica* populations in self-compatibility, floral adaptations for self-fertilization, and vegetative morphology has led to their recognition as distinct land races (Lloyd, 1965). Previous work in this system has shown that populations at the center of the species' range are primarily composed of self-incompatible plants, whereas peripheral sites harbor many self-compatible plants (Map 1 in Lloyd, 1965). Self-compatible populations are thought to be highly self-fertilizing because flowers readily produce seed autonomously, lack spatial separation of the anthers and stigma, and possess many adaptations for self-pollination such as introrse anthers, short petals, short styles, and low pollen to ovule ratios (Rollins, 1963; Lloyd, 1965).

The narrow Moulton Valley of northern Alabama is composed of a rocky bed of limestone that is covered with an extremely thin layer of moist soil (Baskin et al., 1995). In such cedar glades, *L. alabamica* grows and is visited by several species of solitary bees

(*Andrena spp.* and *Halictus ligatus*), which vigorously climb over the central comb containing the stigma and anthers (Lloyd, 1965). At the periphery of the Moulton Valley, the elevation rises 75 m on the Cumberland Plateau to the south and the Highland Rim to the north (Johnston, 1930). These changes in elevation are also associated with reductions in soil moisture and the availability of exposed rock. At the periphery of the species' range, cedar glades are smaller and more disturbed, populations are less dense, and from historical observations, native pollinators appear less common (Lloyd, 1965). All these factors may act in concert to selectively favor self-compatibility at the border of the Moulton Valley.

If the availability or activity of pollinators declines in peripheral populations of *L. alabamica*, then pollen limitation of seed set should be greater in these environments (Herrera et al., 2001; Schueller, 2004). If this hypothesis is true, self-compatible genotypes should produce more seed than self-incompatible genotypes, and this difference should be greatest in peripheral environments. If this hypothesis is not supported, then the evolution of self-compatibility in peripheral environments may be attributed to other ecological forces limiting pollen availability, such as small population size, low plant density, or reduced S-allele diversity (Byers and Meagher, 1992; Reinartz and Les, 1994; Pannell and Barrett, 1998; Fischer et al., 2003). In a reciprocal-transplant experiment, I tested the idea that pollen limitation of seed set is higher at the periphery of the species' range. Sequence diversity was analyzed in one self-incompatible and two peripheral self-compatible populations to determine if patterns of genetic variation were consistent with the role of small population size or historical bottlenecks in peripheral environments.

MATERIALS AND METHODS

Variation in self-compatibility

Leavenworthia alabamica Rollins (Alabama glade cress; Brassicaceae) is a winter annual, endemic to the limestone cedar glades of the Moulton Valley in northern Alabama, though a few populations are found in the Tennessee River Valley near Tuscumbia, AL (Rollins, 1963). Individuals of *L. alabamica* germinate in the late fall and grow very slowly during the winter months. Vegetative growth accelerates when temperatures warm in the late winter and plants begin to flower in early March. Seeds mature on maternal plants and are passively dispersed throughout late April and early May (Solbrig and Rollins, 1977). Pollen dispersal between populations is rare because of the small home-range of native bees (Lloyd, 1965). Gene flow between populations is thought to primarily occur following the dispersal of seeds by intermittent periods of flooding during the spring.

Floral morphology was studied in five self-incompatible populations (*Hatton*, *Isbell*, *Newburg*, *Tharptown*, and *Waco*) and five self-compatible populations (*Landersville, Lebanon, Morgan/Huckaby Bridge, Russellville,* and *Tuscumbia*; see Busch, 2005 for population locations). Plant density and population size were estimated in March 2003 by counting plants within 30 separate 0.093 m^2 quadrats along two linear transects (Krebs, 1999; Table 1). Seeds were collected from these populations in late April and early May of 2003 and germinated in petri plates during October 2003. Seeds and seedlings were grown according to established protocols (Busch, 2005) to flowering in an Indiana University greenhouse.

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Variation in self-compatibility and floral morphology was quantified in the greenhouse because previous work suggested that the self-incompatibility reaction of *L. alabamica* may be influenced by environmental variation (Levin, 1996). Petal length, pistil height, and the height of the paired stamens on the first or second flower produced by plants were measured to the nearest 0.01 mm. The rotation of the anther sacs on the paired stamens was also scored because this trait likely influences the ability of plants to autonomously self-fertilize (Lloyd, 1965). The convention of Rollins (1963) was used to quantify the rotation of the anther sacs away from the pistil according to discrete angles (45°, 90°, 135° or 180°; Lyons and Antonovics, 1991). All six stamens were carefully removed with forceps, and pollen grains were immersed in 5 mL of a 3 : 1 solution of lactic acid and glycerol and vortexed for 30 s, then $1/1000^{th}$ of this solution (5 µL) was placed onto a slide and mixed with 10 µL of Alexander's stain (Kearns and Inouye, 1993). The total number of pollen grains per flower was then estimated from counts of four replicate samples. Ovule number was counted using a dissecting microscope.

Self-compatibility and the rate of autonomous self-fertilization were measured on each plant. To determine the degree of self-compatibility, 10 newly opened flowers were forcibly self-pollinated on each individual. Each individual's degree of self-compatibility was scored as the fraction of hand-self-pollinated flowers that produced fruit (Charlesworth and Yang, 1998). The rate of autonomous self-fertilization was scored as the fraction of undisturbed flowers that produced fruit in a pollinator-free greenhouse. Autonomous self-fertilization in this experiment does not exclude the possibility of agamospermy, or the production of seed through asexual reproduction.

Differences between races in floral traits were evaluated with univariate analyses of variance and Tukey's post-hoc comparisons (Sokal and Rohlf, 1995). To minimize the effect of multiple comparisons, I ran significance tests with an alpha-corrected Bonferroni level of $\alpha = 0.01$. The differences among populations within races were limited, so populations were grouped according to their race as originally designated by Lloyd (1965).

Reciprocal-transplant experiment

A reciprocal-transplant experiment was conducted using two central and two peripheral sites that support self-incompatible (Isbell, Waco; Table 1) and self-compatible plants (Morgan, Tuscumbia; Table 1), respectively. In December 2003, 250 seedlings from each of four populations were removed from their native sites and placed in flats of soil. Efforts were made to minimize variation among individuals in their number of leaves at the start of the experiment. Approximately 50 individuals from each population were then transplanted into linear transects at each experimental site on 13–15 December and marked with rust-resistant nails. In the early part of March, utility crews planned to destroy the Morgan experimental site. Plants at this site were transplanted 500 meters downstream of the natural drainage, into a site supporting *L. alabamica*. Plants were watered at this site for 1 week to ensure that they survived transplantation.

During March and April, floral traits associated with autonomous self-fertilization were measured on all experimental plants. The date of flowering was recorded as the first day an individual produced an open flower. Petal length was recorded to the nearest 0.01 mm with digital calipers. The degree of anther rotation was also scored. Differences between self-incompatible and self-compatible genotypes in the afore-mentioned floral

traits and time to flowering were evaluated within each site with t tests using a Bonferroni-corrected alpha level of $\alpha = 0.01$. The total number of flowers and fruits produced during the lifetime of plants was measured at the end of the flowering season. The proportion of flowers that produced fruit on each individual plant was then counted at each experimental site. At the end of the flowering season, the aboveground biomass of plants was harvested, placed in bags, and dried in an oven at 50°C for 1 week. The number of matured seeds and aborted seeds per fruit were counted for all fruits and averaged for each individual plant.

Fruit and seed set were measured only on transplanted plants in each population. Separate univariate analyses of variance (ANOVAs) were conducted on these measures of seed production: (1) arcsine square-root transformed fruit set and (2) number of matured seeds per fruit. In both ANOVAs, experimental site and self-incompatibility type (i.e., genotype) were fixed effects, and "days to flowering" was used as a continuously distributed covariate. Populations were pooled according to their self-incompatibility type because these populations were randomly sampled to reflect variation caused by the transition from self-incompatibility to self-compatibility, and the differences between populations were limited. A significant interaction between site and genotype would suggest that the benefits of self-compatibility are environment dependent. Significance tests of the difference between self-incompatible and self-compatible genotypes were conducted using a Tukey's post-hoc test (Sokal and Rohlf, 1995). In analyses with a significant site by genotype interaction, the differences between self-incompatible and self-compatible genotypes were evaluated separately within each site. A univariate

ANOVA was also conducted on the number of aborted seeds per fruit to determine if genotypes differed in their propensity to selectively abort developing offspring.

Sequencing neutral genetic diversity

Species of *Leavenworthia* possess only a single copy of the cytosolic phosphoglucose isomerase gene (Liu et al. 1999).Approximately 25 to 30 individuals from one self-incompatible and two self-compatible populations were sampled. Leaves from individual plants were flash frozen with liquid nitrogen, and genomic DNA was extracted using Qiagen DNeasy plant mini kits. An approximately 250-bp region corresponding to intron 12 was amplified using the plus primer AGTATGGCTTCTCCATGGTT (PGIC.P1) and a minus primer ATGTGGACTTGAAATGCTG (PGIC.2PR). Another pair of conserved primers was used to amplify an approximately 650-bp region containing several exons and introns 13, 14, and 15 of PgiC (PGI+14: AGGGAGCTTCAAGCATTGAT; PGI-4: TCGAACGGGAGAGGTAGACCA).

DNA was amplified using the PCR reaction conditions reported for intron 12 and introns 13–15 (Filatov and Charlesworth, 1999; Liu et al., 1999). Organic residues were removed from PCR products by using QIAquick PCR purification kits (Qiagen). DNA templates were cycle-sequenced in 10 µL reactions with 0.0002 µg of the forward primer (PGI.P1 or PGI+14), 1 µL v. 3.1 BigDye terminator ready reaction mix (Applied BioSystems, Foster City, California, USA) and 1.5 mM $MgCl_2$. The procedure consisted of 25 cycles each of 30 s at 96°C, 15 s at 50°C, and 4 min at 60°C. The samples were sequenced with an ABI 3730 automated sequencer (Applied Biosystems). Sequences were aligned in Sequencher v. 4.1 (Gene Codes Corp., Ann Arbor, Michigan, USA).

Individuals heterozygous at sites of insertion–deletion polymorphisms were excluded from analyses because of the inability to score nucleotides.

Haplotype structure was analyzed using Haplotyper software (Niu et al., 2002). Ambiguous base calls were corrected manually. The exons found in the region containing introns 13–15 were identified by alignment with an *L. crassa* mRNA (GenBank accession AF054455) and were removed before further analyses. In some cases, sequencing efforts in intron 12 and introns 13–15 utilized different individuals, so these regions were analyzed separately. The haplotypes sequenced in intron 12 and introns 13–15 are GenBank accessions AY745782–AY745805. Measures of sequence diversity were estimated using DNAsp v. 4.0.0.4 (Rozas et al., 2003). The number of segregating sites per site (p_s) and the populational heterozygosity, or nucleotide diversity per site (π) were calculated. Insertion-deletion polymorphisms were coded as single nucleotide polymorphisms. The variance in nucleotide diversity was directly estimated by sampling with replacement from the original dataset of sequences 500 times. Nucleotide diversity within populations was statistically compared, assuming that the divergence between populations is recent (Innan and Tajima, 2002). Tajima's tests were conducted to test the hypothesis that populations are not at equilibrium between mutation and genetic drift (Tajima, 1989a).

RESULTS

Variation in self-compatibility

Self-incompatible populations of *L. alabamica* are an order of magnitude larger than self-compatible populations ($t = 13.83$, df $= 8$, $P = 0.001$; Table 1). Although not statistically significant, self-incompatible populations also tend to have higher plant

density in comparison to self-compatible populations ($t = 1.586$, df = 8, $P = 0.151$; Table 1). Self-incompatible plants produce very few fruits autonomously in the greenhouse (<2% of all flowers) and have large flowers with extrorse anthers and high pollen to ovule to ratios (Table 2). Although the self-incompatibility reaction of the Brassicaceae is leaky, there are discrete differences in its prevalence between the races. Of the 86 plants from the self-incompatible race (a1) measured in the greenhouse, two individuals produced seed more than 80% of the time following self-pollination, whereas the majority of plants accepted self pollen less than 10% of the time. Therefore, the majority of plants sampled from the a1 race are self-incompatible, although there may be a low frequency of alleles conferring self-compatibility in these populations (2/86 = 0.023).

Populations at the periphery of the Moulton Valley are often found in glades that have experienced disturbance and are less suitable for the growth of *L. alabamica* (Table 1). In these peripheral environments, all plants are self-compatible, have smaller flowers with introrse anthers, lower pollen to ovule ratios, and produce many fruits autonomously (>31% of all flowers; Table 2). The a2 race is an exception to these general trends because plants of this race are capable of autonomous self-fertilization, yet retain large flowers and extrorse anthers. The Russellville and Tuscumbia races of *L. alabamica* are the most self-compatible, have the highest rates of autonomous self-fertilization, and the lowest pollen to ovule ratios (Table 2). The results of the greenhouse experiment strongly support previous work on this species that suggested that the evolution of self-compatibility is associated with a large number of floral adaptations to autonomous self-fertilization in geographically peripheral populations (Lloyd, 1965).

Reciprocal-transplant experiment

In all field sites, plants transplanted from self-compatible populations produced smaller flowers with more introrse anthers compared to plants transplanted from self-incompatible populations (Fig. 1a, b). In the peripheral site which had to be re-transplanted (Morgan), self-incompatible plants flowered earlier and had higher lifetime flower production in comparison to self-compatible genotypes. There were no significant differences between the genotypes at the remaining sites, which were not watered during the spring (Fig. 1c, d). There was a significant interaction between site and genotype on patterns of fruit set (Table 3). This interaction resulted from self-compatible genotypes having a significantly higher fruit set than self-incompatible genotypes in all sites except the peripheral Tuscumbia site (Fig. 2). There was not a significant interaction between site and genotype on patterns of seed production per fruit (Table 4), with self-compatible plants producing a greater number of seeds per fruit in all environments (Fig. 3). Overall, self-compatible genotypes produced 17–22% more seeds per fruit compared to self-incompatible plants in all environments. Although not statistically significant, there was also a trend for self-compatible plants to abort more seeds per fruit in all sites ($F_{1,479} = 3.422$, $P = 0.072$; Fig. 3).

Self-compatibility and sequence diversity

The self-incompatible Waco population had a nucleotide diversity of 1.1% within intron 12 and a nucleotide diversity of 1.8% within introns 13–15 (Table 5). In contrast, the self-compatible Morgan population was monomorphic over all intron sites and the self-compatible Tuscumbia population had a nucleotide diversity of 0.1% in the region containing intron 12 (Table 5). The self-incompatible Waco population had a significantly higher nucleotide diversity compared to both self-compatible populations

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within introns 13–15 ($G(0) = 3.177$, $P = 0.027$) and within intron 12 when compared to the Tuscumbia ($G(0) = 2.372$, $P = 0.041$) and Morgan population, respectively ($G(0) = 2.864$, $P = 0.034$).

In the sequence analysis within the self-incompatible Waco population, I found 11 and 10 haplotypes within introns 12 and introns 13–15, respectively. In contrast, the self-compatible Morgan population has a single intron 12 haplotype that is quite frequent in the self-incompatible Waco population (Table 6). The self-compatible Tuscumbia population possesses the same intron 12 haplotype at a relatively high frequency, but possesses two rare and unique haplotypes in this region. Both of the self-compatible populations were monomorphic for unique haplotypes in the region spanning introns 13–15 (Table 6). In all of the populations, the results of Tajima's tests of neutrality in both regions did not suggest recent changes in effective population size (Table 5). However, the power of this test to detect changes in population size is limited by the low number of polymorphisms observed in self-compatible populations of *L. alabamica* (Tajima, 1989a, 1989b).

DISCUSSION

The pattern of pollen limitation in the reciprocal-transplant experiment is not consistent with the hypothesis that pollinator limitation has favored the evolution of self-compatibility in *L. alabamica*. Pollen limitation of seed set appears to be relatively uniform across the geographical range of this species and unrelated to the appearance of self-compatible races with adaptations for self-fertilization. The spread of alleles conferring self-compatibility is likely driven by other ecological factors that limit population size, plant density, or pollen availability at the margins of the Moulton Valley.

Geographic variation in mating system

Self-incompatibility within the a1 race is caused by single-locus sporophytic self-incompatibility, which has been observed throughout the Brassicaceae and has also been shown to be the ancestral mating system in the genus *Leavenworthia* (Rollins, 1963; Lloyd, 1967; J. Beck, I. Al-Shehbaz, and B. Schaal, Washington University, unpublished data). Although alleles conferring self-compatibility are likely present at low frequency in central populations, natural selection has not caused their spread and fixation. Because complex genetically controlled self-incompatibility systems in plants are more easily lost through mutation than they are gained (Barrett et al., 1996; Kohn et al., 1996), it is likely that self-compatibility has evolved from the self-incompatible condition at the periphery of the species' range. The existence of two reproductive modes in *L. alabamica* also suggests that the loss of the sporophytic self-incompatibility mechanism and subsequent adaptations to self-fertilization may have occurred relatively recently. In support of this idea, a selective sweep favoring self-compatibility in *Arabidopsis thaliana* has likely occurred in the last 17,000 years, suggesting that floral adaptations to self-fertilization may evolve rapidly in the Brassicaceae (Shimizu et al., 2004).

The self-compatible races exhibit varying degrees of floral adaptation to autonomous self-fertilization. The a2 race has relatively large flowers, extrorse anthers, and an intermediate pollen to ovule ratio, whereas the remaining races (a4, Russellville and Tuscumbia) have the smallest flowers with introrse anthers and low pollen to ovule ratios. Once the genetically controlled self-incompatibility system was dissolved in peripheral populations of *L. alabamica*, natural selection likely favored adaptations for self-fertilization and reductions in investment in traits that serve to attract pollinators

(Cruden, 1977; Charlesworth and Charlesworth, 1981; Schueller, 2004). The retention of extrorse anthers and relatively large flowers in race a2 suggests that self-compatibility has evolved recently in this race or that it periodically experiences gene flow with the nearby self-incompatible populations (Lloyd, 1965). These results support findings in other systems of variation among recently derived self-compatible populations in their adaptations to autonomous self-fertilization (Rick et al., 1977, 1979; Barrett and Shore, 1987; Husband and Barrett, 1993; Johnston and Schoen, 1996).

Pollen limitation of seed production

In comparison to self-incompatible plants, self-compatible plants had consistently higher fruit and seed set in all environments. These results are counter to the hypothesis that reduced visitation by native pollinators may drive the evolution of self-fertilization at the periphery of the Moulton Valley in *L. alabamica* (Lloyd, 1965). Although pollen limitation of seed set has been documented in many flowering plant species (Burd, 1994), few studies have tested the hypothesis that lack of pollinators is the agent selectively favoring self-compatible or self-fertilizing plants (Inoue et al., 1996; Fausto et al., 2001; Goodwillie, 2001; Herrera et al., 2001; Elle and Carney, 2003; Schueller, 2004). Results from studies have supported the hypothesis that self-fertilization allows plants to produce extra seed when pollinator activity is low (Inoue et al., 1996; Fausto et al., 2001; Goodwillie, 2001; Elle and Carney, 2003; Davis and Delph, 2005), though other attempts have not found support for the idea that pollinator limitation drives the evolution of autonomous self-fertilization (Leclerc-Potvin and Ritland, 1994; Klips and Snow, 1997; Eckert and Schaefer, 1998; Herrera et al., 2001; Schueller, 2004).

It is possible that natural selection favors self-compatibility in peripheral environments during years of low pollinator activity, which would require multiple seasons of study to detect (Piper et al., 1986; Burd, 1994; Goodwillie, 2001; Herrera et al., 2001; Kalisz et al., 2004; Schueller, 2004). Based on the results of this experiment in a single year, self-incompatible genotypes experienced some pollen limitation of seed set in all environments. These results are perhaps not too surprising because this winter annual flowers during the cold temperatures of March and April, when the foraging activity of their insect pollinators may be limited (Rollins, 1963). The fact that self-compatibility becomes established in peripheral environments is most likely explained by the fact that reductions in population size or density may further limit seed production in a species with genetically controlled self-incompatibility (Byers and Meagher, 1992; Reinartz and Les, 1994; Fischer et al., 2003). Reductions in plant density may be particularly pronounced at the periphery of the Moulton Valley where the environment is less suitable for the growth of *Leavenworthia*. In support of this idea, the two highly self-compatible populations belonging to the a4 race became extinct during the period of study (J. Busch, personal observation). Catastrophic declines in population size have also been implicated in the evolution of self-pollination in *Clarkia xantiana*, an annual plant endemic to woodlands in the Sierra Nevada foothills (Moore and Lewis, 1965; Fausto et al., 2001; Moeller and Geber 2005).

Self-compatibility and sequence diversity

If peripheral populations have been small for long periods of time or undergo frequent bottlenecks in size, then these populations should be genetically depauperate. In this study, a self-incompatible population exhibited relatively high nucleotide diversity

$(1.1\% < \pi < 1.8\%)$, whereas two self-compatible populations maintained much less

genetic variation $(0 < \pi < 0.1\%)$. Since geographically central and peripheral populations

of *L. alabamica* simultaneously differ in size, persistence, and the maintenance of self-

incompatibility, it is hard to attribute reductions in sequence diversity within the self-

compatible populations to the effects of small size per se or increased rates of self-

fertilization (Schoen et al., 1996; Ingvarsson, 2002). Nevertheless, the nearly complete

loss of genetic variation in self-compatible populations of *L. alabamica* supports results

from other studies that have compared genetic diversity in closely related self-

incompatible and self-compatible taxa (Rick et al., 1977, 1979; Hamrick and Godt, 1996;

Savolainen et al., 2000; Charlesworth, 2003).

Sequence diversity within the one self-incompatible and two self-compatible

populations in this experiment is consistent with previous work on the intron 12 region of

PgiC in the genus *Leavenworthia* (Filatov and Charlesworth, 1999; Liu et al., 1999). The

species *L. crassa*, which is the sister taxon of *L. alabamica,* displays similar variation

among populations in the presence or absence of sporophytic self-incompatibility (Lloyd,

1965, 1967). Nucleotide diversities in self-incompatible populations of *L. crassa* range

from 0.5 to 1%, whereas three highly self-compatible populations were completely

monomorphic at intron 12 (Liu et al., 1999). Analyses of intron variation over a broad

2.3-kb region of the *PgiC* gene in the self-incompatible species *L. stylosa* found evidence

of long-term balancing selection, and relatively high nucleotide diversity near 5%

(Filatov and Charlesworth, 1999). In contrast, the close self-compatible relatives *L.*

torulosa and *L. uniflora* lack any within-population variation within intron 12 of *PgiC*

(Liu et al., 1999). These results suggest that the multiple independent derivations of self-

compatibility in the genus *Leavenworthia* are all associated with nearly complete reductions in sequence diversity within populations (Innan and Tajima, 2002; Charlesworth, 2003).

Conclusions

There has long been interest in understanding the ecological factors that favor the evolution of self-compatibility and adaptations to self-fertilization (Darwin, 1876; Stebbins, 1974), which is perhaps the most common transition observed in plants (Barrett et al., 1996; Kohn et al., 1996; Schoen et al., 1997; Barrett, 2002). One recurring theme in the evolution of inbreeding in plants is the association between isolated, marginal, island, or pollinator-poor environments with self-compatible mating systems (Baker, 1955, 1967; Lloyd, 1980; Schueller, 2004; Herlihy and Eckert, 2005). The results of this study suggest that consistent pollen limitation of seed set favors self-compatible genotypes in all populations of *L. alabamica.* Although self-compatibility is selectively favored in all environments, this mode of reproduction appears to invade and spread to high frequency only in peripheral and ecologically marginal environments, which support fewer individuals. These results support the idea that decreased population size or abundance in peripheral, island, or isolated environments may strongly favor the dissolution of genetically controlled self-incompatibility systems in flowering plants (Baker, 1955, 1967; Schoen and Brown, 1991; Pannell and Barrett, 1998).

TABLE 1. Populations of *Leavenworthia alabamica* used to study divergence in mating system. Mating system describes whether individual plants within the population are self-incompatible (SI) or self-compatible (SC). Races denote groups with varying levels of floral adaptations to self-fertilization (Lloyd, 1965). Underlined populations are sites used in the reciprocal-transplant experiment. Density and population size were estimated by quadrat sampling methods.

Population	Mating system	Lloyd race	Ecological description	Average density (individuals/m^2)	Population size
Hatton (H)	SI	a1	medium glade	100.9	25 500
Isbell (I)	SI	a1	medium glade	185.5	30 500
Newburg (N)	SI	a1	large glade	77.1	30 000
Tharptown (T)	SI	a1	glade bordering a farm	19.2	36 500
Waco (W)	SI	a1	large glade and field	94.2	32 500
Landersville (V)	SC	a2	disturbed roadside glade	8.4	2250
Lebanon (L)	SC	a4	farmfield	21.9	2000
Morgan (M)	SC	a4	disturbed roadside ditch	31.3	2200
Russellville (R)	SC	R	small elevated glade	30.0	300
Tuscumbia (Tu)	SC	Tu	disturbed roadside glade	120.7	6400

TABLE 2. Floral trait divergence among the races of *Leavenworthia alabamica*. *N* denotes the numbers of individuals measured in the greenhouse experiment. Races are based upon a historical investigation of the differences among populations in the degree of self-compatibility, associated floral traits, and vegetative morphology (Lloyd, 1965). Self-compatibility is the fraction of hand self-pollinated flowers producing fruit, and autonomy rate equals the fraction of flowers producing fruit in a pollinator-free greenhouse. Pollen to ovule ratio equals the average number of pollen grains per flower divided by the average number of ovules per flower within a race. Standard errors of estimates are reported and superscripts denote significant differences between races at the alpha = 0.01 level.

Lloyd race	N	Self-compatibility	Autonomy rate	Petal length	Stamen height	Pistil height	Anther rotation	Pollen /flower	Ovule /flower	Pollen:ovule ratio
a1	86	$0.06^a \pm 0.014$	$0.02^a \pm 0.006$	$12.62^a \pm 0.144$	$6.45^a \pm 0.060$	$5.65^a \pm 0.075$	$135.00^a \pm 2.354$	$47172^a \pm 1084$	$10.26^a \pm 0.213$	4600
a2	18	$0.67^b \pm 0.084$	$0.33^b \pm 0.048$	$11.58^b \pm 0.311$	$6.46^a \pm 0.237$	$5.98^a \pm 0.296$	$127.50^a \pm 9.095$	$28974^b \pm 1628$	$11.78^{ab} \pm 0.558$	2460
a4	29	$0.68^b \pm 0.046$	$0.35^b \pm 0.036$	$9.15^c \pm 0.106$	$5.74^b \pm 0.061$	$4.66^b \pm 0.072$	$86.90^c \pm 3.823$	$19874^c \pm 760$	$9.38^c \pm 0.287$	2120
Russellville	21	$0.94^{bc} \pm 0.029$	$0.71^c \pm 0.051$	$9.12^c \pm 0.158$	$5.67^b \pm 0.067$	$4.75^b \pm 0.110$	$100.71^b \pm 6.136$	$16151^c \pm 1005$	$8.43^c \pm 0.559$	1920
Tuscumbia	17	$0.81^{bc} \pm 0.042$	$0.47^b \pm 0.057$	$9.41^c \pm 0.165$	$5.96^{ab} \pm 0.063$	$4.88^b \pm 0.094$	$76.76^b \pm 5.126$	$17412^c \pm 1096$	$10.47^a \pm 0.322$	1660

TABLE 3. Univariate ANOVA describing variation in fruit set ($R^2 = 0.156$) in

Leavenworthia alabamica. Fruit set equals the proportion of flowers that produced

fruit in natural populations. Fruit set values were arcsine square-root transformed for

analyses. Genotype refers to whether plants were self-incompatible or self-

compatible.

Source	Type IV sum of squares	df	Mean square	*F*	*P*
Model	25.22	8	3.15	12.74	<0.001
Intercept	19.88	1	19.88	80.32	<0.001
Days to flower	4.85	1	4.85	19.59	<0.001
Site	3.96	3	1.32	5.34	<0.001
Genotype	15.10	1	15.10	61.03	<0.001
Site × genotype	2.17	3	0.73	2.93	0.033
Error	136.12	550	0.25		

TABLE 4. Univariate ANOVA describing variation in the number of matured seeds per fruit in *Leavenworthia alabamica* ($R^2 = 0.365$). Genotype refers to whether plants were self-incompatible or self-compatible.

Source	Type III sum of squares	df	Mean square	*F*	*P*
Model	342.93	8	42.87	34.28	<0.001
Intercept	106.32	1	106.32	85.02	<0.001
Days to flower	32.77	1	32.77	26.21	<0.001
Site	323.37	3	107.79	86.20	<0.001
Genotype	40.82	1	40.82	32.64	<0.001
Site × genotype	4.34	3	1.45	1.16	0.326
Error	597.71	478	1.25		

TABLE 5. Nucleotide variation in introns of *PgiC* within self-incompatible and self-compatible populations of *Leavenworthia alabamica*. Mating system denotes whether populations are self-incompatible (SI) or self-compatible (SC).

[a] number of individuals sequenced per region

[b] number of segregating sites

[c] nucleotide diversity ±1 standard deviation

[d] Tajima's test statistic of neutrality (see Materials and Methods: sequencing neutral genetic diversity for description).

Population	Mating system	*PgiC* intron 12 (211 sites)					*PgiC* introns 13–15 (465 sites)				
		N^a	S^b	π^c	Tajima's D^d	P	N^a	S^b	π^c	Tajima's D^d	P
Waco	SI	22	9	0.011±0.0008	0.026	>0.10	14	24	0.018±0.0026	0.464	>0.10
Morgan	SC	28	0	0	n/a	n/a	27	0	0	n/a	n/a
Tuscumbia	SC	25	2	0.001±0.0005	−1.513	>0.10	29	0	0	n/a	n/a

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TABLE 6. Haplotype structure in populations of *L. alabamica*. Haplotypes within intron 12 and introns 13–15 were identified through independent investigation. A period ('·') denotes nucleotide identity with the topmost haplotype in a region. Nucleotide substitutions are denoted by alternate bases, and insertions (i) and deletions (d) are also shown. '*f*' represents the frequency of a haplotype within each population.

PgiC intron 12 (211 sites)

Population	23	45	61	72	89	91	92	97	165	192	211	*f*
Waco (SI)	G	T	G	G	T	A	A	T	T	G	A	0.204
	.	.	.	.	.	d	.	T	.	.	.	0.318
	.	.	.	.	.	.	.	.	.	A	.	0.114
	.	.	.	.	.	.	.	T	.	A	.	0.114
	.	.	.	.	.	d	.	.	.	.	.	0.045
	.	.	C	.	d	.	T	.	.	.	.	0.045
	.	.	.	.	.	.	.	G	.	.	.	0.045
	.	.	A	d	.	T	.	C	.	.	.	0.045
	.	.	.	.	.	T	.	.	.	.	.	0.027
	.	i	.	.	.	.	.	.	.	.	.	0.027
	.	i	.	.	.	.	.	G	.	.	.	0.027

PgiC introns 13–15 (465 sites)

56	102	123	125	126	137	149	201	205	221	227	228	252	270	281	293	300	304	320	329	343	347	358	370	393	398	*f*
T	T	T	C	T	G	C	T	T	G	T	T	T	C	C	C	C	T	C	G	T	A	T	A	G	A	0.500
.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	T	T	C	T	.	.	.	G	.	.	.	0.107
i	i	i	.	.	G	.	.	G	.	A	.	C	.	A	.	.	C	T	.	.	G	.	G	A	.	0.071
i	i	i	.	.	G	.	.	G	.	A	.	C	.	A	.	.	C	T	.	.	G	.	G	A	G	0.071
.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.071
.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	0.036
i	i	i	A	.	.	.	.	.	A	d	C	.	T	A	.	.	C	T	.	.	G	.	G	A	.	0.036
i	i	i	A	.	.	.	.	.	A	d	C	.	T	A	.	.	C	T	.	.	G	.	G	A	C	0.036
i	i	i	.	d	.	T	.	i	A	d	C	.	T	A	.	.	C	T	.	C	G	.	.	A	.	0.036
i	i	i	.	d	.	T	.	i	A	d	C	.	T	A	.	.	C	T	.	C	G	.	.	A	.	0.036

Morgan (SC)	1.00	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	i	.	.	.	.	1.00
Tuscumbia (SC)	0.92	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	i	.	.	.	.	1.00
	0.04	i	.	.	.	.	.	.	.	.	.	.									
	0.04	.	.	.	.	.	.	.	.	.	.	i									

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Figure 1. Floral traits of self-incompatible and self-compatible genotypes in the reciprocal-transplant experiment. (a) Petal length of the first flower (mm). (b) Degree of anther rotation away from the pistil. (c) Number of days from transplantation to the opening of the first flower. (d) Number of flowers produced over the lifetime of a plant. * denotes a significant difference between genotypes at the $\alpha = 0.01$ level.

Figure 2. Fruit set of self-incompatible and self-compatible genotypes in the reciprocal-transplant experiment. Fruit set values equal the percentage of flowers that matured into fruit. Error bars denote 1 standard error, and * denotes a significant difference between genotypes at the $\alpha = 0.01$ level.

Figure 3. Seed set of self-incompatible and self-compatible genotypes in the field experiment. Bars represent estimated marginal means. Dark portions of the stacked bars represent the average number of matured seeds per fruit, whereas light bars represent the number of aborted seeds per fruit. Self-compatible genotypes matured more seeds ($P < 0.001$; $N = 487$) and showed a trend for greater seed abortion ($P = 0.072$; $N = 489$).
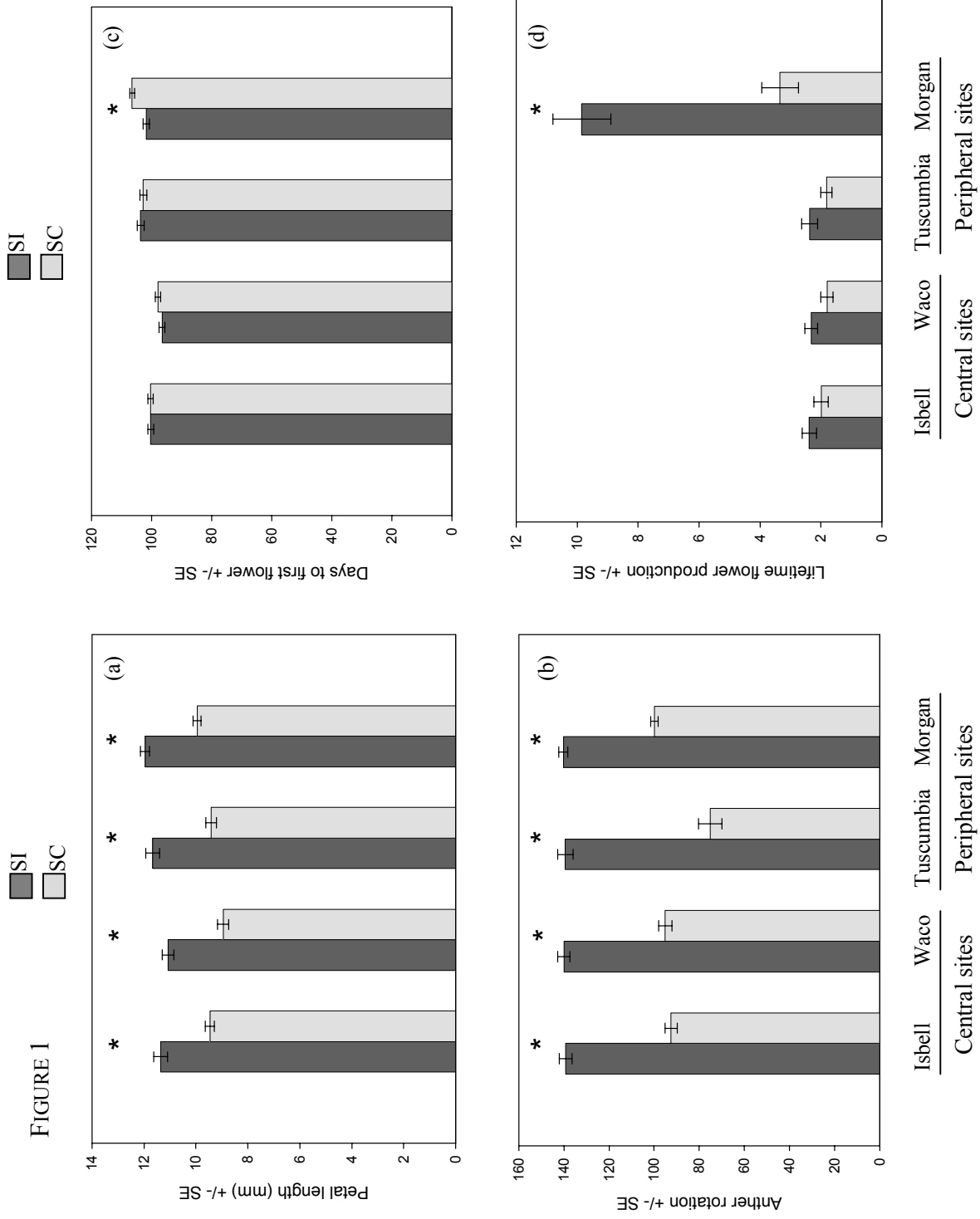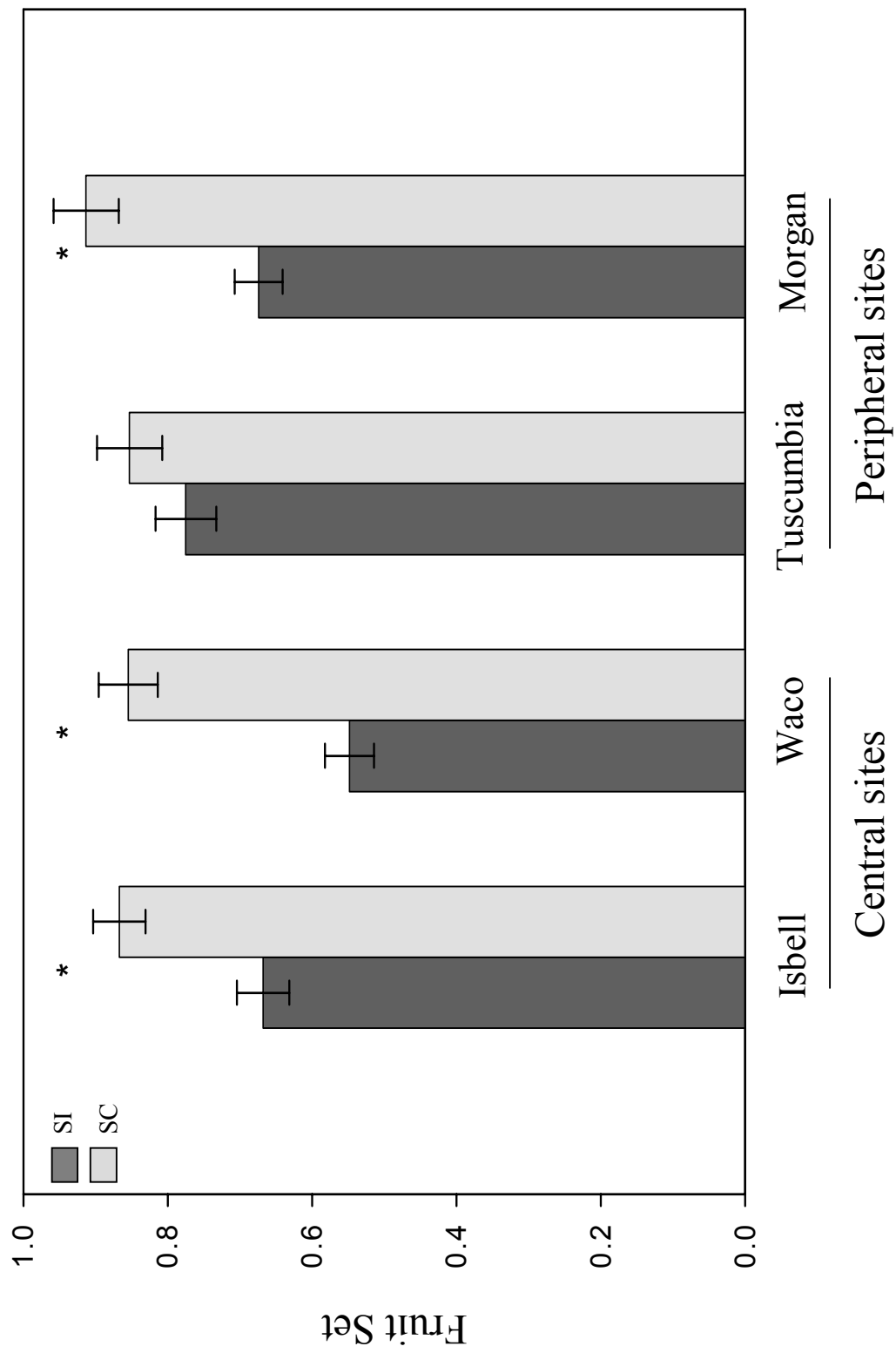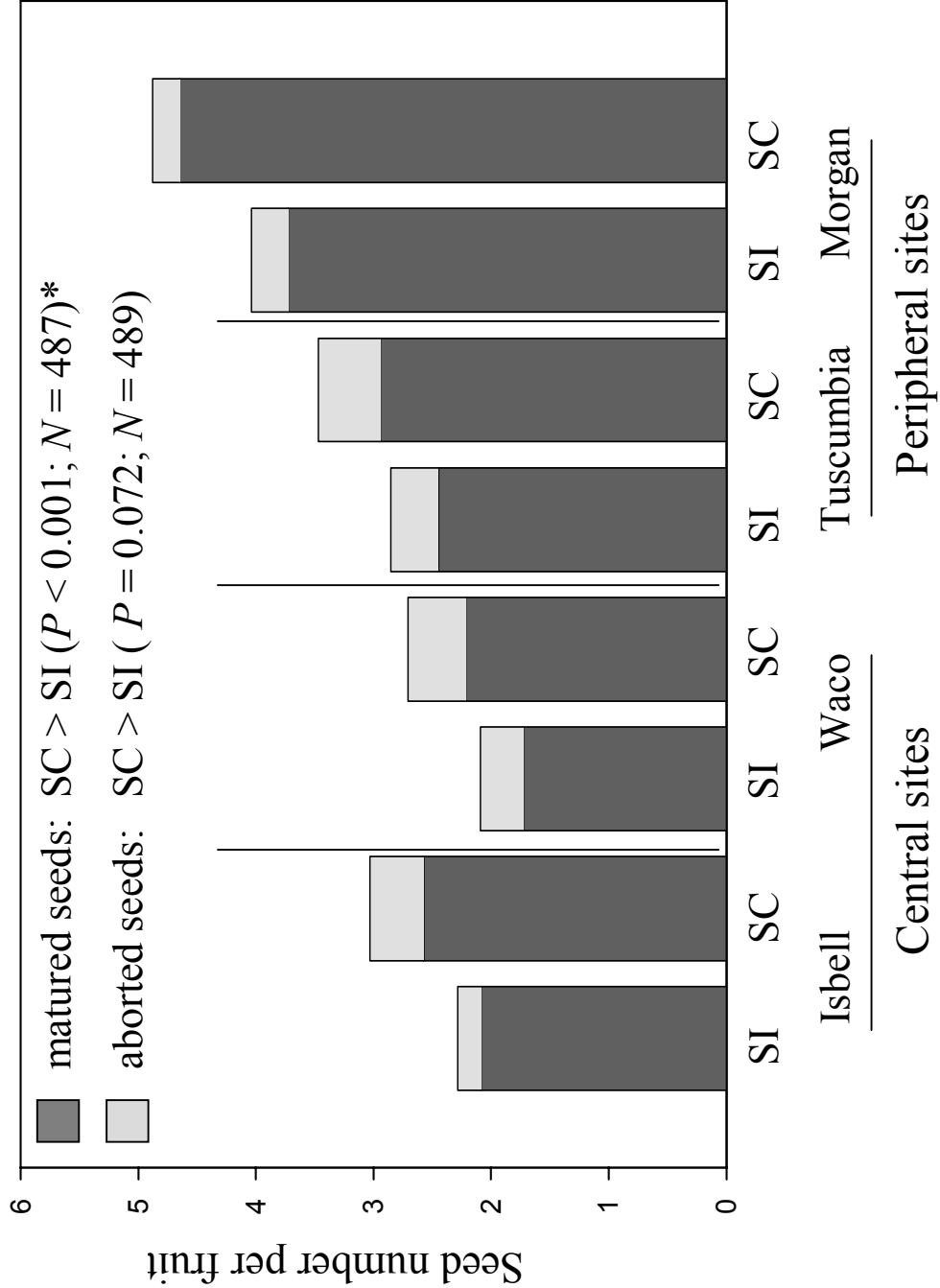
FIGURE 1

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FIGURE 2

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FIGURE 3

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CHAPTER 3

INBREEDING DEPRESSION IN SELF-INCOMPATIBLE AND SELF-COMPATIBLE POPULATIONS OF

LEAVENWORTHIA ALABAMICA

ABSTRACT

Inbreeding depression is one of the leading factors preventing the evolution of self-fertilization in plants. In populations where self-fertilization evolves, theory suggests that natural selection against partially recessive deleterious alleles will reduce inbreeding depression. The purpose of this study was to evaluate this hypothesis by comparing the magnitude of inbreeding depression in self-incompatible and self-compatible populations of *Leavenworthia alabamica*. Within-population crosses were conducted to compare the quantity and quality of offspring produced by outcrossing and self-fertilization. These progeny were grown in a common greenhouse and inbreeding depression was measured in germination, survival, biomass, transition rate to flowering, flower number, petal length, pollen grains/anther, pollen viability, and ovule number.

In comparison to outcrossing, self-fertilization led to the production of fewer and smaller seeds within self-incompatible populations. Moreover, inbreeding depression was observed in 8 of 11 offspring traits within self-incompatible populations of *L. alabamica*. In contrast, there was significant inbreeding depression only in flower number within self-compatible populations. The results of this study are consistent with the idea that self-fertilization selectively removes partially recessive deleterious alleles causing inbreeding depression in natural plant populations. However, in plant species such as *L. alabamica* where self-compatibility may evolve in small populations following long-distance dispersal, declines in inbreeding depression may also be facilitated by genetic drift.

Keywords: deleterious mutations, genetic drift, genetic load, purging, self-fertilization.

Self-fertilization has an innate transmission advantage that should lead to the elimination of cross-fertilization because selfing individuals pass on an extra copy of their chromosomes through seeds (Fisher 1941). However, the initial spread of genes for self-fertilization can be halted by inbreeding depression, or the lowered viability and fecundity of selfed plants (Lloyd 1979; Lande and Schemske 1985; Charlesworth et al. 1990; Jarne and Charlesworth 1993). Genetic models have explored the conditions favoring the spread of genes for self-fertilization in cross-fertilizing populations. In general, rates of self-fertilization will increase in populations whenever selfed plants are at least half as fit as outcrossed plants, if genetic associations between fitness loci and selfing-rate modifier loci are not considered (Lloyd 1979; Lande and Schemske 1985; Campbell 1986; Charlesworth et al. 1990; Uyenoyama and Waller 1991a, 1991b).

Evidence suggests that inbreeding depression is caused mainly by the expression of partially recessive deleterious alleles in the homozygous state (Dudash and Carr 1998; Charlesworth and Charlesworth 1999; Willis 1999). The evolution of self-fertilization should lower the equilibrium frequency of lethal and sublethal mutations that are nearly recessive because these alleles are exposed to natural selection in the homozygous state (Lande and Schemske 1985; Charlesworth et al. 1990; Byers and Waller 1999). This hypothesis has been tested by forcibly self-fertilizing naturally outcrossing families of plants for several generations (Barrett and Charlesworth 1990; Carr and Dudash 1997; Dudash et al. 1997; Willis 1999) or by comparing populations or lineages of plants that have divergent rates of self-fertilization in natural populations (Holtsford and Ellstrand 1990; Carr and Dudash 1996; Johnston and Schoen 1996; Fishman 2001; Takebayashi and

Delph 2001). Results of these studies suggest that inbreeding depression may be reduced by self-fertilization, although purging may not always occur in response to periods of inbreeding (Byers and Waller 1999).

Leavenworthia alabamica is an ideal species in which to test the idea that self-fertilization reduces inbreeding depression in natural plant populations (Lloyd 1965). In this species, there is variation among populations in the prevalence of self-incompatibility. In particular, self-incompatibility predominates at the center of the species range and self-compatibility occurs in smaller populations at the border of the species range (Lloyd 1965). Individuals within self-compatible populations produce smaller flowers, have lower pollen to ovule ratios, and produce a greater proportion of fruit through autonomous self-fertilization (Lloyd 1965; Busch unpublished data). There has likely been a history of natural selection in these populations to circumvent the ancestral sporophytic self-incompatibility reaction found throughout the Brassicaceae and the genus *Leavenworthia* (Bateman 1955; Lloyd 1967). The purpose of this experiment was to test the hypothesis that the evolution of self-compatibility in *L. alabamica* is associated with reductions in the genetic load causing inbreeding depression.

MATERIALS AND METHODS

Study System

Leavenworthia alabamica Rollins (Alabama glade cress; Brassicaceae) is a winter annual endemic to the limestone cedar glades of the Moulton Valley in northern Alabama (Rollins 1963). These cedar glades are typified by exposed and slowly eroding beds of limestone that are covered with a thin and moist layer of soil (Baskin et al. 1995). Seed germination occurs in the late fall following a long period of summer desiccation required

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to break dormancy. Flowering occurs from early March until the middle of April. Seeds

mature within siliques on maternal plants in the early summer. There is likely limited

pollen dispersal between populations because of the short flight distances of native

pollinators (*Andrena spp.* and *Halictus ligatus*) and the relatively large distances between

cedar glades. Seed dispersal is greatly limited because seeds are passively dispersed

following maturation. Nevertheless, the most likely form of gene flow between

populations is thought to occur following the dispersal of seeds by rare flooding events

(Lloyd 1965).

Seed Collection and Growth of Parental Plants

Attempts were made during the spring of 2002 to locate the naturally occurring

populations described by Lloyd (1965). Populations with high rates of autonomous self-

fertilization, small petal size, and low pollen number should have long histories of

inbreeding (Byers and Waller 1999). In this study, I collected seed from five large, cross-

fertilizing populations: *Hatton* (34.50993^{o} N, 87.44204^{o} W)*, Isbell* (34.45725^{o} N,

87.75298^{o} W)*, Newburg* (34.47049^{o} N, 87.57300^{o} W)*, Tharptown* (34.59327^{o} N,

87.57198^{o} W)*, Waco* (34.47866^{o} N, 87.62746^{o} W) and five small, self-fertilizing

populations: *Huckaby Bridge* (34.34532^{o} N, 86.95074^{o} W)*, Landersville* (34.44783^{o} N,

87.39458^{o} W)*, Lebanon* (34.35911^{o} N, 86.97309^{o} W)*, Russellville* (34.53898^{o} N,

87.66992^{o} W)*,* and *Tuscumbia* (34.70833^{o} N, 87.83132^{o} W); (see Lloyd 1965 for details).

The populations used in this study were sampled from the entire species range of *L.*

alabamica and represent all of the known variation in inbreeding. Approximately two

siliques (6-26 seeds) were collected from 50 randomly chosen maternal plants in each of

the ten populations and placed in single envelopes. However, in the very small *Russellville*

population, seeds were only collected from 25 maternal plants in order to assure that my efforts did not drive this isolated population closer to extinction. These seeds were later grown in a greenhouse and used as parents in a crossing design.

Seed envelopes were placed in a 30° C oven for five days and then stored at 22° C for one month to break seed dormancy. Seeds from each maternal family were placed on 4.25 cm diameter filter paper and placed within small petri plates. These dishes were maintained in an incubator with 14 hour days (15° C) and 10 hour nights (12° C), and were moistened daily with 3 mL of autoclaved water. These plants were then grown in a common greenhouse to minimize any potential environmental maternal and paternal effects that would influence offspring performance. Seedlings were transplanted into three inch pots containing a 1:1 ratio of MetroMix (Scotts-Sierra Horticultural Products, Marysville, OH) and sterilized soil following the emergence of both cotyledons. Plants received 14 hours of artificial light in the Indiana University greenhouse to promote flowering.

Crossing Design and Greenhouse Experiment

Plants were used as parents in a crossing design to quantify the fitness effects of outcrossing and selfing. Outcrossed and selfed offspring were generated by outcrossing and forcibly self-fertilizing plants in all populations, respectively. Outcrossed offspring were produced by using randomly chosen pollen donors from within populations. To successfully produce selfed offspring, it was necessary to circumvent the self-incompatibility system active in some populations through bud-pollination. By forcing styles to accept pollen before flowers open, bud-pollinations allow self pollen tubes to evade detection by maternal style proteins that normally inhibit the germination of related

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pollen grains after flowers have opened (Bateman 1955). Bud-pollinations were used in all crosses to ensure that flower damage would not be responsible for the reduced performance of self-fertilized offspring (Cabin et al. 1996). Overall, a total of 435 families were generated by outcrossing and selfing.

Each morning, flowers with visible petal emarginations were chosen for bud-pollinations. All of the sepals, petals, and anthers were then removed with forceps from the bud of the ovule parent. Pollen parents were selected haphazardly, with the restriction that each plant was used as a pollen donor no more than three times. Following pollen transfer, the date of bud-pollination and the identity of the pollen donor were recorded for each flower. Marked fruits were allowed to mature for 1 month and were then collected for storage. These seeds were put in envelopes, placed in a 30° C oven for one week, and then stored at 22° C for three months to ensure that seeds broke their natural dormancy. The number of seeds produced by outcrossing and self-fertilization were counted and weighed to the nearest 0.001 mg.

Families of seeds from each cross combination were given a random number to ensure that biases could not influence the measurement of performance traits. Seeds were germinated during March of 2003 as described for parental plants. Dates of germination were recorded on a daily basis within all families. When germination rates stopped increasing within families, seedlings were selected for transplantation. To avoid potential biases in selection of plants, the three most centrally located seedlings within a petri plate were chosen, although there were often less than three germinated seeds in a family. In such situations, every seedling was transplanted. Seedlings were placed in 3 inch pots

containing a 1:1 mixture of MetroMix and sterilized soil. Plants were watered twice daily and received more than 14 hours of natural light to promote flowering.

Survival was recorded daily while plants matured in the greenhouse. The date of flowering was recorded as the day when the first flower opened on a plant. Days to flowering was measured as the number of days between germination and the opening of the first flower. The few plants that failed to successfully make the transition from the vegetative rosette to flowering were recorded as producing zero flowers. The number of flowers produced in the first two weeks of the flowering period was recorded for all individuals. Flowers were removed on a daily basis to ensure they were not counted more than once. At the end of the first month of flowering, the aboveground biomass of plants was harvested, excluding flowers and fruits. Plants were placed in envelopes, dried in a 60° C oven for two weeks, and weighed to the nearest 0.001 g.

All floral traits were measured on the third flower produced by plants. Petal length was measured with digital calipers to the nearest 0.01 mm. Pollen was collected in two ways to determine the quantity and quality of male gametes. Pollen was collected in the morning immediately following anther dehiscence. One of the four paired anthers was placed in a vial for pollen counting, while another was used for viability estimation. The number of pollen grains was estimated by using the Elzone 280 © automated particle counter. Pollen viability was estimated through the use of Alexander's stain, which colors viable pollen grains red while leaving inviable pollen grains blue (Kearns and Inouye 1993). Viability scores were carried out by tapping single anthers onto plain glass slides three times to release adequate pollen grains. Using a medicine dropper, about 10 μL of Alexander's stain was added to the slide and a cover slip was placed over the sample.

Samples were then briefly warmed over a Bunsen burner flame to catalyze the coloring of viable pollen grains. Counts of inviable and viable pollen grains were performed using a light microscope. For each sample, at least several hundred pollen grains were counted to ensure that this trait was accurately measured. Female gametes were measured by cutting open the pistil with a dissecting needle and counting the number of enlarged ovules under a dissecting microscope.

Estimates of Lifetime Male and Female Fitness

The values for traits measured on all the individuals of a family were combined to generate family averages. Although many traits describe aspects of phenotype (e.g. biomass), some traits are direct components of survival and reproduction (e.g. flower number). It is possible to combine these traits to generate estimates of lifetime male and female fitness for families (e.g. Willis 1999). Male gamete production was calculated as follows: (germination rate) *x* (survival rate) *x* (flower number) *x* (pollen number/anther) *x* (% pollen viability). As a consequence, this composite value estimates the number of viable pollen grains produced, on average, over the lifetime of a plant. In a similar fashion, estimates of female gamete production were obtained for plants: (germination rate) *x* (survival rate) *x* (flower number) *x* (ovule number/flower). This value is a measure of the number of ovules produced over the lifetime of a plant.

Inbreeding Depression Estimates

There was often inadequate germination within families and populations of both types of progeny (i.e. outcrossed and selfed) to accurately estimate lineage or population specific measures of inbreeding depression. However, it was possible to pool populations according to their mating system to determine the effect of long-term inbreeding on fitness.

Therefore, outcrossed (w_o) and selfed (w_s) averages for all traits were pooled within self-incompatible and self-compatible populations, respectively. Inbreeding depression was calculated as $1 - (w_s/w_o)$ when selfed offspring had lower trait values than outcrossed progeny. In contrast, inbreeding depression was calculated as $(w_s/w_i) - 1$ when trait values of selfed plants exceeded that of outcrossed individuals (Agren and Schemske 1993). In all cases except for the trait 'days to flowering,' larger values translate into greater levels of performance. For this trait, longer times to flowering translate into fewer reproductive opportunities within natural populations (O'Neil 1997). Consequently, values of this trait were inversely transformed to produce estimates of 'transition rate to flowering.' This transformed trait describes the rate at which plants make the transition from the vegetative to the reproductive state.

Predictions and Analyses

Self-fertilization should strongly influence inbreeding depression because it exposes a greater fraction of partially recessive deleterious alleles to natural selection in the homozygous state. Therefore, there are two *a priori* predictions for the magnitude of inbreeding depression observed in populations: 1) self-incompatible populations should have inbreeding depression for most traits; and 2) self-compatible populations should have relatively low levels of inbreeding depression because deleterious alleles have been purged by natural selection. Consequently, I evaluated the null hypothesis of no difference between outcrossed and selfed trait averages independently within these two types of populations using planned comparisons (Sokal and Rohlf 1995). The means of outcrossed and selfed trait averages were computed and compared using t-tests with one-sided significance tests. Although most traits were normally distributed, several traits had to be

analyzed using non-parametric methods because of their highly skewed distributions towards lower values. In particular, comparisons of outcrossed and selfed means for germination rate, survival rate, lifetime male fitness, and lifetime female fitness were conducted using Mann-Whitney U test for difference in rank location (Sokal and Rohlf 1995). As for the parametric tests, the null hypotheses were evaluated with one-sided tests of significance. Analyses were conducted in SPSS (version 11.5; Norusis 2000).

RESULTS

Within self-incompatible populations, self-fertilized seeds were smaller (mass = 1.22 mg) compared to outcrossed seeds (mass = 1.34 mg; Table 1). Self-fertilization also led to the production of 48% fewer seeds in these populations (Table 1). Self-incompatible populations exhibited significant inbreeding depression in germination rate, biomass, flower number, petal length, pollen viability, and lifetime male and female fitness (Figure 1; Table 2; Table 3). There was also marginally significant inbreeding depression for survival rate in these populations (t = 1.535; df = 150; P = 0.063). Inbreeding depression values were positive for all traits with the exception of pollen grains/flower, which was slightly negative (δ = -0.02; Table 2). There were large amounts of inbreeding depression in both male and female fitness (δ = 0.54). The only traits for which there was not significant inbreeding depression in self-incompatible populations were transition rate to flowering, pollen number per anther, and ovule number per flower.

There were no significant differences in the size or number of seeds produced by outcrossing or self-fertilization in self-compatible populations (Table 1). Within self-compatible populations, there was marginally significant inbreeding depression (δ = 0.14) in flower number caused by forced self-fertilization (t = 1.583; df = 112; P = 0.058; Table

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2). Of the remaining ten traits, observed inbreeding depression values were positive for petal length, pollen viability, ovule number per flower, and estimates of male and female fitness ($0.01 < \delta < 0.15$). In contrast, germination rate, survival rate, biomass, flowering rate, and pollen number per anther exhibited slightly negative values of inbreeding depression ($-0.02 < \delta < -0.01$; Table 2).

DISCUSSION

Reductions in the quantity and quality of inbred offspring may prevent the evolution of self-fertilization in self-incompatible populations of *L. alabamica*. In these populations, 48% fewer seeds were produced by self-fertilization (Table 1), and there was significant inbreeding depression for 8 of the 11 traits (Table 2). The relatively large 54% declines in male and female fitness caused by self-fertilization are consistent with the idea that inbreeding depression may generate and maintain genetically controlled self-incompatibility systems in plants (Charlesworth and Charlesworth 1979; Lande and Schemske 1985; Charlesworth 1988; Lloyd 1992). In contrast, inbreeding depression was detected for only a single trait in self-compatible populations (Table 2). These results are consistent with a body of data suggesting that self-fertilization purges the genome of strongly deleterious, nearly recessive mutations (Barrett and Charlesworth 1990; Johnston and Schoen 1996; Carr and Dudash 1997; Dudash et al. 1997; Willis 1999). The fact that inbreeding depression for flower number is present in self-compatible populations is also consistent with the idea that purging should preferentially occur for traits expressed early in life (Husband and Schemske 1996).

In *L. alabamica*, the large values of inbreeding depression observed within the large and self-incompatible populations at the center of the species range may help

counteract the transmission advantage enjoyed by rare self-compatible genotypes (Charlesworth and Charlesworth 1979; Uyenoyama et al. 1993). Interestingly, natural selection favoring the transmission of self-compatible genotypes may be enhanced by the benefits of reproductive assurance in populations at the border of the species range (Barrett et al. 1989; Johnston 1998; Fausto et al. 2001; Fishman 2001). In particular, previous work has shown that the appearance of self-compatibility within *L. alabamica* is often associated with smaller and more marginal glade sites that receive fewer visits by native pollinators (Lloyd 1965; Solbrig and Rollins 1977). In these environments where opportunities for cross-fertilization may be limited, self-compatible genotypes may have been favored because of their ability to produce seed autonomously (Baker 1955, 1967; Lloyd 1979; Barrett et al. 1989; Burd 1994; Goodwillie 2001). If reproductive assurance has favored the evolution of self-compatibility at the border of the species range in *L. alabamica*, then reductions in inbreeding depression may likely be caused by the selective removal of partially recessive deleterious alleles in these environments.

Reduced inbreeding depression within self-compatible populations is also consistent with the action of genetic drift in small and/or frequently bottlenecked populations (Wang et al. 1999; Bataillon and Kirkpatrick 2000; Kirkpatrick and Jarne 2000). If the alleles causing inbreeding depression are strongly deleterious, they will be maintained at extremely low frequencies by natural selection, and thus may be easily lost by drift in effectively small populations that have undergone long-distance dispersal or recurrent extinction-recolonization (Barrett et al. 1989; Richards 2000; Carr and Dudash 2003). In *L. alabamica*, self-compatible populations may have been established by rare long-distance seed dispersal, and field observations suggest that self-compatible

populations go extinct at higher rates (Lloyd 1965; Busch, personal observation). Consequently, it is likely that both natural selection and genetic drift reduce inbreeding depression in the self-compatible populations of *L. alabamica*, though the results of this study cannot determine which force has played a more significant role. Nevertheless, genetic drift has been repeatedly implicated as a major force responsible for reductions in the number of selectively neutral polymorphisms within self-fertilizing populations (Schoen and Brown 1991; Hamrick and Godt 1996; Charlesworth 2003).

A previous study measured inbreeding depression within highly self-compatible populations of the species *Leavenworthia uniflora* and *Leavenworthia crassa* (Rollins 1963; Charlesworth et al. 1994). Comparisons between outrossed and selfed progeny in these species suggested a high level of inbreeding depression. In particular, there were 30-41% and 27-49% reductions in total lifetime fitness caused by self-fertilization in *L. uniflora* and *L. crassa*, respectively. These levels of inbreeding depression are similar to those observed within the self-incompatible populations of *L. alabamica*, though they are much higher than those observed within self-compatible populations of this species (Table 2). The fact that *L. uniflora* and *L. crassa* have higher genetic loads compared to the self-compatible populations of *L. alabamica* may suggest that self-fertilization influences inbreeding depression to different extents in closely related plant species (Byers and Waller 1999).

Alternatively, larger estimates of inbreeding depression within the more highly self-fertilizing species *L. uniflora* and *L. crassa* may be the result of differences in the harshness of experimental conditions between studies (Charlesworth et al. 1994). In particular, inbreeding depression was measured within a competitive environment in the

experiments with *L. uniflora* and *L. crassa*, which generally increases the differences in performance between outcrossed and selfed progeny (Dudash 1990; Wolfe 1993; Charlesworth et al. 1994). In light of the ecologically similar habitats experienced by the extant species of *Leavenworthia* (Rollins 1963; Lyons and Antonovics 1991), this issue may be addressed by measuring inbreeding depression in natural populations of self-incompatible and self-compatible taxa. Estimates of inbreeding depression taken from natural populations are required in this and other model systems in order to further understand the effect of deleterious genetic variation on the evolution of plant mating systems (Barrett and Harder 1996; Byers and Waller 1999).

Table 1. The number and mass of seeds produced by outcrossing and self-fertilization in self-incompatible (SI) and self-compatible (SC) populations. All crosses were conducted by using bud pollinations. N represents the number of outcrossed and selfed families used in analyses. Asterisks denote significant differences between selfed and outcrossed seeds (* p < 0.05; *** p < 0.001).

Seed trait	Mating system	Outcrossed	Selfed	t-statistic	N
Seed number/silique	SI	5.04	2.63	12.564***	242
	SC	7.10	7.30	0.777	195
Seed mass (mg)	SI	1.34	1.22	2.012*	241
	SC	1.20	1.28	1.424	195

Table 2. Inbreeding depression in self-incompatible (SI) and self-compatible (SC) populations. Orthogonal contrasts were used to test the one-sided hypothesis that outcrossed offspring have higher trait values than selfed offspring ([+] $p = 0.063$; [++] $p = 0.058$; * $p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$). [†]For traits with non-normal distributions, test statistics were calculated from Mann-Whitney U tests of rank difference. N represents the number of families used to generate means.
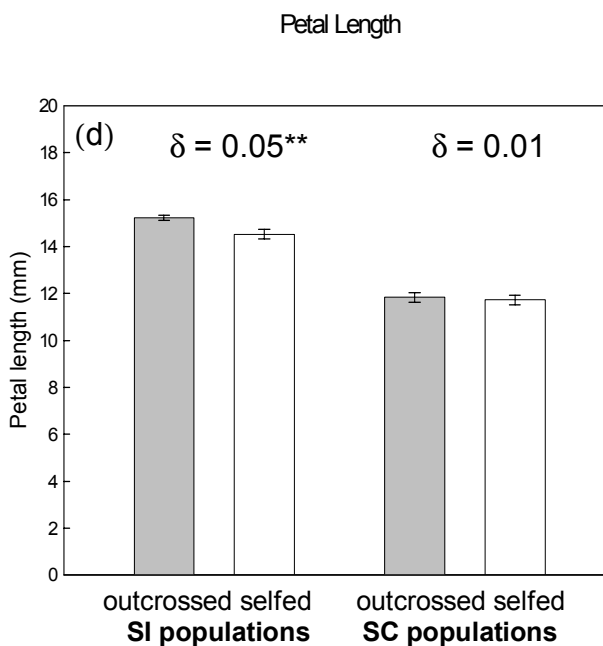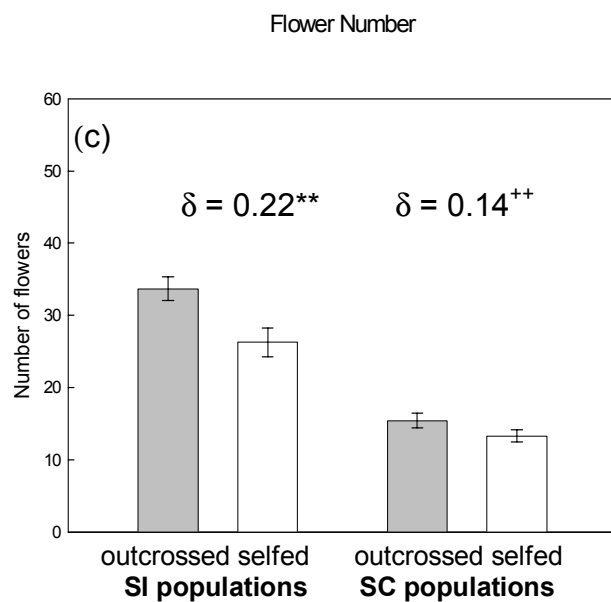
Trait	Mating system	δ	Test statistic	N
Germination rate	SI	0.37**	2.929[†]	240
	SC	-0.01	0.457[†]	195
Survival rate	SI	0.13[+]	1.535[†]	151
	SC	-0.01	0.146[†]	140
Biomass	SI	0.11*	1.707	127
	SC	-0.01	-0.118	113
Transition rate to flowering	SI	0.02	0.832	127
	SC	-0.02	-0.879	113
Flower number	SI	0.22**	2.836	127
	SC	0.14[++]	1.583	113
Petal length	SI	0.05***	3.263	127
	SC	0.01	0.377	113
Pollen number/anther	SI	-0.02	-0.500	126
	SC	-0.02	-0.512	113
Pollen viability	SI	0.12***	3.545	127
	SC	0.02	0.464	113
Ovule number/flower	SI	0.05	1.581	127
	SC	0.03	0.994	113
Lifetime male fitness	SI	0.54***	3.846[†]	239
	SC	0.15	0.432[†]	195
Lifetime female fitness	SI	0.54***	3.817[†]	240
	SC	0.14	0.382[†]	195

Table 3. Germination and survival of outcrossed and selfed offspring in self-incompatible (SI) and self-compatible (SC) populations. N represents the number of outcrossed and selfed families used to generate means.

Trait	Mating system	Outcrossed	Selfed	N
Germination rate	SI	0.368	0.233	240
	SC	0.236	0.238	195
Survival rate	SI	0.794	0.688	151
	SC	0.697	0.702	140

FIGURE LEGENDS

Figure 1 (a-g). Mean trait values for outcrossed and selfed offspring in self-incompatible

(SI) and self-compatible (SC) populations. Inbreeding depression values (δ) were tested

using orthogonal contrasts of outcrossed and selfed trait values (Table 1; [++] p = 0.058; * p

< 0.05; ** 0.001 < p 0.01; *** p < 0.001). Inbreeding depression values equal $1 - (w_s/w_o)$

when selfed offspring had lower trait values than outcrossed progeny and $(w_o/w_s) - 1$ when

trait values of selfed plants exceeded those of outcrossed individuals. Error bars represent

±1 SE.

Biomass

(a) δ = 0.11*   δ = -0.01

Transition Rate to Flowering

(b) δ = 0.02   δ = -0.02

Flower Number

(c) δ = 0.22**   δ = 0.14++

Petal Length

(d) δ = 0.05**   δ = 0.01

Pollen Number

(e)

δ = -0.02   δ = -0.02

Pollen number/anther

1600
1400
1200
1000
800
600
400
200
0

outcrossed selfed   outcrossed selfed
SI populations   **SC populations**

Pollen Viability

(f)   δ = 0.12***   δ = 0.02

% Viable pollen

1.2
1.0
0.8
0.6
0.4
0.2
0.0

outcrossed selfed   outcrossed selfed
SI populations   **SC populations**

Ovule Number

(g)

δ = 0.04   δ = 0.03

Ovule number/flower

16
14
12
10
8
6
4
2
0

outcrossed selfed   outcrossed selfed
SI populations   **SC populations**

CHAPTER 4

ISOLATION INCREASES THE LOCAL DRIFT LOAD IN A POPULATION OF THE ENDEMIC

FLOWERING PLANT *LEAVENWORTHIA ALABAMICA*

Evolution (2005, *in review*)

ABSTRACT

Mildly deleterious mutations are thought to play a major role in the extinction of natural populations, especially those that are small, isolated, or inbred. Self-fertilization should reduce the effective size of populations and simultaneously reduce migration between populations. As a result, long-term self-fertilization should cause populations to harbor substantial local drift loads caused by the fixation of mildly deleterious mutations. I experimentally tested this hypothesis by comparing the average fitness of offspring from within- and between-population crosses to quantify heterosis, or the increase in fitness caused by the masking of locally common deleterious alleles in the heterozygous state. There was little heterosis observed in crosses between five large, self-incompatible populations and two of the three small, self-fertilizing populations of *L. alabamica*. However, the most geographically isolated population with the longest history of self-fertilization (Tuscumbia) exhibited a 110.2% increase in germination and a 73.6% increase in fitness, which is consistent with a sizeable local drift load. The finding of substantial heterosis in an isolated population with a long history of inbreeding suggests that the fixation of deleterious mutations may threaten the persistence of self-fertilization in nature, but not immediately following the evolution of this mating system.

KEYWORDS: *deleterious mutation, extinction, fitness, genetic drift, heterosis, inbreeding, mutational meltdown*

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Deleterious alleles are the most common class of mutations in natural populations (Keightley and Lynch 2003). These harmful alleles are primarily recessive in their effects and are maintained in populations by a balance between natural selection and recurrent deleterious mutation (Lynch et al. 1999). In infinitely large populations, all deleterious mutations are kept at a low frequency determined by the antagonistic balance between mutation and natural selection (Haldane 1937; Muller 1950). However, in finite populations, some fraction of deleterious mutations may have sufficiently weak effects on fitness such that their change in frequency may be influenced by chance events, or genetic drift (Kimura et al. 1963; Lynch and Gabriel 1990). In small populations, a large number of these "effectively neutral" mutations may actually become fixed in populations in response to genetic drift. As the effective size of a population declines, a greater fraction of the segregating genetic load within a population is fixed by genetic drift, being converted into a "local drift load" that may significantly reduce population fitness (Lande 1994; Lynch et al. 1995a; Wang et al. 1999; Bataillon and Kirkpatrick 2000; Whitlock et al. 2000). Mildly deleterious mutations may even endanger the persistence of extremely small populations if these alleles reduce fitness to the point where reproduction no longer replaces parents, such that a "mutational meltdown" occurs (Lynch and Gabriel 1990; Lynch et al. 1995a; Saccheri et al. 1998).

The connectedness of populations through migration also influences their long-term effective size and thus the efficacy of purifying selection. In metapopulations connected by a large amount of migration, selection may keep mildly deleterious mutations within demes at frequencies similar to those of a large unstructured population (Higgins and Lynch 1999; Glemin et al. 2003). In contrast, a large degree of isolation should cause

populations to experience fitness loss as these mutations drift to high frequencies, especially when migration rates are low ($Nm < 1$; Whitlock et al. 2000). Under realistic scenarios for mutation rates to deleterious alleles, the effects of these alleles on fitness, and rates of migration, theory suggests that fitness losses within finite populations could range from as low 5% to as high as 60% (Crow 1948; Whitlock et al. 2000; Theodorou and Couvet 2002; Glemin et al. 2003; Rose and Rousset 2004). In support of theory, empirical crosses between small and/or isolated populations often uncover a substantial local drift load since interpopulation hybrids can mask the effects of partially recessive deleterious alleles in the heterozygous state (Crow 1948; Levin 1984; van Treuren et al. 1993; Hauser and Loeschcke 1994; Ouborg and van Treuren 1994; Heschel and Paige 1995; Richards 2000; Paland and Schmid 2003).

Although the local drift load is obviously an important issue for the loss of endangered natural populations (Lande 1988; Lande 1994; Westermeier et al. 1998), it may also play a major role in the decline of highly self-fertilizing plant lineages (Charlesworth et al. 1993; Lynch et al. 1995b). Although the evolution of self-fertilizing strategies is a common trend in flowering plants, most species utilize outcrossing to some degree (Vogler and Kalisz 2001; Barrett 2002). One explanation for this apparent paradox is that the fixation probabilities of mildly deleterious alleles are elevated in self-fertilizing populations, through any of the following mechanisms: 1) single self-fertilizing individuals may found new populations, facilitating population bottlenecks (Baker 1955); 2) purging of the segregating deleterious load causing inbreeding depression by selection in self-fertilizing populations will reduce the effective population size; 3) lack of effective recombination may not allow the generation of offspring with fewer deleterious mutations

(Heller and Maynard Smith 1979); 4) elevated linkage disequilibrium should increase the effects of linked selection in self-fertilizing populations (Nordborg et al. 2002); and 5) migration of pollen between populations will attenuate as investment in pollen declines with increased rates of self-fertilization (Charlesworth and Charlesworth 1981). All of these factors may be operating singly or in concert within self-fertilizing populations to reduce the efficacy of purifying selection and thereby increase the local drift load. If this occurs, then the maintenance of outcrossing may in part be explained by declines in the fitness of self-fertilizing plant lineages (Charlesworth et al. 1993; Lynch et al. 1995b).

Leavenworthia alabamica is an excellent system for testing the hypothesis that small and self-fertilizing natural populations suffer from substantial local drift loads. Populations at the center of the species range are large, consist of individuals with sporophytic self-incompatibility, and maintain high levels of inbreeding depression (Rollins 1963; Lloyd 1965; Busch 2005a,b; Figure 1). In contrast, self-compatibility evolves at the margins of the Moulton Valley or outside of this region, in which populations are approximately an order of magnitude smaller, genetically depauperate, and maintain low levels of inbreeding depression (Busch 2005a,b). In theory, a reduction in the effective size of self-fertilizing populations should reduce fitness as mildly deleterious alleles stochastically reach high frequency in response to genetic drift (Lynch et al. 1995a; Wang et al. 1999; Bataillon and Kirkpatrick 2000). This experiment tests the prediction that the magnitude of heterosis caused by the masking of locally common deleterious alleles will be greatest in the small and self-fertilizing populations of *L. alabamica*.

METHODS

Study System

Leavenworthia alabamica Rollins (Alabama glade cress; Brassicaceae) is a winter annual endemic to the limestone cedar glades of northern Alabama. Most populations are restricted to the narrow Moulton Valley, though several are found in the Tennessee River Valley near the town of Tuscumbia (Figure 1). Populations vary in the possession of sporophytic self-incompatibility, which is the ancestral mating system in the genus (Rollins 1963; Lloyd 1965; Beck et al. unpublished data). Self-compatibility has evolved at least four independent times within the genus *Leavenworthia* (Lloyd 1965; Beck et al. unpublished data). In *L. alabamica*, self-compatibility evolves at the margins of the Moulton Valley or outside this region, where populations are smaller and inhabit more disturbed glade sites (Busch 2005b). Lloyd (1965) recognized a single self-incompatible race (a1) and four self-compatible races (a2, a4, Russellville, and Tuscumbia) following studies of self-compatibility and floral morphology. The a2 race retains adaptations for outcrossing, whereas the a4, Russellville and Tuscumbia races have adaptations for autonomous self-fertilization, suggesting a long history of inbreeding (Busch 2005b).

Crossing Design and Greenhouse Experiment

Seeds were collected during the spring of 2004 from five self-incompatible populations of the a1 race (*Hatton*, *Isbell*, *Newburg*, *Tharptown*, and *Waco*), one population from the a4 race (*Morgan/Huckaby Bridge*), and one population from each of the Russellville and Tuscumbia races. The geographic locations of these populations are reported elsewhere (Busch 2005a). Seeds from 25-30 families within each population were germinated in petri dishes maintained in an incubator with 14 hour days (18° C) and

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10 hour nights (8° C). Seedlings were transplanted to three inch pots containing a 1:1 ratio of sterilized soil to MetroMix (Scotts-Sierra Horticultural Products, Marysville, OH) following the emergence of both cotyledons. Plants received 14 hours of artificial light in an Indiana University greenhouse to promote flowering. These plants were then mated with each other to produce outcrossed and hybrid progeny. Outcrossed offspring were created by mating each plant to three random pollen donors from the same population. Hybrid offspring were created by mating plants to three random pollen donors from other populations of the same mating system. There were the following numbers of outcrossed and hybrid families from each population: Hatton (25/25); Isbell (23/22); Newburg (18/16); Tharptown (24/23); Waco (24/24); Morgan (25/25); Russellville (22/22); and Tuscumbia (22/22). Overall, there were 627 individual plants that germinated from 320 families at the outset of the experiment.

Offspring were germinated during November of 2004 as described for the parental plants. Dates of germination were recorded on a daily basis within all families. When germination rates stopped increasing within families, seedlings were selected for transplantation. To avoid potential biases in selection of plants, the two most centrally located seedlings within a petri plate were transferred to pots. Seedlings were placed in 3 inch pots containing a 1:1 ratio of sterilized soil to MetroMix. Plants were watered twice daily and received more than 14 hours of natural light to promote flowering. Survival was recorded daily while plants matured in the greenhouse. The date of flowering was recorded as the day when the first flower opened on a plant. Days to flowering was measured as the number of days between germination and the opening of the first flower. The number of flowers produced in the first two weeks of the flowering period was

recorded for all individuals. At the end of the experiment, plants were placed in envelopes, dried in a 60° C oven, and weighed to the nearest 0.001 g.

Petal length of the third flower was measured with digital calipers to the nearest 0.01 mm. The fourth flower was collected in the morning immediately following anther dehiscence to estimate pollen number. These flowers were immersed in 5 mL of a 3:1 solution of lactic acid to glycerol and the number of pollen grains in 10 uL of well-mixed solution was counted using a compound microscope. This number was multiplied by 500 to estimate the number of pollen grains per flower. Pollen viability was estimated through the use of Alexander's stain, which colors viable pollen grains red while leaving inviable pollen grains blue (Kearns and Inouye 1993). Viability scores were carried out by tapping single anthers onto plain glass slides three times to release adequate pollen grains. Counts of inviable and viable pollen grains were performed using a compound microscope. The pistil of each flower was split lengthwise with forceps and ovule number was counted using a dissecting microscope.

Population Mean Fitness and Heterosis

The values for all traits measured on individuals within a family were combined to generate family averages. Multiplicative fitness was estimated for each family by combining aspects of survival and reproduction (Willis 1999). In particular, the fitness (w_i) of the *ith* family equaled the product of each fitness component:

$$w_i = \textit{(germ. rate}_i\textit{)} * \textit{(surv. rate}_i\textit{)} * \textit{(flower \#}_i\textit{)} * \left[\left(\frac{male_i}{male} + \frac{female_i}{female} \right) \right]$$

where *male$_i$* and *female$_i$* are the total number of viable pollen grains and ovules per flower for the *ith* family, respectively. Since all individuals have one paternal and one maternal

parent, it was necessary to standardize the male and female fitness components so that they contributed equally to the total fitness of the population. The average male ($\overline{male}$) or female ($\overline{female}$) fitness of a population was calculated by pooling together offspring created from within- and between-population crosses. The average fitness of the hybrid and outcrossed families was then calculated within each population.

Heterosis is typically defined as the relative increase in population mean fitness caused by increased heterozygosity in hybrids (Whitlock et al. 2000; Theodorou and Couvet 2002; Glemin et al. 2003). As a result, heterosis was defined as the increase in mean fitness of hybrid individuals relative to the mean fitness of individuals produced by random mating within a population:

$$H_j = \left(\frac{\overline{w_j}(hybrid) - \overline{w_j}(outcrossed)}{\overline{w_j}(outcrossed)} \right)$$

where $\overline{w_j}(hybrid)$ and $\overline{w_j}(outcrossed)$ represent the mean fitness of hybrid and outcrossed individuals within the *jth* population. The mean values for outcrossed and hybrid individuals within all eight populations are reported in the appendix. To statistically evaluate whether heterosis was significant, the average trait values of hybrid and outcrossed individuals within a population were compared using t-tests (Sokal and Rohlf 1995). Positive values of *H* suggest that fitness is increased in hybrid offspring, whereas negative values of *H* suggest that outbreeding depression occurs in hybrids. Analyses of germination and survival rates were conducted using arcsine square-root transformed values. All statistical analyses were conducted in SPSS (Norusis 2000).

The average heterosis, or increase in the performance of hybrids relative to randomly outcrossed progeny, was -0.1% in the large and self-incompatible populations of *L. alabamica* (Figure 2). Within these populations, there was a significant difference between the mean value of hybrid and outcrossed offspring for only eight of the 88 trait comparisons (Table 1). Overall, most of the heterosis values were near zero, suggesting little genetic differentiation among populations. Specifically, over all five of the self-incompatible populations, 30 of the 88 heterosis values were slightly negative, whereas the remaining values range were slightly positive (Table 1; Figure 2). Similarly, the average heterosis observed in the small and self-compatible populations of *L. alabamica* was 3.0% (Figure 2). There was a significant difference between the mean value of hybrid and outcrossed plants for only 6 of the 33 trait comparisons (Table 1). Overall, there were 14 slightly negative, 18 slightly positive heterosis values, and one very large and positive heterosis value (Figure 2). This large heterosis value represents a 110.2% increase in the germination of hybrid seed within the geographically isolated Tuscumbia population (Figure 3a,b).

There were no significant differences between the multiplicative fitness of hybrids and outcrossed individuals within the five large and self-incompatible populations (Figure 4). In two of these populations, there was slight outbreeding depression for fitness ($H = -0.066$), whereas there was slightly positive heterosis for fitness in the remaining populations ($H = 0.004, 0.037,$ and 0.203). In two of the three small and self-fertilizing populations (Morgan and Russellville), there was slight heterosis for fitness ($H = 0.133$) and slight outbreeding depression ($H = -0.131$), although these values were not

significantly different from zero. The large magnitude of heterosis for fitness (73.6%) in

the Tuscumbia population is driven by the 110.2% increase in the germination rates of

hybrids compared to offspring created by random outcrossing within the population (Table

1; Figure 3).

DISCUSSION

Heterosis, or the increase in fitness observed in hybrids, occurs whenever genetic

drift causes deleterious mutations to stochastically reach relatively high frequencies in

finite populations. The magnitude of heterosis, therefore, reflects the number and

frequency of deleterious mutations fixed by genetic drift, which is a function of the size of

a local population and its connectedness to the remainder of the metapopulation through

migration (Whitlock et al. 2000; Theodorou and Couvet 2002; Glemin et al. 2003; Rose

and Rousset 2004). The results of this study demonstrate little difference between all of

the self-incompatible and two of the self-fertilizing plant populations in their local drift

load. Nevertheless, the finding of 73.6% heterosis for fitness in one of the self-fertilizing

populations (Tuscumbia) is among the largest values observed in crosses between natural

plant populations (Keller and Waller 2002; Tallmon et al. 2004).

The Tuscumbia population of *L. alabamica* occurs in the Tennessee River Valley,

which is separated from the remainder of populations in the Moulton Valley by a narrow

region of dry and elevated soil that does not support the growth of *Leavenworthia*

(Johnston 1930; Figure 1). Interestingly, historical work suggests that the Tuscumbia

population may have became reproductively isolated relatively long ago, when the species

migrated into northern Alabama from larger cedar glades situated to the north in Tennessee

(pp. 7-14, Lloyd 1965). This scenario of current and historical reproductive isolation

between Tuscumbia and the remainder of the species is supported by the fact that this population is genetically distinct from other populations of this species. In particular, a study of neutral genetic variation at the *PgiC* locus found a novel substitution at this locus that has reached fixation in the Tuscumbia population, whereas the Morgan population is more recently derived from the self-incompatible populations (Busch 2005b). This pattern of geographical and genetic data suggests that the accumulation of mutations in inbreeding plant populations may operate on relatively long time scales (Lynch and Gabriel 1990; Charlesworth et al. 1993; Lynch et al. 1995b; Wang et al. 1999; Higgins and Lynch 2001).

Previous work in *L. alabamica* has shown that self-fertilization tends to evolve in populations occurring at the periphery of the Moulton Valley, where populations are smaller and undergo extinction and recolonization (Busch 2005b; Figure 1). The effective size of these already small populations should therefore be substantially reduced by fluctuations in population number (Rice 2004). As a result, the evolutionary transition from self-incompatibility to that of self-fertilization in *L. alabamica* should lead to the fixation of more deleterious mutations by genetic drift within populations (Charlesworth et al. 1993; Lynch et al. 1995b; Wang et al. 1999). The fact that two recently founded self-fertilizing populations of *L. alabamica* have not experienced mutation accumulation suggests that self-fertilization in and of itself does not diminish the power of selection to keep weakly deleterious mutations at low frequencies, and may not endanger population fitness over a relatively short time scale (Lynch and Gabriel 1990; Charlesworth et al. 1993; Lande 1994; Lynch et al. 1995a,b; Schultz and Lynch 1997).

The magnitude of fitness lost in response to genetic drift is of great interest because this factor can theoretically reduce fitness to such an extent that populations may

experience extinction (Lynch and Gabriel 1990; Lande 1994; Lynch et al. 1995a). Since

no biological organisms are infinitely abundant, recent work has attempted to determine

whether the local drift load can endanger the persistence of natural animals or plants,

especially those populations that have recently become fragmented (Saccheri et al. 1998;

Westermeier et al. 1998; Keller and Waller 2002; Tallmon et al. 2004). Increasing

migration between populations of prairie chickens (Westermeier et al. 1998), adders

(Madsen et al. 1999), wolves (Vila et al. 2002), and many plants (Levin 1984; van Treuren

et al. 1993; Hauser and Loeschcke 1994; Ouborg and van Treuren 1994; Heschel and Paige

1995; Richards 2000; Paland and Schmid 2003) often leads to substantial heterosis (>50%)

in the survival and reproduction of hybrids. This emerging trend suggests that genetic drift

may reduce the power of purifying selection in small natural populations and that

migration is essential to counteract declines in fitness caused by the random fixation of

weakly harmful mutations.

Table 1. Heterosis observed for all traits measured in hybrid and outcrossed individuals. Heterosis values equaled:

$$\left(\overline{hybrid}\middle/\overline{outcrossed}\right)-1.$$

P-values indicate significant differences: * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$).

Trait	Self-incompatible populations					Self-compatible populations		
	Hatton	Isbell	Newburg	Tharptown	Waco	Morgan	Russellville	Tuscumbia
Seed #/silique	0.076	0.162	-0.057	0.120	-0.152	-0.032	-0.021	-0.113
Seed mass	0.009	-0.082	-0.109	-0.036	-0.085	**0.125***	0.044	0.092
% Germination	-0.141	0.179	0.408	0.087	-0.070	0.100	-0.096	**1.102*****
% Survival	0.019	-0.048	**-0.133***	-0.091	-0.060	0.000	0.000	0.067
Biomass	-0.007	0.034	-0.043	0.021	-0.056	-0.026	**-0.234*****	-0.126
Flowering rate	**-0.040***	-0.029	0.044	0.018	0.031	0.047	**0.066***	-0.013
Flower number	-0.059	-0.108	0.046	-0.217	0.060	-0.151	-0.312	0.255
Petal length	0.005	0.034	-0.003	**0.085*****	0.007	0.038	**0.049***	-0.007
Pollen #/flower	0.023	0.014	-0.052	0.079	-0.113	0.129	-0.044	0.088
Pollen viability	-0.010	-0.002	-0.002	0.004	-0.004	**0.030****	0.004	0.015
Ovule #/flower	**0.162***	**0.190****	-0.031	-0.057	-0.080	0.057	-0.082	-0.077

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FIGURE LEGENDS

Figure 1. (a) The range of *L.alabamica*, which is endemic to the limestone cedar glades of northern Alabama. (**b**) The geographical location of *L. alabamica* populations, with large self-incompatible (black dots) and small self-compatible populations (white dots). All five of the studied self-incompatible populations belong to the a1 race, which is localized within the oval shown at the center of the Moulton Valley. The three self-compatible populations used in this experiment belong to the Tuscumbia, Russellville, and a4 races. The geological regions that do not support the growth of *L. alabamica* are shown in gray. The a2 race was not included in this study because it is self-compatible yet retains adaptations for outcrossing. This figure was modified from Lloyd (1965).

Figure 2. Distribution of heterosis values for all traits measured in large/self-incompatible (dark bars) and small/self-compatible populations (light bars). The mean heterosis observed in both types of populations was not significantly different from zero, and the distributions were not different in mean, dispersion, or skew (Kolmogorov Smirnov $Z = 0.853$; N = 88; $P < 0.0460$).

Figure 3. Germination rates in families from the geographically isolated Tuscumbia population. (a) Outcrossed offspring (average germination = 0.38). (b) Hybrid offspring (average germination = 0.80).

Figure 4. Heterosis for multiplicative fitness in the populations of *L. alabamica*. See methods for the definition of multiplicative fitness. Light and dark bars represent the average fitness of hybrids and randomly outcrossed individuals, respectively. The total number of families compared in each population equaled: Hatton (N =50); Isbell (N=45); Newburg (N=34); Tharptown (N=47); Waco (N=48); Morgan (N=50); Russellville (N=44); Tuscumbia (N=44). Error bars indicate ± 1 SE. * (*P* < 0.05); ** (*P* < 0.01); *** (*P* < 0.001).

APPENDIX. The average fitness of outcrossed and hybrid offspring within all of the populations in this experiment. Standard errors are given in parentheses below each mean value.

FIGURE 1

(a)

(b)

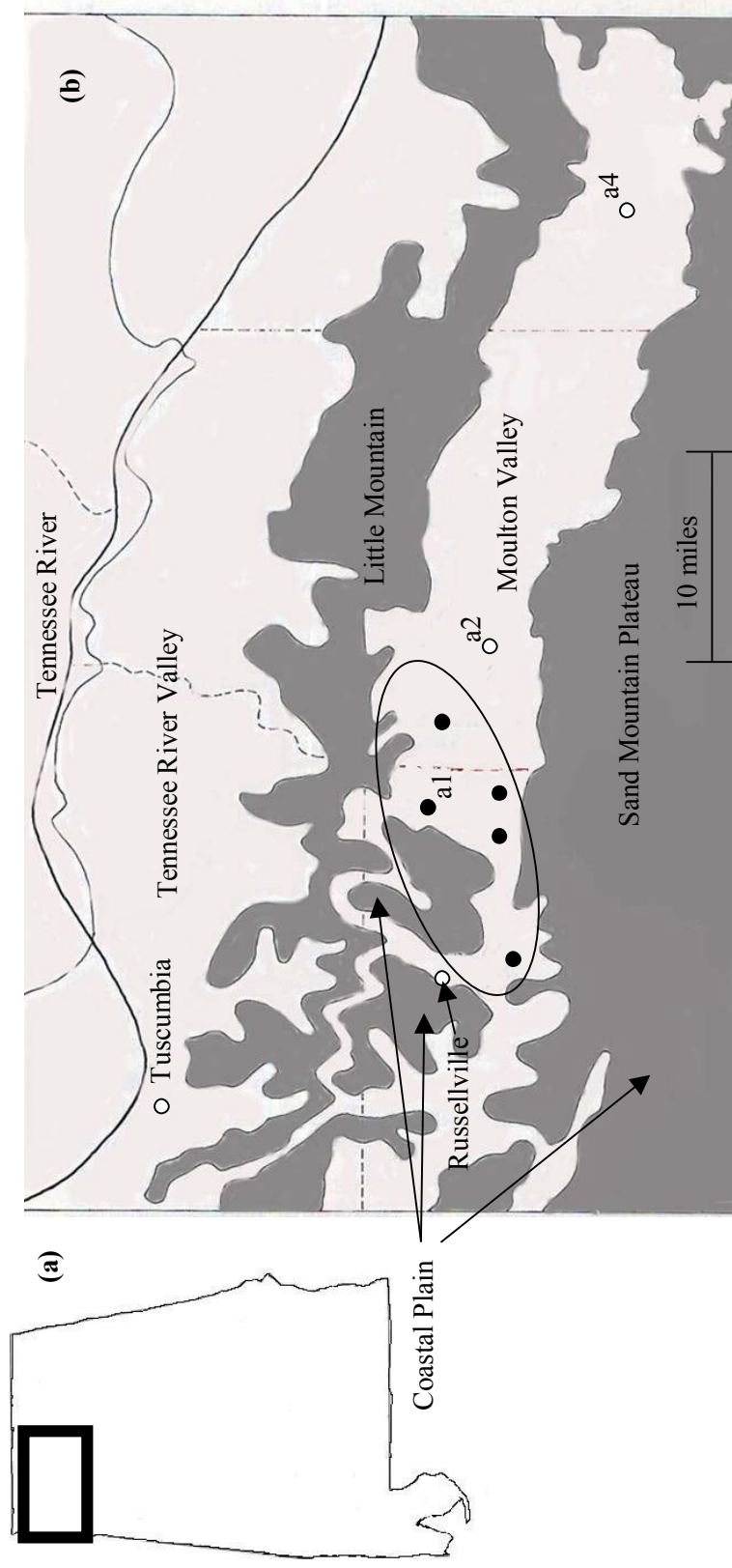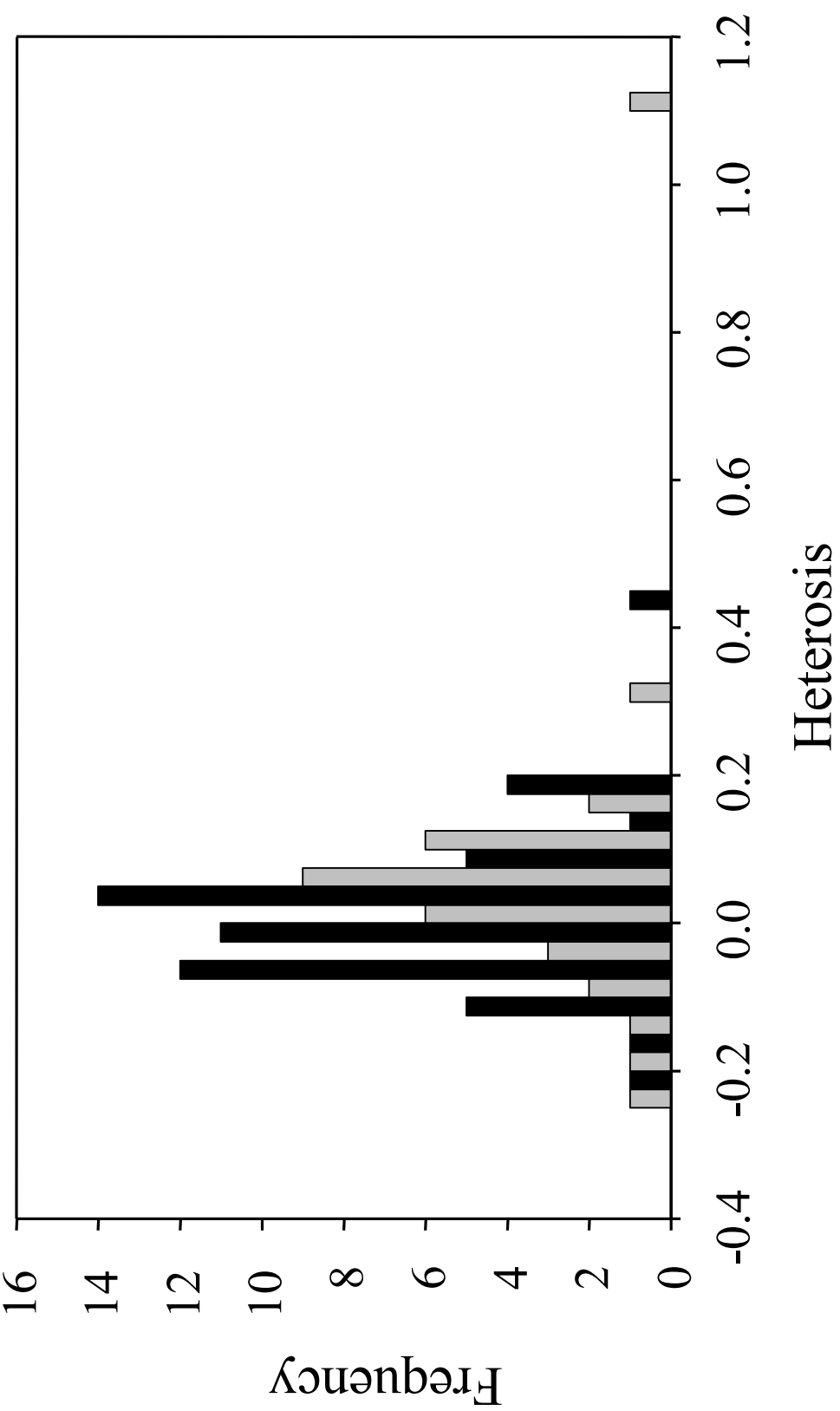Tuscumbia
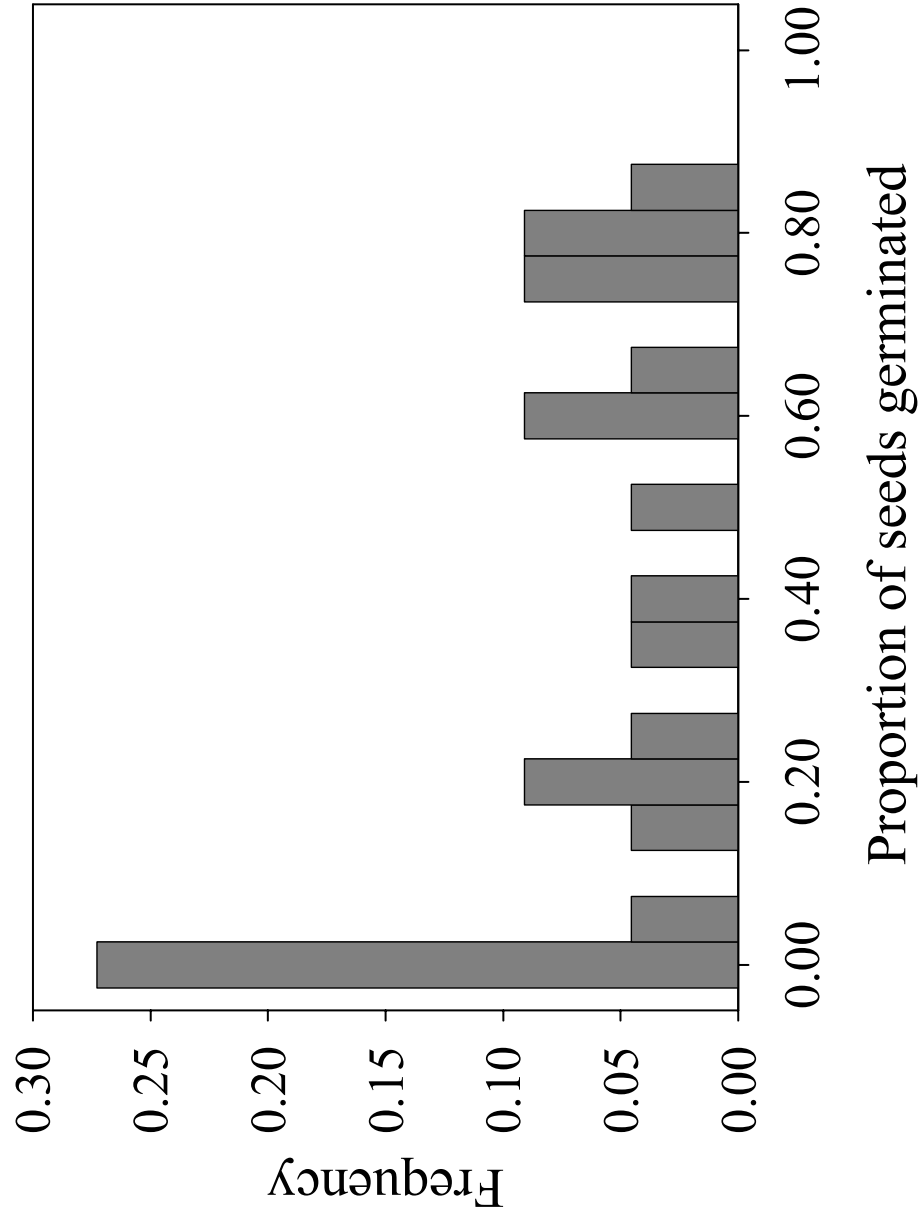
Tennessee River

Tennessee River Valley

Coastal Plain

Russellville

Little Mountain

a1

a2

Moulton Valley

a4

Sand Mountain Plateau

10 miles

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FIGURE 2

Frequency

Proportion of seeds germinated
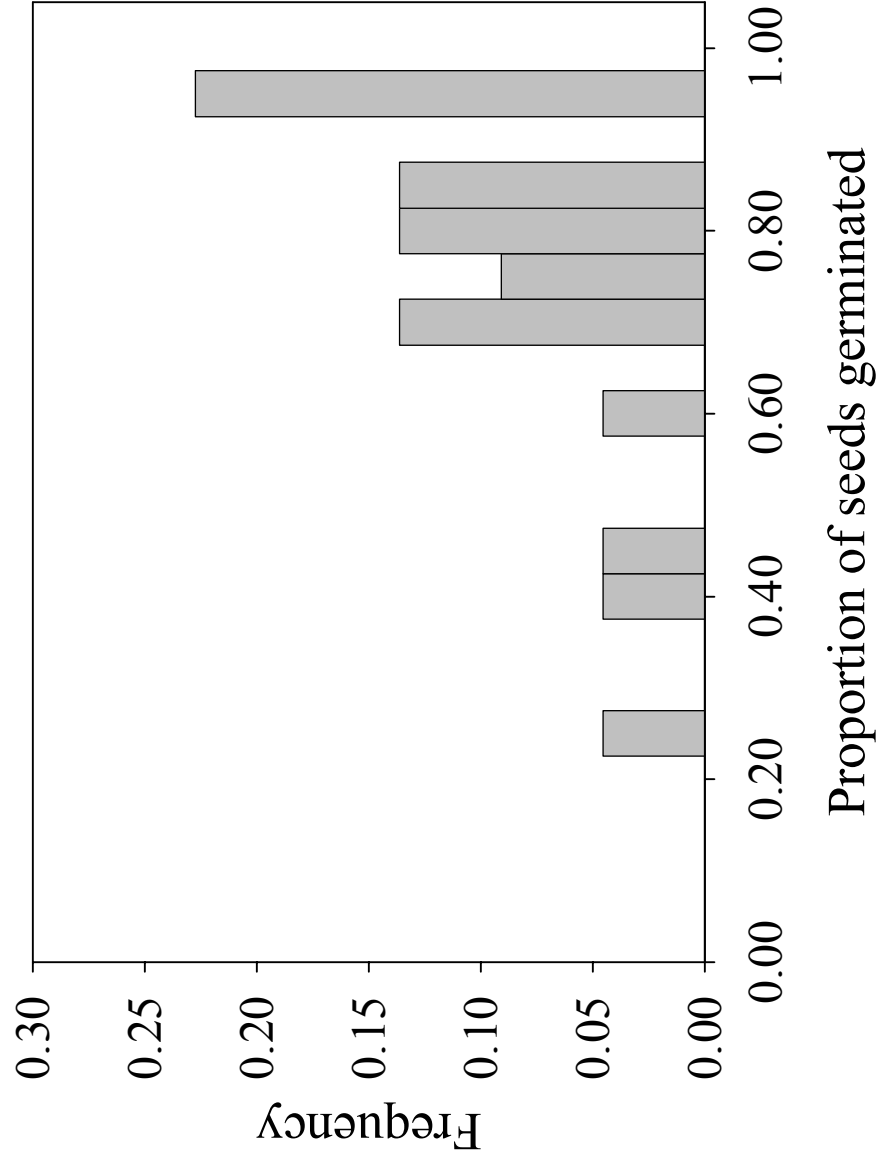
Figure 3b

F<small>IGURE</small> 4
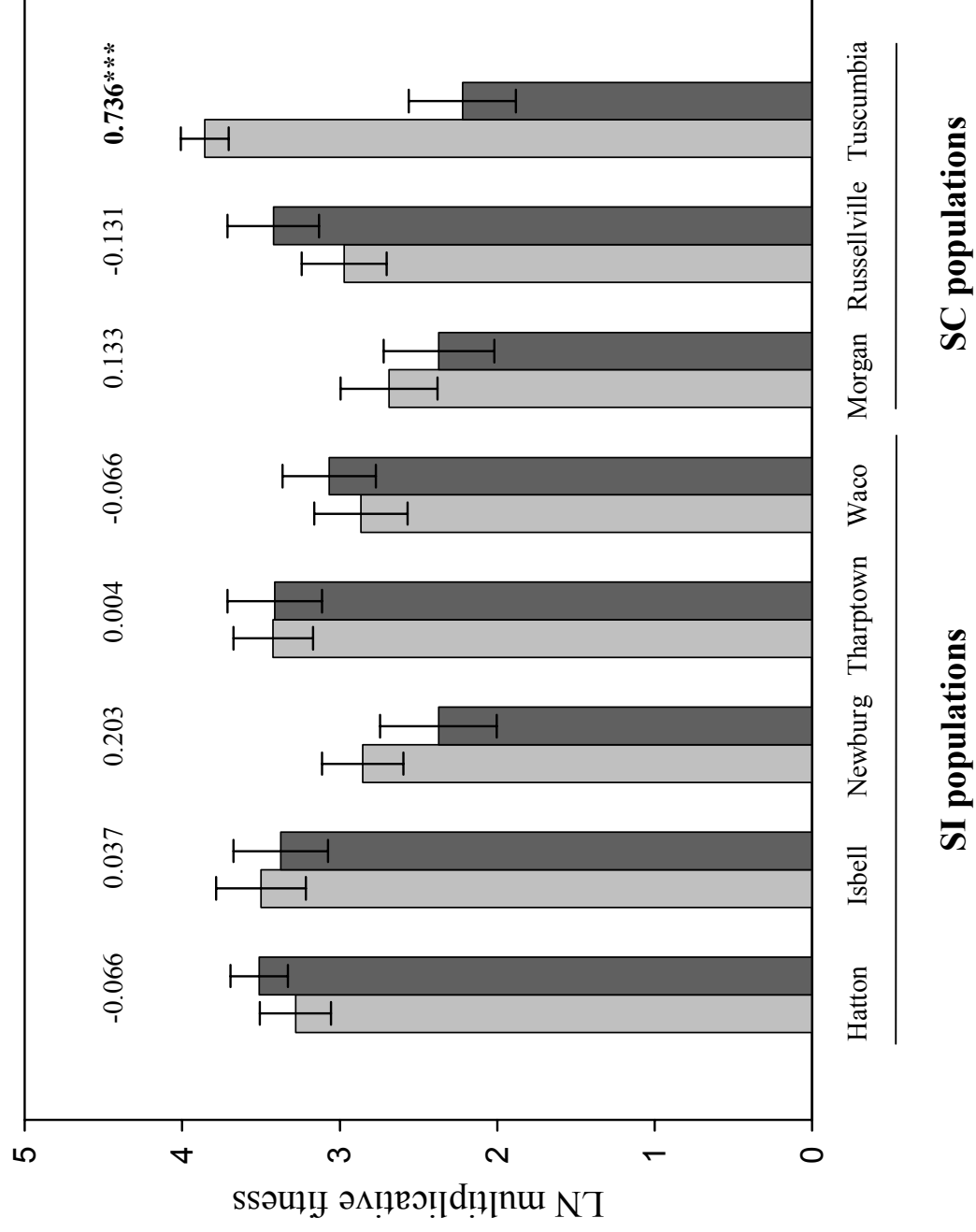
APPENDIX

Trait	Hatton		Isbell		Newburg		Tharptown		Waco	
	outcrossed	*hybrid*	*outcrossed*	*hybrid*	*outcrossed*	*hybrid*	*outcrossed*	*hybrid*	*outcrossed*	*hybrid*
Seed #/silique	4.53 (0.030)	4.87 (0.273)	4.91 (0.471)	5.70 (0.494)	4.95 (0.500)	4.67 (0.385)	5.43 (0.451)	6.08 (0.448)	5.29 (0.385)	4.49 (0.419)
Seed mass	1.72 (0.080)	1.73 (0.077)	1.28 (0.087)	1.17 (0.084)	1.71 (0.099)	1.53 (0.122)	1.62 (0.106)	1.56 (0.083)	1.70 (0.087)	1.56 (0.103)
% Germination	0.76 (0.039)	0.65 (0.053)	0.57 (0.065)	0.68 (0.062)	0.36 (0.078)	0.50 (0.080)	0.59 (0.071)	0.64 (0.060)	0.67 (0.061)	0.62 (0.071)
% Survival	0.92 (0.047)	0.94 (0.046)	0.95 (0.034)	0.90 (0.056)	1.00 (0.000)	0.87 (0.059)	1.00 (0.000)	0.91 (0.053)	0.89 (0.065)	0.83 (0.063)
Biomass	0.84 (0.054)	0.83 (0.065)	0.77 (0.071)	0.80 (0.053)	1.04 (0.059)	0.99 (0.080)	0.85 (0.041)	0.86 (0.050)	1.14 (0.072)	1.07 (0.113)
Flowering rate	0.02 (0.001)	0.017 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)
Flower number	34.17 (2.779)	32.15 (2.767)	39.28 (3.260)	35.05 (2.458)	31.96 (5.431)	33.43 (4.308)	39.17 (5.485)	30.67 (1.450)	30.78 (3.488)	32.62 (3.049)
Petal length	14.88 (0.140)	14.96 (0.316)	14.48 (0.233)	14.97 (0.216)	15.46 (0.319)	15.41 (0.220)	14.25 (0.266))	15.46 (0.225)	15.24 (0.221)	15.35 (0.178)
Pollen #/flower	35020 (1779)	35824 (1890)	41530 (2026)	42105 (2132)	46372 (2529)	43947 (4828)	38377 (2467)	41425 (2656)	43942 (1910)	38969 (3452)
Pollen viability	0.97 (0.013)	0.96 (0.011)	0.96 (0.010)	0.96 (0.011)	0.97 (0.012)	0.97 (0.010)	0.95 (0.011)	0.96 (0.010)	0.95 (0.015)	0.94 (0.015)
Ovule #/flower	7.52 (0.373)	8.74 (0.351)	8.93 (0.233)	10.63 (0.432)	11.29 (0.384)	10.93 (0.565)	10.79 (0.374)	10.17 (0.502)	11.53 (0.364)	10.60 (0.479)

Trait	Morgan		Russellville		Tuscumbia	
	outcrossed	*hybrid*	*outcrossed*	*hybrid*	*outcrossed*	*hybrid*
Seed #/silique	4.75 (0.271)	4.60 (0.375)	6.82 (0.489)	6.68 (0.408)	5.82 (0.377)	5.16 (0.343)
Seed mass	1.62 (0.062)	1.82 (0.054)	1.40 (0.064)	1.46 (0.063)	1.28 (0.054)	1.39 (0.046)
% Germination	0.43 (0.075)	0.48 (0.068)	0.60 (0.071)	0.54 (0.072)	0.38 (0.070)	0.80 (0.041)
% Survival	1.00 (0.000)	1.00 (0.000)	1.00 (0.000)	1.00 (0.000)	0.938 (0.043)	1.00 (0.000)
Biomass	1.15 (0.060)	1.12 (0.058)	1.27 (0.070)	0.975 (0.046)	1.16 (0.102)	1.02 (0.064)
Flowering rate	0.01 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)	0.02 (0.001)
Flower number	33.12 (3.327)	28.13 (3.171)	46.93 (6.761)	32.28 (3.721)	33.86 (4.228)	42.50 (5.552)
Petal length	11.13 (0.160)	11.56 (0.143)	11.14 (0.164)	11.68 (0.154)	11.84 (0.180)	11.76 (0.129)
Pollen #/flower	18147 (1366)	20496 (1555)	21048 (1231)	20125 (887)	17411 (1604)	18945 (1394)
Pollen viability	0.96 (0.004)	0.98 (0.004)	0.98 (0.006)	0.98 (0.004)	0.97 (0.009)	0.98 (0.007)
Ovule #/flower	11.09 (0.327)	11.73 (0.255)	12.75 (0.383)	11.70 (0.397)	11.50 (0.421)	10.62 (0.372)

CHAPTER 5

CONCLUSIONS

Biologists are keenly interested in understanding the costs and benefits of mating systems because patterns of reproduction strongly influence the evolutionary process. The primary goal of this dissertation was to use the species *Leavenworthia alabamica* as a study system in which to understand the selective agents shaping variation in the mating system in a flowering plant. Much of the present work was made possible by a history of work on the phenotypic adaptations associated with the evolution of self-compatibility throughout the genus *Leavenworthia* (Rollins 1963; Lloyd 1965; Solbrig and Rollins 1977) and the consequences of the transition to inbreeding on neutral genetic variation (Lyons and Antonovics 1991; Charlesworth and Yang 1998; Filatov and Charlesworth 1999; Liu et al. 1999). Taken together, this body of work indicated that the transition to self-compatibility was likely driven by the benefits of reproductive assurance, although the exact agent of selection was unclear (Rollins 1963; Lloyd 1965). In addition, deleterious mutations should play a large role in the maintenance of self-incompatibility, although it was unclear whether the patterns in *Leavenworthia* supported theoretical expectations (Charlesworth et al. 1994).

In the central portion of the geographical range of *L. alabamica*, populations are large, relatively stable, and are composed of large flowered individuals that retain the mechanism of self-incompatibility found throughout the genus. Interestingly, there is a low frequency of individuals that are self-compatible in these populations. Lloyd (1965) originally suggested that the maintenance of self-incompatibility in these populations was likely caused by inbreeding depression, though he had limited evidence to evaluate this hypothesis. This dissertation provides strong support for the hypothesis that partially recessive deleterious alleles may limit the spread of self-compatibility alleles in the

relatively large and stable populations of *L. alabamica*. This pattern also lends support to a large body of theoretical work suggesting that the outcome of mating-system evolution may be dictated by the ability of inbreeding depression to counteract the transmission advantage of alleles causing self-fertilization (Lande and Schemske 1985; Campbell 1986; Charlesworth et al. 1990; Uyenoyama and Waller 1991a, 1991b).

The ecological correlates of self-compatibility in *L. alabamica* suggest that this purely genetic model may not fully describe the factors important in the evolution of mating systems in flowering plants (chapter 2). Following their taxonomic studies of the genus *Leavenworthia*, Rollins (1963) and Lloyd (1965) originally suggested that the evolution of self-compatibility occurred at least four independent times among the eight extant species in the genus. They also concluded that self-compatibility evolved to provide plants with reproductive assurance since it was associated with floral adaptations for autonomous seed production. This pattern of parallel mating-system evolution was thought to be driven by the fact that pollinators were inefficient pollen vectors during the cold temperatures of late March, when these winter annuals begin to flower (Rollins 1963). Although this factor may contribute to pollen limitation in all of the populations of *L. alabamica*, the results of this dissertation do not support the idea that reductions in pollinator availability explain the spread and fixation of self-compatibility alleles. If this were true, then I would have observed more pronounced pollen limitation of seed set in the environments that normally support populations with fully self-compatible mating systems.

The most compelling ecological correlate of the mating system in *L. alabamica* is the association of self-compatible populations with marginal and highly disturbed

environments. Interestingly, two of the five self-compatible populations of this species went extinct during the period of study, suggesting that the transition to inbreeding may be associated with bouts of extinction and recolonization. In light of this ecological trend, it is likely that small population size or bottlenecks may intensify the benefits of reproductive assurance to such an extent that self-compatible genotypes may be favored by natural selection in *L. alabamica*. Small population size should play an especially prominent role in limiting seed production in species with genetically controlled self-incompatibility, since S-allele diversity is strongly related to the long-term effective size (Byers and Meagher 1992; Demauro 1993; Reinartz and Les 1994; Pannell and Barrett 1998; Fischer et al. 2003; Wright 1964). More generally, the results of this dissertation support the role of colonization in the evolution of self-compatibility, a pattern in mating-system evolution known as Baker's Law (Baker 1955; Baker 1967).

Studies of neutral genetic variation at *PgiC* provide strong evidence for the role of periods of mate limitation in the evolution of self-compatibility throughout the genus *Leavenworthia*. The species *Leavenworthia crassa*, which is the sister taxon of *L. alabamica*, displays similar variation among populations in the presence or absence of sporophytic self-incompatibility (Lloyd 1965, Lloyd 1967). The self-incompatible populations of this species maintain nucleotide diversities near 1%, whereas three highly self-compatible populations are completely monomorphic at intron 12 (Liu et al., 1999). The wholly self-incompatible species *Leavenworthia stylosa* supports extremely high nucleotide diversity near 5% (Filatov and Charlesworth, 1999). In contrast, the close self-compatible relatives *Leavenworthia torulosa* and *Leavenworthia uniflora* lack any within-population variation within intron 12 of *PgiC* (Liu et al., 1999). Overall, these

results demonstrate that the transition to self-compatibility in the genus *Leavenworthia* is repeatedly associated with the nearly complete loss of genetic variation, which is expected following catastrophic crashes in population size (Innan and Tajima, 2002; Charlesworth, 2003).

The relatively low inbreeding depression found in the highly self-fertilizing populations of *L. alabamica* also supports a body of evidence suggesting that long periods of inbreeding may selectively purge the genetic load caused by strongly deleterious, primarily recessive mutations (Byers and Waller 1999; Charlesworth and Charlesworth 1999). However the results of this study are not wholly consistent with those observed in another study of inbreeding depression in the genus *Leavenworthia,* which found relatively high inbreeding depression in the highly self-fertilizing populations of *L. crassa* and *L. uniflora* (Charlesworth et al. 1994). In particular, these self-fertilizing populations maintained inbreeding depression that was two to three times as great as that observed in the self-fertilizing populations of *L. alabamica*. This discrepancy may suggest that the phenomenon of purging may be an inconsistent force in mating-system evolution, or that there were differences in the environmental expression of inbreeding depression across studies. This latter hypothesis is likely since a competitive environment was used in the growth of *L. crassa* and *L. uniflora*, which has repeatedly been shown to increase inbreeding depression (Dudash 1990; Wolfe 1993).

By combining studies of the ecological correlates of self-fertilization (chapter 2) with studies of inbreeding depression in *L. alabamica* (chapter 3), this dissertation provides a synthetic framework for understanding the outcome of mating-system evolution in the wild. The patterns observed in this species also provide insight into the

likely scenario of phenotypic adaptation that accompanies the evolution of self-compatibility (chapter 2). The self-incompatible populations of *L. alabamica* consist of many large-flowered individuals with extrorse anthers, which likely serve to enhance the export of pollen by insect vectors. One self-compatible race (a2) of this species retains these aspects of floral morphology, suggesting that self-compatibility alleles may have only recently been established in this locale. However, several geographically isolated races (a4, Russellville, and Tuscumbia) have undergone a battery of adaptations for securing self-pollination and a high rate of autonomous seed production such as short petals, introrse anthers, and little separation of the stigma and anthers. These highly self-fertilizing races also exhibit reduced pollen to ovule ratios, which reflect reductions in the allocation to male reproduction that commonly occurs in highly inbreeding populations (Charlesworth and Charlesworth 1981). In general, these results strongly support a history of work on the strong association between the mating system and floral morphology in the genus *Leavenworthia* (Rollins 1963; Lloyd 1965; Solbrig and Rollins 1977; Lyons and Antonovics 1991).

One of the major goals of this dissertation was to elucidate a mechanism for why self-fertilizing populations may be short-lived evolutionary phenomena (Stebbins 1957). Inbreeding populations may go extinct at elevated rates because the transition to self-fertilization may cause a greater fraction of deleterious mutations to become effectively neutral or will cause the stochastic loss of the most-fit genotypes (Heller and Maynard Smith 1979; Charlesworth et al. 1993; Lynch et al. 1995b). This hypothesis has never been tested in natural plant populations that vary in their rate of self-fertilization. The results of this dissertation demonstrate that all of the self-incompatible and two of the

three self-compatible populations of *L. alabamica* have similar mutational loads depressing fitness (chapter 4). However, the most reproductively isolated and putatively oldest population of self-compatible plants exhibited a dramatic increase in fitness caused by the masking of locally common deleterious alleles. This result suggests that mutation accumulation may play some role in the elimination of self-fertilization in nature, but this process may operate over a long time scale as mutations stochastically fix in highly inbred populations.

In *L. alabamica*, it is also possible that the ecological agent favoring the evolution of self-compatibility may also drive populations to extinction. Put another way, self-compatibility may be favored at the individual level because it provides reproductive assurance in the smallest and most disturbed environments, and these populations may be more likely to experience extinction because they undergo frequent and catastrophic reductions in size (Moeller and Geber 2005). In such a situation, mutation accumulation may not strongly influence the outcome of mating-system evolution because this process may operate on a time scale irrelevant to the appearance and loss of self-fertilization. Interestingly, this conclusion parallels the argument that ecological and demographic factors are more likely to threaten the viability of threatened or endangered populations since mutation accumulation operates over relatively long time scales (Lande 1988; Lande 1994). Nevertheless, the finding of a substantial local drift load in the most reproductively isolated population supports the idea that mutation accumulation can significantly reduce fitness, and that migration may strongly counteract the loss of fitness in finite natural populations (Keller and Waller 2002; Tallmon et al. 2004).

In conclusion, the results of research on *L. alabamica* improve our understanding of mating-system evolution because they simultaneously consider all of the factors thought to be relevant in favoring or disfavoring self-fertilization in the wild. Although genetic factors are clearly relevant to the evolution of self-fertilization, ecological factors limiting pollen availability likely play a larger role (Uyenoyama et al. 1993). The evolution of self-compatibility in this and other species of *Leavenworthia* is strongly associated with rarity and the loss of genetic variation (Innan and Tajima 2002). This pattern suggests that mating-system evolution may often be driven by natural selection favoring alleles because they provide reproductive assurance, especially in the smallest of populations. Although the factors favoring self-fertilization are fairly well-understood, there is a greater need to identify the factors limiting the long-term success of self-fertilizing populations. The results of this dissertation are illuminating because they demonstrate that the evolution of self-fertilization does not immediately accelerate mutation accumulation. Self-fertilization may often be an evolutionary "dead-end" because this mating-system evolves in inherently marginal, peripheral, or isolated environments which experience extinction by ecological means in the short-term and inevitable extinction by the consequences of genetic drift in the long-term.

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Education

2005   Ph.D.   Indiana University
                  Major: Evolution   Minor: Plant Sciences
                  Advisor: Lynda Delph
                  "The evolution of self-compatibility and its genetic
                  consequences in *Leavenworthia alabamica* (Brassicaceae)"

2000   B.A.   University of Chicago
                  Ecology and Evolution with Honors
                  Advisors: Joy Bergelson and Ellen Simms
                  "Genetic variation in defensive traits of *Brassica rapa*"

Professional Experience

2005-present	Postdoctoral researcher, McGill University, Schoen Laboratory
2000-2005	Graduate student, Indiana University, Delph Laboratory
2000 summer	Research technician, Wright State University, Cipollini Laboratory
1998-2000	Research assistant, University of Chicago, Bergelson Laboratory
1998 summer	NSF REU, University of Chicago, Simms Laboratory
1997-1998	Research assistant, University of Chicago, Simms Laboratory
1997 summer	Field research assistant, Argonne National Laboratory

Teaching Experience

2005 spring	Lecturer, *Evolution*, Indiana University
2001 fall	Associate instructor, *Biological Mechanisms,* Indiana University
1998 spring	Teaching assistant, *Evolutionary Biology*, University of Chicago
1998 winter	Teaching assistant, *Environmental Ecology*, University of Chicago

Awards and Fellowships

2005-2007	Tomlinson Fellowship, McGill University
2004-2005	Chancellor's Fellowship, Indiana University
2000-2003	National Science Foundation Graduate Research Fellowship
2000	Young Botanist Award, Botanical Society of America
1999	Howard Hughes Medical Institute Summer Support Grant
1996-2000	National Merit Scholarship

Research Grants

2003	Indiana Academy of Sciences Grant ($1,730) *The evolution of self-fertilization and its consequences in an endemic plant*
2002	Sigma Xi Grant-in-aid of Research ($800)

> *The evolutionary breakdown of self-incompatibility in*
> *Leavenworthia alabamica*

2001   Indiana University Graduate Student Support Grant ($250)

Professional Memberships

Botanical Society of America, Society for the Study of Evolution

Service

Reviewer for: *American Journal of Botany*
Evolution
Evolutionary Ecology Research
Heredity
Plant Biology

Presentations

2005 summer   Annual Meeting, Society for the Study of Evolution
Local drift load in populations of an endemic plant
(Leavenworthia alabamica)

2004 fall   Invited Seminar, University of Virginia
Maintenance and loss of self-incompatibility in Leavenworthia
alabamica

2004 summer   Annual Meeting, Society for the Study of Evolution
Reproductive assurance and the evolution of self-compatibility in
Leavenworthia

2003 fall   Departmental Seminar, Indiana University
Genetic consequences of self-fertilization in Leavenworthia

Publications

Anderson, I.A., and J.W. Busch. 2006. Relaxed pollinator-mediated selection weakens floral integration in self-compatible taxa of *Leavenworthia* (Brassicaceae). American Journal of Botany (*in prep*).

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pathogens in plants. Evolution 58: 2584-2590.

Cipollini, D.F., J.W. Busch, K. Stowe, E. Simms, and J. Bergelson. 2003. Genetic variation and relationships of constitutive and herbivore-induced glucosinolates, trypsin inhibitors, and herbivore resistance in *Brassica rapa.* Journal of Chemical Ecology 29: 285-302.

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